Methods in
Molecular Biology 2962

Springer Protocols

Laurent Delpy *Editor*

RNA Analysis

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, UK

For further volumes:
http://www.springer.com/series/7651

For over 35 years, biological scientists have come to rely on the research protocols and methodologies in the critically acclaimed *Methods in Molecular Biology* series. The series was the first to introduce the step-by-step protocols approach that has become the standard in all biomedical protocol publishing. Each protocol is provided in readily-reproducible step-by-step fashion, opening with an introductory overview, a list of the materials and reagents needed to complete the experiment, and followed by a detailed procedure that is supported with a helpful notes section offering tips and tricks of the trade as well as troubleshooting advice. These hallmark features were introduced by series editor Dr. John Walker and constitute the key ingredient in each and every volume of the *Methods in Molecular Biology* series. Tested and trusted, comprehensive and reliable, all protocols from the series are indexed in PubMed.

RNA Analysis

Methods and Protocols

Edited by

Laurent Delpy

CNRS UMR 7276 - INSERM U 1262, University of Limoges, Limoges, France

Humana Press

Editor
Laurent Delpy
CNRS UMR 7276 - INSERM U 1262
University of Limoges
Limoges, France

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-0716-4725-7 ISBN 978-1-0716-4726-4 (eBook)
https://doi.org/10.1007/978-1-0716-4726-4

© The Editor(s) (if applicable) and The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

This work is subject to copyright. All rights are solely and exclusively licensed by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, expressed or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Humana imprint is published by the registered company Springer Science+Business Media, LLC, part of Springer Nature.
The registered company address is: 1 New York Plaza, New York, NY 10004, U.S.A.

If disposing of this product, please recycle the paper.

Preface

RNA plays an essential role in gene expression as a crucial intermediary between DNA and proteins. Beyond this fundamental role, RNA molecules display remarkable diversity and regulatory functions that influence cell fate. Advances in RNA research have shed light on the vast landscape of regulatory and non-coding RNAs, highlighting their importance in development, homeostasis, and disease. In addition, the emerging field of epitranscriptomics has revealed that dynamic and reversible chemical modifications of RNA molecules can influence their fate and function.

From transcription to degradation, the RNA life cycle is tightly controlled by a complex network of molecular mechanisms involving numerous post-transcriptional modifications and a myriad of RNA-binding proteins (RBPs) that recognize specific RNA motifs and structures and orchestrate the fate of RNA molecules by regulating their processing, transport, stability, and degradation. Similarly, RNA capping and polyadenylation protect RNAs from degradation, and influence their intracellular location and mRNA translation.

Pre-mRNA splicing, which induces the removal of introns, is catalyzed by the spliceosome, a giant ribo-nucleoprotein (RNP) complex. Fine-tuning of exon composition by alternative splicing or alternative polyadenylation results in multiple transcript isoforms, with major influence on gene expression. During RNA splicing, RBPs are deposited at the exon-exon junction (EJC) and play a critical role in nonsense-mediated mRNA decay (NMD), which serves to eliminate defective transcripts, thereby maintaining cellular homeostasis.

Technological advances in RNA sequencing have propelled the field forward, enabling researchers to map transcriptomes with unprecedented resolution. High-throughput sequencing has facilitated the discovery of novel RNA species, including variant transcripts and fusion transcripts, which play critical roles in cancer biology and other genetic disorders. The sequencing of 16S rRNA is also an essential tool for microbial community profiling. Similarly, in immunology, RNA sequencing has revolutionized our ability to study the immunoglobulin repertoire, providing insights into antibody diversity and the adaptive immune response. These applications highlight the broad impact of RNA research across biological disciplines, from fundamental molecular biology to biomedical and environmental sciences.

This volume of *Methods in Molecular Biology* brings together cutting-edge technologies for RNA analysis, covering fundamental techniques and innovative approaches that continue to expand our understanding of RNA biology. The aim of this volume is to provide detailed, step-by-step protocols for selected techniques used by molecular and computational biologists to isolate, amplify, detect, modify, and analyze RNA to gain insight into a wide range of cellular functions.

The experimental procedures documented in this volume are the result of the concerted efforts of many contributors. It is hoped that these products of exceptional quality will inspire students, technicians, and senior scientists, and will contribute to the expansion of knowledge in this dynamic field of RNA research in the coming years.

Limoges, France ***Laurent Delpy***

Contents

Preface . . . . . *v*
Contributors . . . . . *ix*

PART I REVERSE TRANSCRIPTION AND RNA CAPTURE METHODS

1 Guidelines for RNA Analysis by Reverse Transcription Quantitative Polymerase Chain Reaction 3
Antoine Praité, Jean-Marie Lambert, Laurent Delpy, and Sandy Al Hayek

2 Rare Transcripts Quantification by Strand-Specific RT-qPCR 15
Ophélie Alyssa Martin, Sandrine Le Noir, and Eric Pinaud

3 Detection of Cancer-Associated Gene Fusion by Ligation-Dependent RT-PCR 25
Vinciane Rainville, Marie-Delphine Lanic, Fanny Drieux, Nicolas Piton, David Rizzo, Fabrice Jardin, and Philippe Ruminy

4 Isolation of a Specific Transcript Using Biotinylated Antisense Probes 39
Antoine Praité and Laurent Delpy

PART II HIGH-THROUGHPUT SEQUENCING METHODS

5 Transcriptome-Wide Characterization of Protein:RNA Interactions with UV-Crosslinking Immunoprecipitation and Sequencing 51
Manuel D. Diaz-Muñoz and Ines C. Osma-Garcia

6 Targeted 16S rRNA Gene Sequencing for Water Samples 65
Mélanie Pimenta, Margaux Gaschet, and Sylvain Meyer

7 RNA-Based High-Throughput Sequencing of the Human Immunoglobulin Repertoire 83
Séléna Teillaud, Sébastien Bender, and Virginie Pascal

8 5′RACE Method for RNA-Based Sequencing of the Mouse Immunoglobulin Repertoire 103
Sandy Al Hayek, Antoine Praité, Jean-Marie Lambert, Sébastien Bender, and Laurent Delpy

9 A Wet Lab Procedure for Gene Fusion Analysis by Targeted RNA Next-Generation Sequencing in Lung Cancer 121
Sylvain Lacorre, Maéva Chaumet, Murat Sahin, Emilie Guérin, Paco Derouault, Léa Darnaud, Alexandre Perani, and Karine Durand

10 Simultaneous Profiling of Transcripts and Genomic Regions Associated with Nuclear Bodies Using the RNA–DNA High-Salt Recovered Sequence (RD-HRS) Method 141
Nathan Lecouvreur, Cosette Rebouissou, Annick Lesne, and Thierry Forné

Part III RNA Maturation: Splicing, Polyadenylation, and Epitranscriptomic Modifications

11 Functional Evaluation of Splice Variants Using a Minigene Strategy 155
Angélique Nizou, Lana Mahfoud, Alexandre Janin, and Anne-Sophie Lia

12 Design and Evaluation of a Truncated Protein Induction Strategy by Antisense Oligonucleotide-Mediated Exon Skipping 171
Pierre Hirt, Sandrine Le Noir, Laurent Delpy, and Eric Pinaud

13 Modifying 3′-End RNA Isoforms Using Antisense Oligonucleotides 185
Antoine Praité, Catherine Horiot, Anne Marchalot, Sandy Al Hayek, and Laurent Delpy

14 A Mass Spectrometry-Based Pipeline for Analyzing RNA Modifications in Cells and Tissues 199
Aurore Attina, Jérôme Vialaret, Christophe Hirtz, and Alexandre David

Part IV RNA Surveillance: Nonsense-Mediated mRNA Decay (NMD)

15 Inhibition of Nonsense-Mediated Decay in Dorsal Root Ganglion Neurons .. 217
Maddy R. Koch, Jenna B. Demeter, Rebecca Chase, Nikesh P. Kunder, Tzu-Fang Lou, Tarjani N. Shukla, and June Bryan I. de la Peña

16 Knockdown of Gene Expression Following Activation of the NMD Pathway Using Splice-Switching Oligonucleotides 237
Pierre Hirt, Anne Marchalot, Catherine Horiot, Jérôme Moreaux, and Laurent Delpy

17 Screening Methods for NMD Inhibitors or Readthrough Activators 255
Selma Nedjma and Fabrice Lejeune

Index *267*

Contributors

Sandy Al Hayek • *UMR CNRS 7276, Inserm 1262, Université de Limoges, Limoges, France; CRIBL lab (Control of the B-cell Immune Response and Lymphoproliferations), Limoges, France; Team 3, BioPIC (Biology of Plasma Cells, Immunopathology and Cancer), Limoges, France*

Aurore Attina • *IRMB-PPC, INM, CHU Montpellier, INSERM, Université de Montpellier, CNRS, Montpellier, Occitanie, France*

Sébastien Bender • *Immunology Laboratory of Dupuytren Hospital University Center (CHU) of Limoges, Limoges, France; UMR CNRS 7276, Inserm 1262, Université de Limoges, Limoges, France; CRIBL lab (Control of the B-cell Immune Response and Lymphoproliferations), Limoges, France; Team 3, BioPIC (Biology of Plasma Cells, Immunopathology and Cancer), Limoges, France*

Rebecca Chase • *Department of Genetics and Department of Psychiatry, Washington University School of Medicine, St. Louis, MO, USA*

Maéva Chaumet • *Cancer Molecular Genetics Platform, Department of Pathology, CHU Dupuytren, Limoges, France*

Léa Darnaud • *Cancer Molecular Genetics Platform, Department of Pathology, CHU Dupuytren, Limoges, France*

Alexandre David • *IRMB-PPC, INM, CHU Montpellier, INSERM, Université de Montpellier, CNRS, Montpellier, Occitanie, France; IRCM, Université de Montpellier, ICM, Inserm, Montpellier, Occitanie, France*

June Bryan I. de la Peña • *Department of Anesthesiology and Critical Care Medicine, School of Medicine, University of New Mexico Health Sciences Center, Albuquerque, NM, USA*

Laurent Delpy • *UMR CNRS 7276, Inserm 1262, Université de Limoges, Limoges, France; CRIBL lab (Control of the B-cell Immune Response and Lymphoproliferations), Limoges, France; Team 3, BioPIC (Biology of Plasma Cells, Immunopathology and Cancer), Limoges, France*

Jenna B. Demeter • *Department of Anesthesiology and Critical Care Medicine, School of Medicine, University of New Mexico Health Sciences Center, Albuquerque, NM, USA*

Paco Derouault • *Bioinformatics Platform, CHU Dupuytren, Limoges, France*

Manuel D. Diaz-Muñoz • *Toulouse Institute for Infectious and Inflammatory Diseases (INFINITy), Inserm UMR1291, CNRS UMR5051, University Paul Sabatier, CHU Purpan, Toulouse, France*

Fanny Drieux • *INSERM U1245, Centre Henri Becquerel, University of Rouen, Rouen, France; Department of Pathology, Centre Henri Becquerel, Rouen, France*

Karine Durand • *Cancer Molecular Genetics Platform, Department of Pathology, CHU Dupuytren, Limoges, France; UMR INSERM 1308 CAPTuR (Contrôle de l'Activation cellulaire, Progression Tumorale et Résistance thérapeutique), Faculty of Medicine, Limoges University, Limoges, France*

Thierry Forné • *IGMM, Univ Montpellier, CNRS, Montpellier, France*

Margaux Gaschet • *Univ. Limoges, RESINFIT, INSERM U1092, Limoges, France*

Emilie Guérin • *Sequencing Platform, CHU Dupuytren, Limoges, France*

Pierre Hirt • *Laboratoire Contrôle de la Réponse Immune B et des Lymphoprolifération (CRIBL), CNRS Unité Mixte de Recherche 7276, INSERM Unité 1262, Université de Limoges, Limoges, France*

Christophe Hirtz • *IRMB-PPC, INM, CHU Montpellier, INSERM, Université de Montpellier, CNRS, Montpellier, Occitanie, France*

Catherine Horiot • *UMR CNRS 7276, Inserm 1262, Université de Limoges, Limoges, France; CRIBL lab (Control of the B-cell Immune Response and Lymphoproliferations), Limoges, France; Team 3, BioPIC (Biology of Plasma Cells, Immunopathology and Cancer), Limoges, France*

Alexandre Janin • *Biochemistry and Molecular Biology Department, Hospices Civils de Lyon and Claude Bernard University – Lyon I, Lyon, France*

Fabrice Jardin • *INSERM U1245, Centre Henri Becquerel, University of Rouen, Rouen, France; Department of Hematology, Centre Henri Becquerel, Rouen, France*

Maddy R. Koch • *Department of Anesthesiology and Critical Care Medicine, School of Medicine, University of New Mexico Health Sciences Center, Albuquerque, NM, USA*

Nikesh P. Kunder • *Department of Laboratory Medicine, University of Texas at M.D. Anderson Cancer Center, Houston, TX, USA*

Sylvain Lacorre • *Cancer Molecular Genetics Platform, Department of Pathology, CHU Dupuytren, Limoges, France*

Jean-Marie Lambert • *UMR CNRS 7276, Inserm 1262, Université de Limoges, Limoges, France; CRIBL lab (Control of the B-cell Immune Response and Lymphoproliferations), Limoges, France; Team 3, BioPIC (Biology of Plasma Cells, Immunopathology and Cancer), Limoges, France; Université Côte d'Azur, CNRS, Molecular and Cellular Pharmacology Institute, Valbonne, France*

Marie-Delphine Lanic • *INSERM U1245, Centre Henri Becquerel, University of Rouen, Rouen, France*

Sandrine Le Noir • *Laboratoire Contrôle de la Réponse Immune B et des Lymphoprolifé rations (CRIBL), CNRS Unité Mixte de Recherche 7276, INSERM Unité 1262, Université de Limoges, Limoges, France*

Nathan Lecouvreur • *IGMM, Univ Montpellier, CNRS, Montpellier, France*

Fabrice Lejeune • *University of Lille, CNRS, Inserm, UMR9020-U1277 – CANTHER – Cancer Heterogeneity Plasticity and Resistance to Therapies, Lille, France*

Annick Lesne • *IGMM, Univ Montpellier, CNRS, Montpellier, France; Sorbonne Université, CNRS, Laboratoire de Physique Théorique de la Matière Condensée, LPTMC, Paris, France*

Anne-Sophie Lia • *UR20218 – NeurIT, University of Limoges, Limoges, France; Biochemistry and Molecular Genetics Department, Limoges Hospital, Limoges, France; Bioinformatics Department, Limoges Hospital, Limoges, France*

Tzu-Fang Lou • *Office of Research and Innovation, University of Texas at Dallas, Richardson, TX, USA*

Lana Mahfoud • *UR20218 – NeurIT, University of Limoges, Limoges, France*

Anne Marchalot • *UMR CNRS 7276, Inserm 1262, Université de Limoges, Limoges, France; CRIBL lab (Control of the B-cell Immune Response and Lymphoproliferations), Limoges, France; Team 3, BioPIC (Biology of Plasma Cells, Immunopathology and Cancer), Limoges, France; Department of Medicine Huddinge, Center for Infectious Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden*

Ophélie Alyssa Martin • *Laboratoire Contrôle de la Réponse Immune B et des Lymphoprolifération (CRIBL), CNRS UMR 7276, INSERM U1262, Université de Limoges, Limoges, France*
Sylvain Meyer • *Univ. Limoges, RESINFIT, INSERM U1092, Limoges, France*
Jérôme Moreaux • *Institute of Human Genetics, Unité Mixte de Recherche, Centre National de la Recherche Scientifique, Université Montpellier, Montpellier, France*
Selma Nedjma • *University of Lille, CNRS, Inserm, UMR9020-U1277 – CANTHER – Cancer Heterogeneity Plasticity and Resistance to Therapies, Lille, France*
Angélique Nizou • *UR20218 – NeurIT, University of Limoges, Limoges, France*
Ines C. Osma-Garcia • *Toulouse Institute for Infectious and Inflammatory Diseases (INFINITy), Inserm UMR1291, CNRS UMR5051, University Paul Sabatier, CHU Purpan, Toulouse, France*
Virginie Pascal • *Immunology Laboratory of Dupuytren Hospital University Center (CHU) of Limoges, Limoges, France; CNRS UMR-7276, INSERM U1262, Team 3 BioPIC of CRIBL, University of Limoges, Limoges, France*
Alexandre Perani • *Cancer Molecular Genetics Platform, Department of Pathology, CHU Dupuytren, Limoges, France; UMR INSERM 1308 CAPTuR (Contrôle de l'Activation cellulaire, Progression Tumorale et Résistance thérapeutique), Faculty of Medicine, Limoges University, Limoges, France*
Mélanie Pimenta • *Univ. Limoges, RESINFIT, INSERM U1092, Limoges, France*
Eric Pinaud • *Laboratoire Contrôle de la Réponse Immune B et des Lymphoprolifération (CRIBL), CNRS Unité Mixte de Recherche 7276, INSERM Unité 1262, Université de Limoges, Limoges, France*
Nicolas Piton • *Department of Pathology, CHU Rouen, Rouen, France*
Antoine Praité • *UMR CNRS 7276, Inserm 1262, Université de Limoges, Limoges, France; CRIBL lab (Control of the B-cell Immune Response and Lymphoproliferations), Limoges, France; Team 3, BioPIC (Biology of Plasma Cells, Immunopathology and Cancer), Limoges, France*
Vinciane Rainville • *INSERM U1245, Centre Henri Becquerel, University of Rouen, Rouen, France*
Cosette Rebouissou • *IGMM, Univ Montpellier, CNRS, Montpellier, France*
David Rizzo • *CNRS UMR-7276, INSERM U1262, Team 1 2MB2C of CRIBL, University of Limoges, and Hematology Laboratory of Dupuytren Hospital University Center (CHU) of Limoges, Limoges, France*
Philippe Ruminy • *INSERM U1245, Centre Henri Becquerel, University of Rouen, Rouen, France*
Murat Sahin • *Cancer Molecular Genetics Platform, Department of Pathology, CHU Dupuytren, Limoges, France*
Tarjani N. Shukla • *Science and Technology, 59th Medical Wing (59 MDW)/Chief Scientist's Office, Defense Health Agency, JBSA-Lackland AFB, San Antonio, TX, USA*
Séléna Teillaud • *Immunology Laboratory of Dupuytren Hospital University Center (CHU) of Limoges, Limoges, France*
Jérôme Vialaret • *IRMB-PPC, INM, CHU Montpellier, INSERM, Université de Montpellier, CNRS, Montpellier, Occitanie, France*

Part I

Reverse Transcription and RNA Capture Methods

Chapter 1

Guidelines for RNA Analysis by Reverse Transcription Quantitative Polymerase Chain Reaction

Antoine Praité, Jean-Marie Lambert, Laurent Delpy, and Sandy Al Hayek

Abstract

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) stands as an essential molecular biology tool for analyzing gene expression, offering a precise and accurate measure of gene expression levels. Its significance is critical in unraveling cellular processes and deciphering the complexities of disease mechanisms. This chapter details a comprehensive methodology for obtaining high-quality RNA from cell culture, followed by RNA extraction and column-based purification. After these steps, reverse transcription (RT) converts the RNA into complementary DNA (cDNA) before the quantitative PCR (qPCR) is performed. Protocols utilizing oligo(dT) and random hexamers for cDNA synthesis are described. Furthermore, we employed two different assays for qPCR: TaqMan and SYBR Green, to ensure a thorough examination of gene expression. These techniques are a valuable resource for researchers who want to study gene expression in a wide range of biological contexts.

Key words Reverse transcription, Quantitative polymerase chain reaction, Cell culture, RNA purification, Gene expression, Random primers, Oligo(dT)

1 Introduction

Reverse transcription quantitative PCR (RT-qPCR) has become a pivotal method for gene expression analysis due to its precision, sensitivity, and adaptability. By combining real-time quantification with accurate measurement of gene expression levels, RT-qPCR excels in both specificity and sensitivity [1–6]. This method is particularly valuable for validating specific genes identified through high-throughput studies like microarrays or RNA sequencing (RNA-seq). While these high-throughput methods provide comprehensive transcriptomic data across thousands of genes, they often require significant bioinformatics expertise for interpretation, which can make routine validation more complex [7, 8]. RT-qPCR

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_1,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

addresses these challenges by offering a cost-effective alternative that rapidly quantifies gene expression with high precision.

During the reverse transcription process, two types of primers are commonly employed: oligo(dT) and random primers [9–13]. These primers serve as initiators for the synthesis of complementary DNA (cDNA) from the RNA template. Oligo(dT) primers are short oligonucleotide sequences, typically 12–20 nucleotides long, containing only deoxythymidine (dT) residues to specifically target RNA molecules with a poly(A) tail. On the other hand, random primers are typically 6-mer or 9-mer deoxyribonucleotide mixtures of a random sequence. Their versatility lies in their capability to initiate cDNA synthesis from any region of the RNA template, providing a nonspecific approach to amplify all RNA molecules in a sample. This characteristic makes random primers suitable for obtaining a global perspective on overall gene expression, allowing researchers to capture a broader snapshot of the transcriptome.

Current qPCR techniques rely on real-time quantification of fluorescence emitted during the amplification process. These fluorescence signals, emitted by experiment-specific reporters, indicate the amount of PCR product, providing a measure of the initial copy number [3, 6, 14–18]. Probe-based experiments are widely used in real-time PCR, with some techniques incorporating a fluorophore and quencher for sequence-specific hybridization. Despite its advantages in specificity and reduced risk of nonspecific amplification, this approach is comparatively costly due to the need for custom probe design. In contrast, other techniques offer a more cost-effective option by using a double-stranded DNA-binding dye that emits fluorescence upon binding to amplified DNA [13]. The amount of emitted fluorescence is proportional to the DNA amount. While these assays are versatile and straightforward in primer design, they are susceptible to nonspecific binding and may require post-amplification melting curve analysis for specificity confirmation.

In this chapter, we present a comprehensive methodology for obtaining high-quality RNA, detailing protocols for cell culture, RNA extraction, column-based purification, reverse transcription, and qPCR (Fig. 1). Our workflow encompasses three human cell lines: SU-DHL-6 (a B-cell lymphoma model), U266 (a myeloma cell line), and HEK293 (an embryonic human kidney cell line). We describe the use of oligo(dT) primers, which specifically target polyadenylated RNA transcripts, and random hexamers for nonspecific priming during reverse transcription. For the qPCR step, we use TaqMan probes to quantify glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and SYBR Green to detect hypoxanthine-guanine phosphoribosyltransferase (*HPRT*).

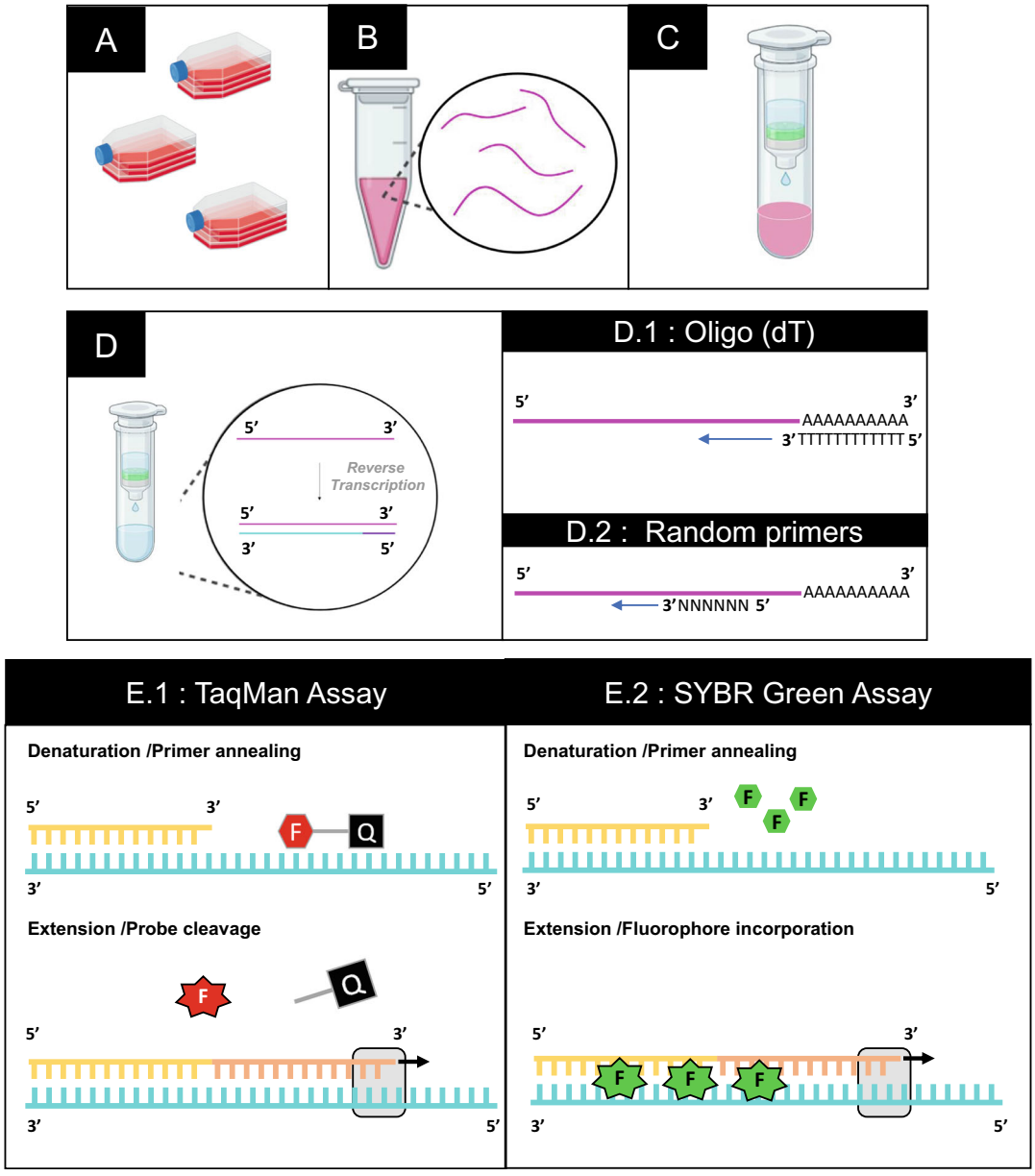


Fig. 1 Experimental workflow from cell culture to qPCR. **a** Cell culture of three different cell lines: HEK293, SU-DHL-6, and U266B1. **b** RNA extraction using the Trizol-based method. **c** RNA purification using column-based techniques. **d** cDNA synthesis from purified RNA using two priming strategies: oligo(dT) (D.1) or random primers (D.2). **e** qPCR is performed using TaqMan assays for *GAPDH* (E.1) and SYBR Green assays for *HPRT* (E.2)

2 Materials

2.1 Cell Culture

1. SU-DHL-6: human diffuse large B-cell lymphoma cell line.
2. U266B1 (U266): human multiple myeloma cell line.
3. HEK293: human embryonic kidney 293 cell line.
4. RPMI-1640 complete growth medium: RPMI-1640 medium, 10–20% fetal bovine serum (FBS).
5. Dulbecco's Modified Eagle Medium (DMEM) high glucose complete growth medium: DMEM medium, 20% FBS, and 1 mM sodium pyruvate.
6. 1× phosphate-buffered saline (PBS), pH 7.4.
7. 70% ethanol.
8. 15 mL sterile conical tubes.
9. Appropriate cell culture flasks.
10. 2× Trypan blue for cell counting.
11. Counting machine.
12. Centrifuge.
13. Incubator at 37 °C with a 5% CO_2 in a humidified atmosphere.
14. 0.25% trypsin-EDTA.

2.2 RNA Extraction and Purification

1. TRIzol™ reagent.
2. Absolute ethanol (molecular biology grade).
3. 95% molecular biology grade ethanol.
4. Direct-zol™ RNA Microprep Kit with Direct-zol™ RNA Pre-Wash solution (prepared in 95% ethanol according to the manufacturer's recommendation), RNA wash buffer (prepared in 95% ethanol), 6 U/μL DNase I solution (prepared in DNase/RNase-free water), and DNA digestion buffer.
5. Zymo-Spin™ IC Columns (included in the Direct-zol™ RNA Microprep kit).
6. Collection tubes (included in the Direct-zol™ RNA Microprep kit).
7. 1.5 mL sterile Eppendorf tubes.
8. Microcentrifuge.

2.3 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

1. 200 U/μL SuperScript® IV Reverse Transcriptase (SSIV).
2. 5× SSIV buffer.
3. 100 mM DTT.
4. 10 mM dNTP mix.
5. 50 μM Oligo(dT)20 primer.

6. 50 μM random hexamers.
7. 40 U/μL RNaseOUT™ Recombinant Ribonuclease Inhibitor.
8. RNA template.
9. 2× SensiFAST Probe Hi-ROX Kit (Bioline).
10. TaqMan™ *GAPDH* assay: primers and probe.
11. 2× SensiFAST SYBR Hi-ROX Kit (Bioline).
12. 10 μM *HPRT* forward primer.
13. 10 μM *HPRT* reverse primer.
14. Thermocycler and real-time PCR apparatus.
15. Centrifuge.
16. Nanodrop machine.

3 Methods

3.1 Cell Culture

All these steps are performed within a tissue culture hood under sterile conditions. Media are stored at 4 °C until use. Cell cultures are regularly tested for mycoplasma contamination using enzymatic and/or PCR assays.

1. Before the experiments, store all cell vials at −80 °C (*see* **Note 1**).
2. To achieve rapid thawing and preserve cells, thaw the vials for approximately 2 min in a 37 °C water bath (*see* **Note 2**).
3. Upon removal from the bath, decontaminate the vials with 70% ethanol.
4. Prior to cell culture, warm the complete growth medium at 37 °C for about 30 min.
5. Transfer the vial contents into a centrifuge tube containing 5–10 mL of 1× PBS or complete culture medium.
6. Centrifuge at 300 × *g* for 5 min.
7. Resuspend the cell pellet using the recommended complete medium.
8. Carefully dispense the resuspended cells into a new culture flask.
9. Place culture flasks in an appropriate incubator at 37 °C with a 5% CO_2.
10. For optimal cell growth, renew the medium every 2 to 3 days (*see* **Note 3**).
11. For adherent cells (i.e., HEK293 cell line) subculturing (*see* **steps 11–22** in Subheading 3.1), remove and discard the old culture medium.

12. Rinse the cell layer briefly with 1× PBS.
13. Add 2–3 mL of 0.25% trypsin-EDTA solution to fully cover the cell layer.
14. Incubate at 37 °C for 2–3 min, or until the cells detach.
15. Neutralize the trypsin by adding 6–8 mL of complete growth medium containing FBS.
16. Transfer the cell suspension to a centrifuge tube.
17. Centrifuge at 300 × *g* for 5 min.
18. Discard the supernatant.
19. Resuspend the cell pellet in fresh complete growth medium.
20. Plate the cells in new culture flasks.
21. Place culture flasks in an incubator at 37 °C with 5% CO_2.
22. Change the medium every 2–3 days to maintain optimal growth.
23. For cell counting, thoroughly resuspend the contents of the cell flasks using gentle pipetting.
24. Mix together equal volumes of cell suspension and 2× Trypan blue viability marker.
25. Use a specific slide, usually provided with the counting machine, for cell counting and proceed accordingly.

3.2 RNA Extraction and Column-Based Purification

1. For this experiment we used 1 ×10^6 cells (*see* **Note 4**).
2. Centrifuge the cell suspension at 300 × *g* for 5 min.
3. Remove the supernatant.
4. Perform cell lysis by resuspension with 700 µL of TRIzol® (*see* **Note 5**).
5. Add an equal volume of absolute ethanol and shake vigorously.
6. Transfer the mixture into a Zymo-Spin™ IC Column with a collection tube (*see* **Note 6**).
7. Centrifuge at 12,000 × *g* for 30 s.
8. Move the column to a new collection tube.
9. Add 400 µL of Direct-zol™ RNA Wash buffer to the Zymo-Spin™ IC Column.
10. Prepare the DNase I solution by mixing 5 µL of DNase I solution (6 U/µL) with 35 µL of DNA digestion buffer.
11. Mix gently by inversion.
12. Add the mix directly to the Zymo-Spin™ IC Column.
13. Incubate at room temperature for 15 min.
14. Introduce 400 µL of Direct-zol™ RNA PreWash buffer to the column.

15. Centrifuge at 12,000 × *g* for 30 s.
16. Discard the flow-through.
17. Repeat **Steps 9–11** in Subheading 3.2.
18. Add 700 µL of RNA wash buffer to the column.
19. Centrifuge at 12,000 × *g* for 1 min to ensure thorough removal of the wash buffer.
20. Transfer the column into a 1.5 mL RNase-free tube.
21. To elute RNA, carefully add 15 µL of DNase/RNase-Free Water directly to the column matrix in contact with the column filter (*see* **Note 7**).
22. Incubate 1 min and then centrifuge at 12,000 × *g* for 1 min (*see* **Note 8**).
23. Place RNA samples on ice.
24. Measure RNA concentration using a NanoDrop machine.

3.3 Reverse Transcription

Volumes used here are for 20 µL reactions.

1. To initiate the experiment, meticulously prepare different sets of tubes with Oligo(dT) Mix or random primers and with (+RT) or without (-RT: negative control) SSIV Reverse Transcriptase.
2. Take 1 µg of previously purified RNA sample (*see* **Step 23** in Subheading 3.2) in duplicate and adjust to a final volume of 11 µL with DNase/RNase-free water (*see* **Note 9** for lower amounts).
3. Mix 1 µL dNTP (final concentration: 0.5 mM) along with either 1 µL Oligo(dT) or 1 µL random primers (final concentration: 2.5 µM). Add 2 µL of this mix to the previously prepared RNA samples. Mix thoroughly and centrifuge briefly to remove drops from the lid or sides.
4. Incubate the mixture (RNA, dNTP, and Oligo(dT) or random primers) at 65 °C for 5 min in a thermocycler.
5. Incubate on ice for at least 1 min.
6. To prepare the RT reaction mix, briefly vortex the 5× SSIV buffer and combine the following components: 4 µL of 5× SSIV, 1 µL DTT (final concentration: 5 mM), 1 µL RNaseOUT™ (Recombinant RNase Inhibitor, *see* **Note 10**), and 1 µL SSIV Reverse Transcriptase for +RT reactions. Alternatively, 1 µL DNase/RNase-free water for -RT reactions.
7. Mix thoroughly and centrifuge briefly to remove drops from the lid or sides.

8. Combine the +RT and -RT tubes with the annealed RNA (*see* **Step 5** in Subheading 3.2).
9. For the random primer mix only, perform an additional incubation for 10 min at 23 °C.
10. Incubate both random primer and Oligo(dT) mixes for 10 min at 50–55 °C.
11. Incubate the mixes for 10 min at 80 °C to inactivate the SSIV reverse transcriptase and stop the reaction.
12. Store the prepared samples at −20 °C until further use or proceed directly with the qPCR.

3.4 Quantitative Polymerase Chain Reaction (qPCR)

1. For qPCR analysis using the TaqMan™ *GAPDH* assay, prepare a mix with the following reagents for a 20 µL qPCR reaction: 10 µL 2× SensiFAST Probe Hi-ROX Mix, 1 µL TaqMan™ *GAPDH* assay (with forward and reverse primers and a fluorescent TaqMan™ probe), 1 µL of cDNA template (*see* **Note 11** for low cDNA concentrations) and adjust to 20 µL with DNase/RNase-free water.
2. Alternatively, for qPCR analysis using SYBR Green chemistry (i.e., for *HPRT*), prepare a mix with the following reagents for a 20 µL qPCR reaction: 10 µL 2× SensiFAST SYBR Hi-ROX Mix, 0.8 µL of *HPRT* Forward Primer (final concentration 400 nM), 0.8 µL of *HPRT* Reverse Primer (final concentration 400 nM), 1 µL of cDNA Template (*see* **Note 11** for low cDNA concentrations), and adjust to 20 µL with DNase/RNase-free water.
3. Centrifuge briefly to ensure proper mixing of reagents before loading.
4. Load the plates into the thermocycler using the following parameters according to a standard qPCR program (*see* **Note 12**): 95 °C for 2 min (for initial polymerase activation) and 40 cycles of amplification (10 s of denaturation at 95 °C followed by 30 s of annealing/extension at 60 °C). Then, cool to 15 °C until the samples are recovered.
5. For SYBR Green assays, add a dissociation step at the end of the qPCR run to perform a melting curve analysis and verify the specificity of the amplified products.
6. After qPCR, check the amplification curves for both +RT and -RT samples (Fig. 2). The cycle threshold (Ct) for -RT samples is usually >33 Ct.

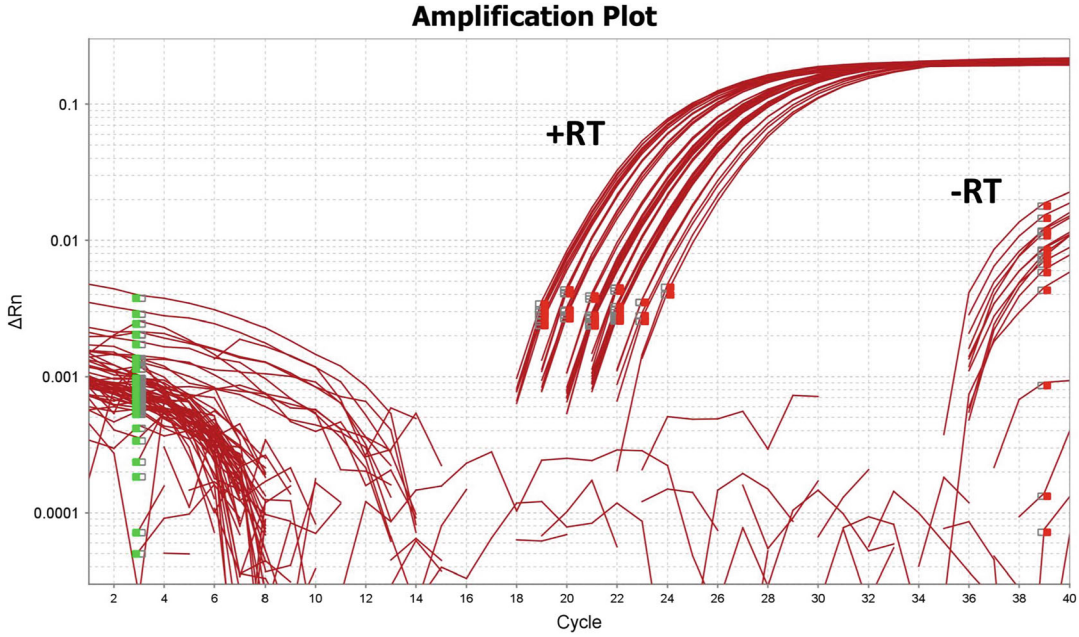


Fig. 2 Typical amplification curves obtained by qPCR. Classic qPCR profile depicting the amplification curves for +RT (with SSIV reverse transcriptase) and -RT (negative controls without reverse transcriptase) samples. Relative fluorescence (ΔRn; Y-axis) and the number of amplification cycles (X-axis) are shown

4 Notes

1. Cells may also be stored in liquid nitrogen instead of −80 °C for long-term storage. To ensure optimal viability levels, promptly thaw cell vials and initiate cell culture upon receipt.
2. To minimize the risk of contamination, make sure the cap stays out of the water during the thawing process.
3. Regularly check for cell concentration in the flasks since cells may grow quickly and acidification of the medium can become toxic.
4. When working with 1×10^6 cells from HEK293, U266, or SU-DHL-6 lines, we usually obtained RNA yields around 5 µg that are below the column's maximum binding capacity of 10 µg. To achieve efficient RNA recovery, ensure proper handling and avoid overloading the column with higher-yield samples.
5. For RNA extraction, we used 700 µL of TRIzol per 1×10^6 cells, which is slightly below the 1 mL recommended by the manufacturer (Thermo Fisher Scientific) for $5–10 \times 10^6$ cells. This volume was sufficient to ensure effective lysis and RNA recovery under our experimental conditions. For optimal

results, ensure that the volume of TRIzol is adequate for the number of cells to achieve complete cell lysis.

6. The maximum capacity of each column is 700 µL. When dealing with volumes exceeding the maximum capacity, adopt a two-step loading approach.
7. The elution volume can be adjusted based on the expected RNA concentration, but 15 µL serves as a good starting point. The manufacturer does not recommend eluting in volumes less than 10 µL.
8. At this step, columns are centrifuged in open-cap tubes. Be careful, as the caps may break during centrifugation, and make sure to label the tubes clearly. Ensure complete liquid passage through the column matrix to avoid obstruction.
9. The SSIV Reverse Transcriptase (200 U/µL) exhibits sensitivity to a broad range of RNA inputs, from 10 pg to 5 µg. In this study, 1 µg of total RNA was utilized.
10. The addition of an RNase inhibitor is optional but proves beneficial to prevent RNA degradation.
11. For low cDNA concentrations, the volume of cDNA template can be increased up to 9 µL for TaqMan™ qPCR assays and up to 8.4 µL for SYBR Green qPCR assays.
12. The parameters of the qPCR program can be optimized on purpose.

Acknowledgments

This work was supported by grants from INCa (PLBIO2022-110, INCA_16734), ANR (2017-CE15-0024-01), Ligue Contre le Cancer (comités Corrèze, Haute-Vienne), Fondation Française pour la Recherche contre le Myélome et les Gammapathies monoclonales (FFRMG), and Région Nouvelle-Aquitaine (AAP ESR 2020 and 2024). AP and SAH were funded by Ministry of Higher Education, Research and Innovation (MESRI), and the INCa agency, respectively.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR—a perspective. J Mol Endocrinol 34:597–601. https://doi.org/10.1677/jme.1.01755
2. Bustin SA, Benes V, Garson JA et al (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622.

https://doi.org/10.1373/clinchem.2008.112797

3. Bachman J (2013) Reverse-transcription PCR (RT-PCR). Methods Enzymol 530:67–74. https://doi.org/10.1016/B978-0-12-420037-1.00002-6
4. Deepak S, Kottapalli K, Rakwal R et al (2007) Real-time PCR: revolutionizing detection and expression analysis of genes. Curr Genomics 8: 234–251
5. Artika IM, Dewi YP, Nainggolan IM et al (2022) Real-time polymerase chain reaction: current techniques, applications, and role in COVID-19 diagnosis. Genes 13:2387. https://doi.org/10.3390/genes13122387
6. Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–39. https://doi.org/10.1677/jme.0.0290023
7. Okoniewski MJ, Miller CJ (2006) Hybridization interactions between probesets in short oligo microarrays lead to spurious correlations. BMC Bioinform 7:276. https://doi.org/10.1186/1471-2105-7-276
8. Saal LH, Troein C, Vallon-Christersson J et al (2002) BioArray software environment (BASE): a platform for comprehensive management and analysis of microarray data. Genome Biol. 3:software0003.1-software0003.6 3:SOFTWARE0003
9. Martín-Alonso S, Frutos-Beltrán E, Menéndez-Arias L (2021) Reverse transcriptase: from transcriptomics to genome editing. Trends Biotechnol 39:194–210. https://doi.org/10.1016/j.tibtech.2020.06.008
10. Rodríguez A, Rodríguez M, Córdoba JJ, Andrade MJ (2015) Design of primers and probes for quantitative real-time PCR methods. In: Basu C (ed) PCR primer design. Springer, New York, NY, pp 31–56
11. Stangegaard M, Dufva IH, Dufva M (2006) Reverse transcription using random pentadecamer primers increases yield and quality of resulting cDNA. BioTechniques 40:649–657. https://doi.org/10.2144/000112153
12. Wacker MJ, Godard MP (2005) Analysis of one-step and two-step real-time RT-PCR using SuperScript III. J Biomol Tech JBT 16: 266–271
13. Yu B, van Tol HTA, Stout TAE, Roelen BAJ (2021) Reverse transcription priming methods affect normalisation choices for gene expression levels in oocytes and early embryos. Mol Hum Reprod 27:gaab040. https://doi.org/10.1093/molehr/gaab040
14. Green MR, Sambrook J (2018) Quantification of RNA by real-time reverse transcription-polymerase chain reaction (RT-PCR). Cold Spring Harb Protoc 2018:pdb.prot095042. https://doi.org/10.1101/pdb.prot095042
15. Higuchi R, Fockler C, Dollinger G, Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnol Nat Publ Co 11:1026–1030. https://doi.org/10.1038/nbt0993-1026
16. Marinowic DR, Zanirati G, Rodrigues FVF et al (2021) A new SYBR Green real-time PCR to detect SARS-CoV-2. Sci Rep 11: 2224. https://doi.org/10.1038/s41598-021-81245-0
17. Navarro E, Serrano-Heras G, Castaño MJ, Solera J (2015) Real-time PCR detection chemistry. Clin Chim Acta Int J Clin Chem 439:231–250. https://doi.org/10.1016/j.cca.2014.10.017
18. Tajadini M, Panjehpour M, Javanmard SH (2014) Comparison of SYBR Green and TaqMan methods in quantitative real-time polymerase chain reaction analysis of four adenosine receptor subtypes. Adv Biomed Res 3:85. https://doi.org/10.4103/2277-9175.127998

Chapter 2

Rare Transcripts Quantification by Strand-Specific RT-qPCR

Ophélie Alyssa Martin, Sandrine Le Noir, and Eric Pinaud

Abstract

B lymphocytes development is associated with significant immunoglobulin (Ig) gene recombination leading to the establishment of a highly diverse and specific Ig repertoire. Accessibility of Ig genes is tightly regulated and associated with the production of sterile transcripts (noncoding), including low abundant anti-sense (AS) transcripts. Initially described by Corcoran's lab, the method used to quantify such transcripts at Ig loci is based on strand-specific reverse transcription (RT) from RNA and can be applied to any other cell types. The detection of strand-specific transcripts requires specific experimental controls run in order to exclude false signals that could arise from RT random priming. This chapter details the different steps of the whole procedure, from RNA isolation (optimized to a small number of cells) and multiplex RT reaction to cDNA amplification, including experimental controls.

Key words RNA isolation, Antisense transcripts, Multiplex RT-qPCR, Strand-specific, B lymphocytes

1 Introduction

B cell development is marked by different Ig gene recombination events required for efficient immune response. In bone marrow, V(D)J recombination is the assembly of different gene segments (V: variable, D: diversity, and J: joining) with the aim to produce a highly diverse set of B cell receptors that can recognize many antigens. It has been shown that the sequential order of V(D)J recombination is tightly regulated at the transcriptional level. Within the Ig heavy chain locus (*IgH*), accessibility of D_H, J_H, and V_H segments matches with the occurrence of "germline" sense (S) and antisense (AS) transcripts that take place specifically within the clusters of rearranging segments of *IgH*. The generation of such sterile transcripts facilitates first D_H to J_H and second V_H to DJ_H recombination events [1–3]. Ig gene remodeling continues in peripheral lymphoid organs upon antigen binding that leads to somatic hypermutation (SHM) and class switch recombination (CSR). By introducing point mutations in variable regions, SHM increases Ig affinity for antigen, while CSR allows the expression of

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_2,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

a new Ig isotype with an effector function adapted to the antigen type. S and AS transcription not only occur during early stages of B cell development but are also observed in activated cells undergoing both SHM and CSR, as these transcripts mark their respective target DNA regions [2, 4, 5]. While AID (activation-induced cytidine deaminase), which initiates SHM and CSR, acts preferentially on single-stranded DNA, it has been proposed that regions of convergent transcription (overlapping sense S and AS transcripts) could serve as preferential sites of AID target activity [4].

However, AS transcripts are low in abundance, probably transient, and processed by RNA exosome [6], making their quantification challenging. Above-mentioned studies used strand-specific reverse transcription (RT) as a reliable method to detect AS transcripts that are then amplified by qPCR [1, 7]. Recently, we have been applying this protocol and detecting low-abundant AS transcripts from rare sorted mice B cell populations (activated germinal center B cells undergoing SHM) [5]. In this chapter, we described the different experimental steps to be followed in order to detect strand-specific transcripts using multiplex-RT, leading to the generation of cDNAs from both the template of interest and a housekeeping template within the same reaction. In addition to material savings, the multiplexing step allows a more accurate quantification using a housekeeping/normalizing template produced in parallel with the template of interest in a single RT reaction. Even though this protocol has been widely used in B cells, it can be applied in other cell types.

2 Materials

2.1 Laboratory Equipment

1. Microcentrifuge.
2. Chemical hood.
3. PCR and qPCR devices.
4. Spectrophotometer (e.g., Nanodrop 2000).
5. Automated electrophoresis system (e.g., Bioanalyzer 2100).

2.2 RNA Isolation and RT-qPCR

1. RNA Later or equivalent (*see* **Note 1**).
2. Acid-guanidinium-phenol reagent.
3. Chloroform.
4. Isopropanol.
5. 100% and 70% ethanol.
6. Direct-zol™ RNA MicroPrep kit with Direct-zol™ RNA Pre-Wash buffer and RNA Wash buffer (Zymo Research).

7. Deoxyribonuclease (DNase) I amplification grade (Invitrogen), with 10× DNase I Reaction Buffer and 25 mM EDTA (pH 8.0).
8. SuperScript IV Reverse Transcriptase kit (Invitrogen) (*see* **Note 2**).
9. Primers, 10 μM (IDT Integrated DNA Technologies).
10. dNTP, 10 mM each.
11. RNaseOUTTM (Invitrogen).
12. 20×qPCR probes (IDT Integrated DNA Technologies).
13. 2× Premix Ex TaqTM (Probe qPCR), ROX Plus: qPCR TaqMan reagents (Takara).
14. Nuclease-free water.

3 Methods

3.1 Primer Design for Strand-Specific Reverse Transcription

1. Use any free open-source primer design software (e.g., Primer3; https://primer3.org/) providing primer proposition for a specified region of interest.
2. To quantify antisense transcripts, select a primer complementary to the predicted antisense transcript (Fig. 1).

3.2 Total RNA Isolation

If the number of cells is not a limitation, the classical RNA isolation procedure using the phase separation method can be applied as described in **steps 1–15** in Subheading 3.2. If the aim is to extract total RNA from rare ex vivo cell populations ($\leq 10^5$ cells), it is recommended to use a column-based method limiting loss of material at the interface during the classic phase separation procedure (*see* **steps 16–30** in Subheading 3.2).

1. Resuspend pelleted cells (e.g., 10^7 cells) in 1 mL of acid–guanidinium–phenol reagent.
2. Homogenize immediately by pipetting up and down a few times until the cells pellet is completely dissociated.
3. Incubate at room temperature for 5 min.

STOP POINT: If not used immediately, lysed samples could be stored at −80 °C.

4. Add to the tube 200 μL of chloroform and shake it vigorously.
5. Incubate at room temperature for 5 min.
6. Centrifuge at maximum speed for 15 min at 4 °C.
7. Three phases can be distinguished after centrifugation: the aqueous upper phase contains RNA. Transfer the aqueous phase into a new, clean tube.

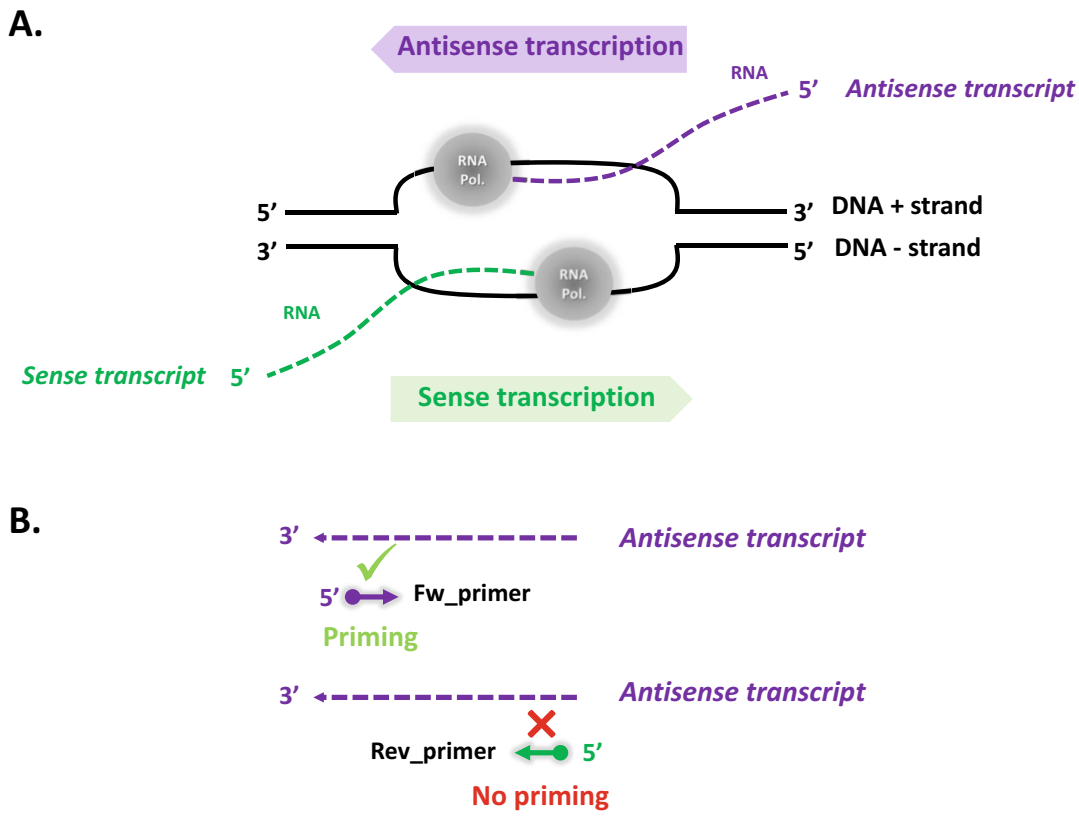


Fig. 1 **a** Schematic representation of sense and antisense transcription. **b** Primer design for strand-specific synthesis. Reverse transcription in the presence of sense primer (called Fw_primer in this figure) is leading to antisense transcript priming and antisense cDNA template synthesis. By contrast, the addition of antisense primer (called Rev_primer in this figure) will not lead to antisense cDNA template synthesis, as Rev_primer cannot be hybridized to the antisense transcript

8. Add to the aqueous phase 500 µL of isopropanol and shake the tube thoroughly.
9. Incubate at room temperature for 5 min.
10. Centrifuge at maximum speed for 10 min at 4 °C.
11. Discard the supernatant without perturbing the pellet.
12. Add to the pellet 1 mL of 70% ethanol.
13. Centrifuge at 7500 × *g* for 10 min at 4 °C.
14. Carefully discard the supernatant and keep the tube open at room temperature until the pellet is dry (approximately 10 min).
15. Resuspend the pellet in 30 µL of nuclease-free water.

STOP POINT: If not used immediately, RNA should be stored at −80 °C.

16. Resuspend pelleted cells (e.g., 10^5 cells) in 100 μL of acid–guanidinium–phenol reagent.
17. Homogenize immediately by pipetting up and down a few times until the cells pellet is completely dissociated.
18. Add to the tube 100 μL of 100% ethanol and mix vigorously.
19. Transfer the entire volume (200 μL) into the dedicated column in a collection tube (provided in the Direct-zol™ RNA MicroPrep kit or equivalent).
20. Centrifuge the column at maximum speed for 30 s at room temperature.
21. Transfer the column into a new collection tube.
22. Add to the column 400 μL of Direct-zol™ RNA PreWash buffer.
23. Centrifuge the column at maximum speed for 30 s at room temperature.
24. Discard the flow-through and repeat **steps 22–24** in Subheading 3.2.
25. Add to the column 700 μL of RNA wash buffer.
26. Centrifuge the column at maximum speed for 2 min at room temperature.
27. Transfer the column into a clean collection tube.
28. Add to the center of the column 15 μL of nuclease-free water.
29. Centrifuge the column at maximum speed for 30 s at room temperature.
30. The flow-through contains eluted RNA.

STOP POINT: If not used immediately, eluted RNA should be stored at −80 °C.

3.3 DNase I Treatment

Before proceeding to RT and amplification, residual DNA trace from RNA samples is eliminated by DNase I treatment. All the reagents mentioned below are supplied in the DNase I, Amplification Grade kit, apart from nuclease-free water.

1. Add to 1 μg of RNA (*see* **Note 3**): 1 μL of 10× DNase I reaction buffer, 1 μL of 1 U/μL DNase I, and nuclease-free water up to 10 μL.
2. Incubate at room temperature for 15 min.
3. Add 1 μL of 25 mM EDTA (pH 8).
4. Incubate at 65 °C for 10 min.

STOP POINT: If not used immediately, DNase I-treated RNA should be stored at −80 °C.

3.4 RNA Quality Control

1. Measure RNA concentration (for reliable measurements, RNA concentration should be at least ≥20 ng/μL).
2. Check RNA purity using a spectrophotometer (e.g., NanoDrop2000) and integrity using a gel electrophoresis system (e.g., 2100 Bioanalyzer). Reference values for high-quality RNA are usually >1.7 for RNA Integrity Number (RIN) and around 2 for A260/A280 and A260/A230 absorbance ratios. In contrast, RIN < 4, A260/A280 < 1.6, and A260/A230 < 1.3 are considered to be poor quality RNA.

3.5 Multiplex-Strand-Specific Reverse Transcription

RT was performed on 250 ng of total RNA with specific primers matching the region of interest and the housekeeping region. All the reagents mentioned below are supplied in the kit SuperScript IV Reverse Transcriptase unless otherwise specified (*see* **Note 2**).

1. To 250 ng of total RNA (Fig. 2), add 0.5 μL of 10 μM-specific primer (*see* **Note 4**), 0.5 μL of 10 μM housekeeping primer (*see* **Note 5**), 1 μL of dNTP mix (10 mM each), and nuclease-free water up to 13 μL.
2. Include a condition with no primer added (*see* **Note 6** and Fig. 2).
3. Incubate at 65 °C for 5 min, then on ice for at least 1 min.
4. Add 4 μL of 5× First Strand buffer, 1 μL of 0.1 M DTT, 1 μL of 40 U/μL RNaseOUT™, and 1 μL of 200 U/μL SuperScript IV RT to the tube.
5. Include a control with no SuperScript IV RT added (*see* **Note 7**).
6. Mix by pipetting up and down a few times.
7. Incubate at 55 °C for 10 min.
8. Incubate at 80 °C for 10 min.

STOP POINT: If not used immediately, cDNA should be stored at −20 °C.

3.6 qPCR Reaction

qPCR can be performed using either TaqMan or the SYBR green method. Our particular purpose was to detect low-abundance AS transcripts and required high detection specificity. TaqMan technology, based on sequence-specific fluorescent probes, improved detection specificity in comparison to SYBR green technology that used a nonspecific dsDNA binding dye. We usually perform our qPCR experiments using TaqMan method. Moreover, multiple fluorochromes can be used to tag the probes, giving the opportunity to set up a multiplex assay (*see* **Note 8**).

1. Prepare the qPCR reaction mix as 10 μL of 2×Premix Ex Taq™ (Probe qPCR) ROX Plus, 1 μL of 20× probe specific to the

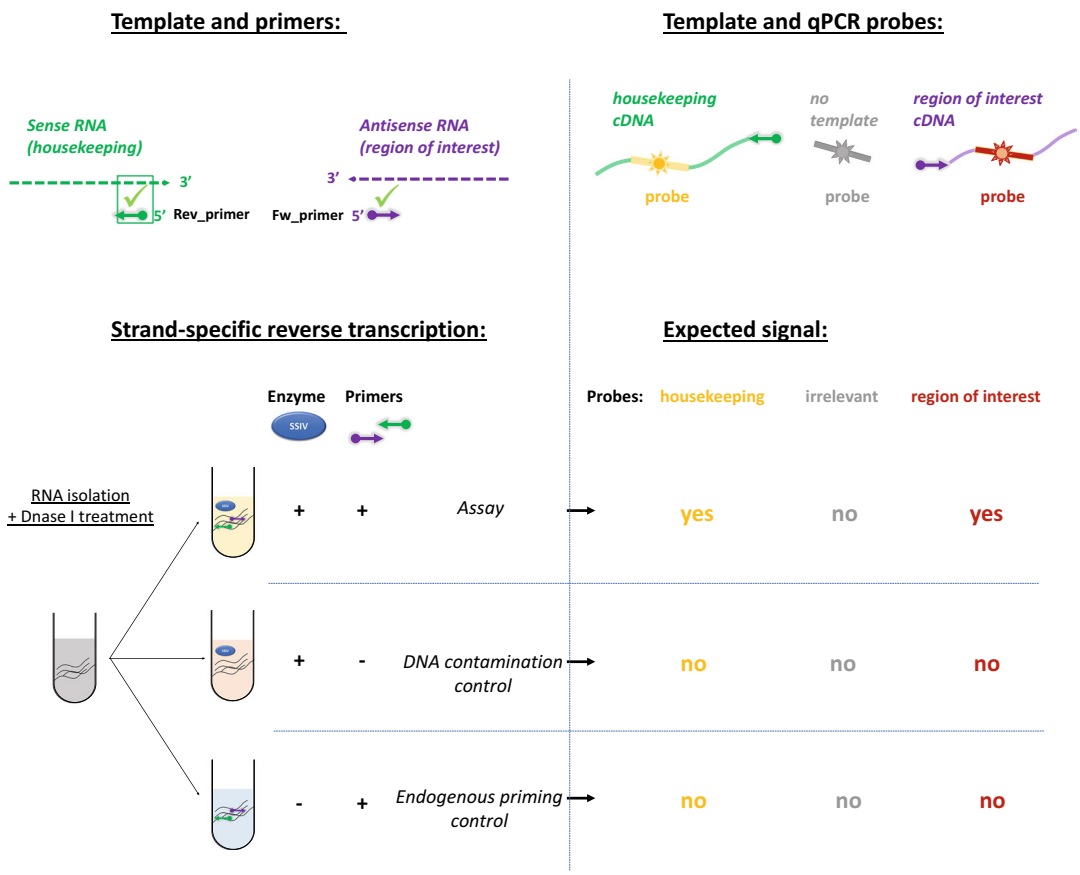


Fig. 2 General experiment procedure from RNA isolation to qPCR amplification. For each RNA sample (right), three reverse transcription reactions are conducted (middle). One reaction included both strand-specific primers that hybridize to the region of interest and to the housekeeping region for normalization. Performing the reverse transcription reaction for both the region of interest and the housekeeping gene within the same tube provides an internal standardization control that is used for relative expression analysis. Two control reactions are also performed with either no primers or no reverse transcriptase (SSIV) added, providing a measurement of endogenous primer and DNA contamination, respectively. Finally, synthesized cDNA is quantified by qPCR using specific probes for the region of interest (red) and the housekeeping region (yellow) (left). A negative control qPCR probe is included (represented in gray and called irrelevant probe), and due to its location should not detect any cDNA template. The expected amplification signal for the different experimental conditions is depicted (*see* yes/no). SSIV stands for SuperScript IV Reverse Transcriptase

region of interest, 1 μL of 20× probe specific to the housekeeping template, and nuclease-free water up to 19 μL.

2. Distribute 19 μL of qPCR reaction mix in tubes compatible with the available qPCR machine.
3. Add 1 μL of cDNA template (12.5 ng) (final volume per well is 20 μL). Include a no-template control.
4. Run the amplification program (*see* **Note 9**).
5. Assess amplification curves (*see* **Note 10**).

4 Notes

1. If cells of interest are collected through flow sorting, add 2 mL of RNA later (or equivalent) to the collection tube in order to better preserve RNA. Then centrifuge cells and discard the supernatant before proceeding to RNA isolation. We have noticed that using polypropylene tubes minimizes cells lost during collection and centrifugation steps.
2. SuperScript IV Reverse Transcriptase enzyme is recommended for strand-specific transcription assay since it is suitable to synthesize cDNA at a temperature up to 55 °C that would reduce random (endogenous) priming.
3. DNase I treatment could be scaled up or down according to RNA quantity.
4. As strand-specific detection required the addition of a primer matching the region of interest. Forward primers are designed to detect antisense transcripts (Fig. 1).
5. In order to quantify antisense transcripts' relative expression, we developed a multiplex-RT reaction that includes a primer to generate cDNA from a housekeeping gene used for analysis/normalization purposes. Within the same RT reaction, specific primers for both the region of interest and the housekeeping gene are provided (Fig. 2).
6. Low abundant transcripts quantification required the addition of a control cDNA template generated through RT reaction ran without adding primers. This control provides a measurement of endogenous priming that could be considered as "false" priming background (Fig. 2).
7. Include a control reaction with no reverse transcriptase added in order to measure potential DNA contamination in your RNA sample.
8. In order to optimize the use of cDNA templates, multiple qPCR reactions could be run within the same tube if using qPCR probes individually labeled with unique fluorophore. This allows the measurement of different targets simultaneously (e.g., combine a FAM/TAMRA labeled probe, used for the detection of the housekeeping cDNA, with another NED/NFQ* labeled probe, used for the detection of cDNA of interest; *NFQ: nonfluorescent quencher). The appropriate combination of fluorescently labeled probes depends on the qPCR device (filters availability); read carefully qPCR device manual.
9. PCR program setup depends on your qPCR reagents and qPCR device; check carefully protocols and device manual.
10. To conclude about assay reliability, different qPCR probes need to be included for quantification, as described in Fig. 3:

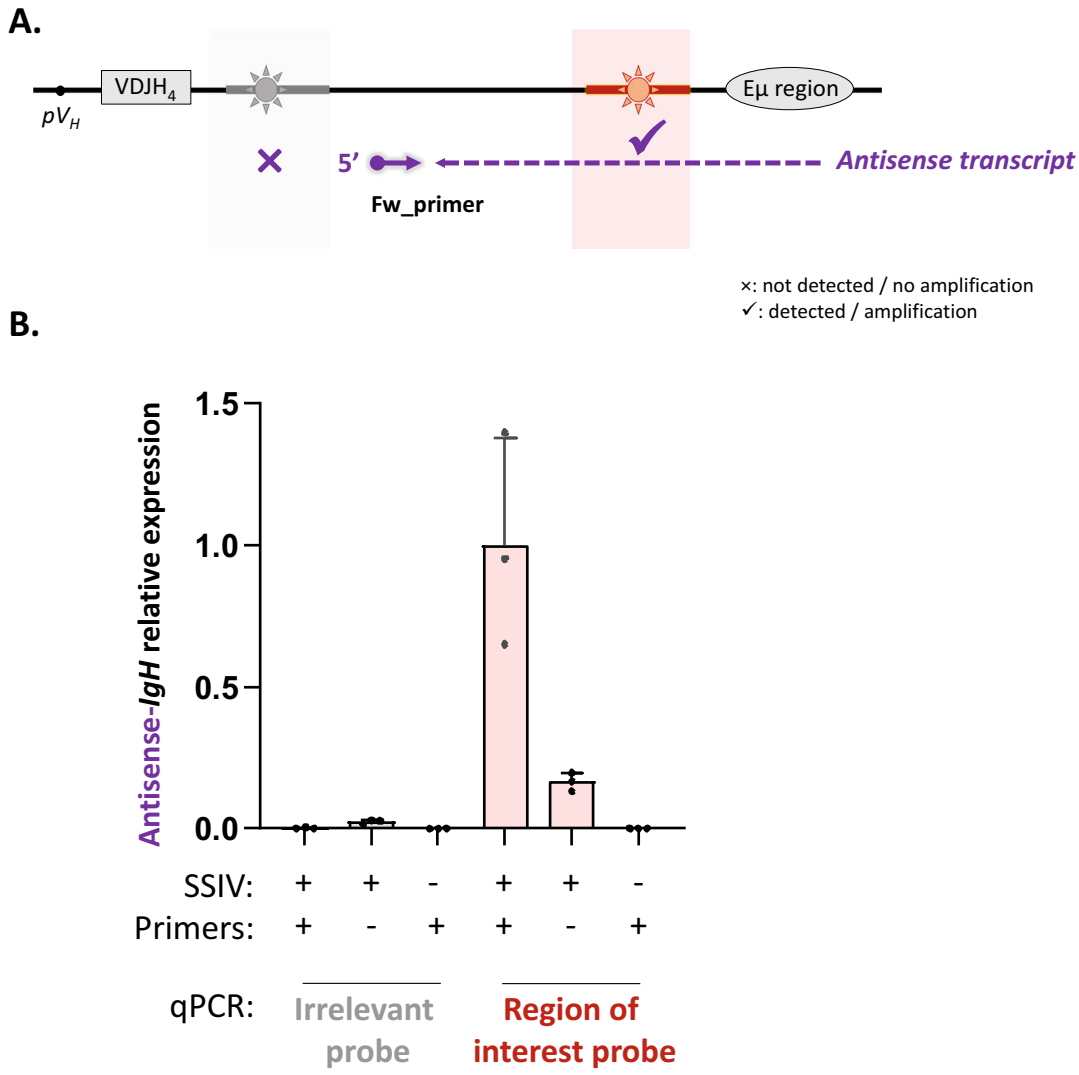


Fig. 3 Schematic representation of antisense template synthesized at the *IgH* locus. **a** An antisense (purple) template, covering an intronic region of interest targeted by SHM within the *IgH* locus, was generated by reverse transcription. The antisense template was then amplified by qPCR using the two probes (irrelevant and region of interest) as described in Fig. 2. **b** Actual relative quantification by qPCR for *IgH* antisense cDNA obtained in CH12 B cells (in vitro stimulated with anti-CD40, 200 ng/mL; mIL-4, 5 ng/mL; and rhTGFβ, 1 ng/mL) from three independent replicates (mean +/− SD). As expected, no amplification signal could be detected using the irrelevant control probe (gray, left part of the graph). In contrast, amplification signal was observed only by using the second qPCR probe matching the region of interest (red, right part of the graph). In the absence of SuperScript IV Reverse Transcriptase (SSIV), no signal could be detected. The signal associated with the no primer + SSIV condition results from endogenous priming. Analysis was performed by comparing threshold qPCR cycle (Ct) values according to the $2^{-(\Delta\Delta Ct)}$ conventional method. For samples where no Ct values were observed (undetermined Ct), we have assigned an arbitrary Ct value of 40. Antisense *IgH* quantification was relative to *Gapdh* expression used for normalization, as mentioned in Fig. 2

(i) a positive control probe that should detect antisense cDNA template (probe 2, Fig. 3) and (ii) a negative control probe that, because of its position, could not hybridize antisense cDNA template (Fig. 3: antisense transcript cannot be amplified with probe 1). The more primer/probe couples are added, the more reliable your assay is.

Acknowledgments

This work was supported by the Fondation Pour la Recherche Médicale, the "Institut français en Grèce" (to EP), La Ligue Contre le Cancer (comités 87, 23 to EP and SLN), Agence Nationale de la Recherche (ANR-21-CE15-0001-0), and Fondation ARC (ARCPJA2023070006749).

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Bolland DJ, Wood AL, Afshar R et al (2007) Antisense intergenic transcription precedes *Igh* D-to-J recombination and is controlled by the intronic enhancer Eμ. Mol Cell Biol 27:5523–5533. https://doi.org/10.1128/MCB.02407-06
2. Perlot T, Li G, Alt FW (2008) Antisense transcripts from immunoglobulin heavy-chain locus V(D)J and switch regions. Proc Natl Acad Sci 105:3843–3848. https://doi.org/10.1073/pnas.0712291105
3. Afshar R, Pierce S, Bolland DJ et al (2006) Regulation of IgH gene assembly: role of the Intronic enhancer and 5′DQ52 region in targeting DHJH recombination. J Immunol 176:2439–2447. https://doi.org/10.4049/jimmunol.176.4.2439
4. Meng F-L, Du Z, Federation A et al (2014) Convergent transcription at intragenic super-enhancers targets AID-initiated genomic instability. Cell 159:1538–1548. https://doi.org/10.1016/j.cell.2014.11.014
5. Martin OA, Thomas M, Marquet M et al (2023) The IgH Eμ-MAR regions promote UNG-dependent error-prone repair to optimize somatic hypermutation. Front Immunol 14. https://doi.org/10.3389/fimmu.2023.1030813
6. Laffleur B, Basu U, Lim J (2017) RNA exosome and non-coding RNA-coupled mechanisms in AID-mediated genomic alterations. J Mol Biol 429:3230–3241. https://doi.org/10.1016/j.jmb.2016.12.021
7. Bolland DJ, Wood AL, Johnston CM et al (2004) Antisense intergenic transcription in V(D)J recombination. Nat Immunol 5:630–637. https://doi.org/10.1038/ni1068



Chapter 3

Detection of Cancer-Associated Gene Fusion by Ligation-Dependent RT-PCR

Vinciane Rainville, Marie-Delphine Lanic, Fanny Drieux, Nicolas Piton, David Rizzo, Fabrice Jardin, and Philippe Ruminy

Abstract

Ligation-mediated RT-PCR is a rapid and highly sensitive solution to detect the presence of gene fusions in cancers. Here, we provide a detailed protocol which allows the detection of hundreds of these tumor markers in a single assay, in a rapid turnaround time.

Key words Gene fusion, Translocation, Cancer, Diagnosis

1 Introduction

Many cancers, including leukemia, sarcoma, and some solid tumors, harbor recurrent chromosomal rearrangements that result in the creation of gene fusions [1]. Many of these fusions are translated into hybrid proteins, often transcription factor or tyrosine kinases, directly involved in the transformation process. In many instances, their detection can be essential for an accurate diagnosis, like for example for the *EWSR1::FLI1* fusion which is present in Ewing sarcoma [2]. They can also guide treatment decision making, like for the recurrent rearrangements of the *ABL1* gene in leukemia and the *ALK* gene in lung cancers which can be targeted by specific pharmacological inhibitors [3].

Different approaches can be used to detect these genomic markers in routine clinical diagnosis. Historically, fluorescent in situ hybridization (FISH) was considered as the gold standard. However, most commercial FISH probes can detect only one rearrangement, or the rearrangements of a single gene, and multiple assays can be necessary to characterize a tumor sample. FISH also suffers from a lack of sensitivity, particularly for the detection of small size paracentric chromosomal inversion, like the *EML4::ALK*

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_3,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

fusion in lung cancer [4]. As an alternative, molecular solutions based on RT-PCR have been developed, for example in acute leukemia. However, multiple individual assays are here again often necessary to detect the multiple rearrangements recurrent in these diseases.

More recently, multiplex strategies have been developed to allow the detection of a large diversity of fusion gene in a single assay. In this context, we have demonstrated that ligation-dependent RT-PCR (LD-RTPCR) can be an elegant solution to detect simultaneously and in a rapid turnaround time a very large number of these markers, with both very high sensitivity and specificity, even starting from formalin-fixed and paraffin embedded (FFPE) tumor biopsies [5–10]. Here, we describe the different steps that allow the setup of a LD-RTPCR assay able to detect hundreds of different fusion mRNAs from a tumor sample (Fig. 1).

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents.

2.1 LD-RTPCR Probes and RT-PCR

1. All LD-RTPCR probes are single-stranded DNA oligonucleotides. We typically order lyophilized custom DNA oligonucleotides purified following a standard desalting procedure, in individual tubes. A synthesis scale of 25 nmoles is sufficient to test hundreds of thousands of samples. Importantly, all 3′ probes have to be phosphorylated at their 5′ end to allow the ligation reaction. As shown in Fig. 2, all probes are made of a gene-specific sequence and an additional tail, as detailed later in the LD-RTPCR probes design section of this manuscript.
2. Nuclease-free water.
3. TE20.2 solution: Tris, 20 mM, EDTA, 2 mM, pH 8.
4. TE10.1 solution: Tris, 10 mM, EDTA, 1 mM, pH 8.
5. The reagents we use for total RNA extraction depends on the nature of the tumor sample. For leukemic cells, which are extemporaneously separated on a Ficoll gradient, we use the Invitrogen TRIzol™ reagent. For fresh tumor tissues, we use the Maxwell Simply RNA Cells or Tissue kit. For FFPE biopsies, we use the Maxwell RSC RNA FFPE Kit.
6. Depending on the nature of tumor samples, we use two different methods for reverse transcription. For leukemic cells, we use the following Invitrogen™ M-MLV reverse transcriptase reagents: M-MLV Reverse Transcriptase, dNTPs set, Random Hexamers, 5× First Strand Buffer, DTT solution. For tissue biopsies (fresh or FFPE), where the percentages of tumor cells

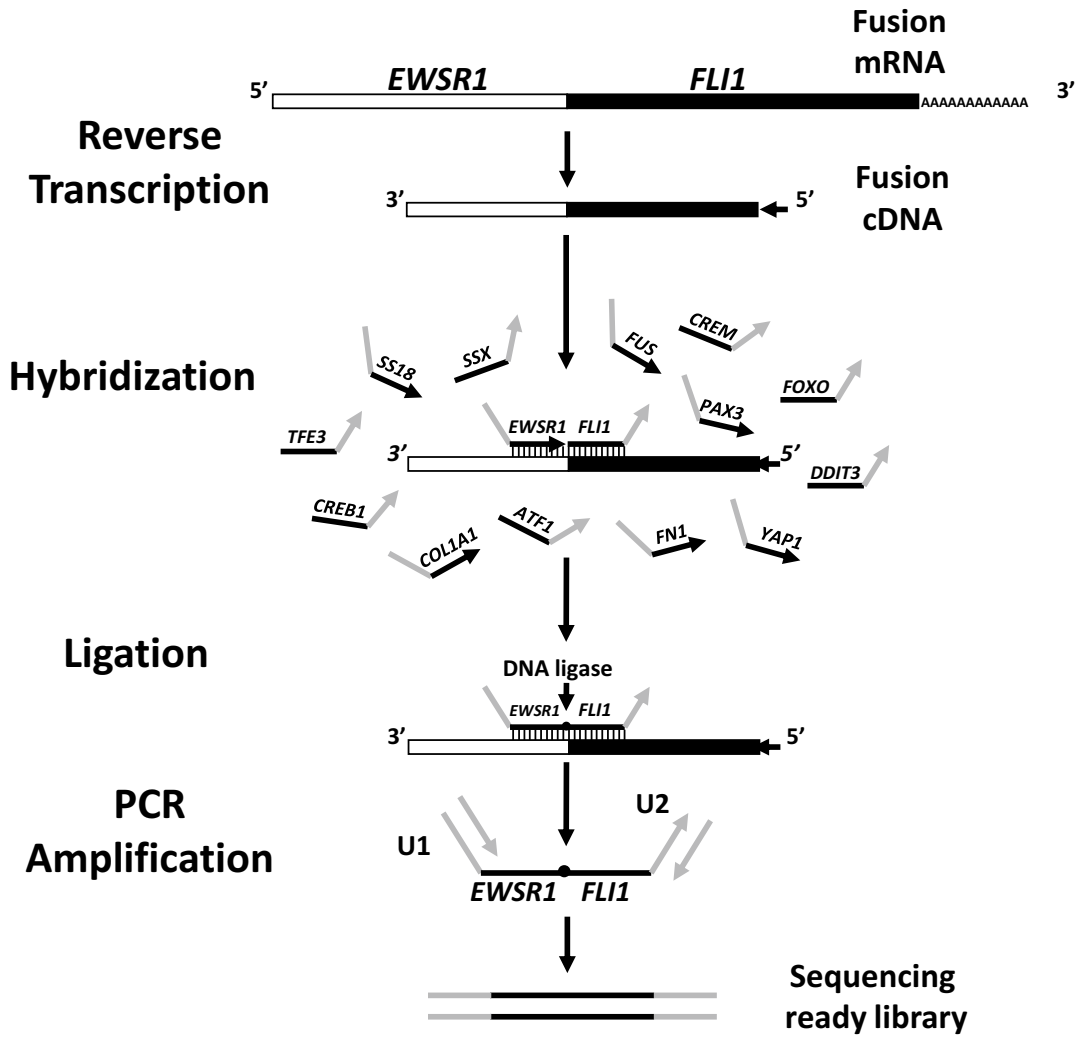


Fig. 1 LD-RTPCR workflow. Four steps are necessary to detect a fusion transcript from an RNA sample. RNA is first reverse transcribed into cDNA. LD-RTPCR probes are next hybridized on fusion cDNAs and ligated using a thermostable DNA ligase. The last step consists of PCR amplification, to obtain a product that can be analyzed by sequencing

can be low, we use the highly efficient Invitrogen™ SuperScript® VILO™ cDNA synthesis kit, that contains 5× VILO™ Reaction Mix and SuperScript® Enzyme mix, to increase the sensibility of the reaction (*see* **Note 1**).

7. At the hybridization and ligation steps, we use the SALSA® MLPA® Reagent Kit (MRC-Holland) that contains all reagents for multiplex ligation-dependent probe amplification (MLPA): SALSA MLPA Buffer, SALSA Ligase Buffer A, SALSA Ligase Buffer B, SALSA Ligase-65.

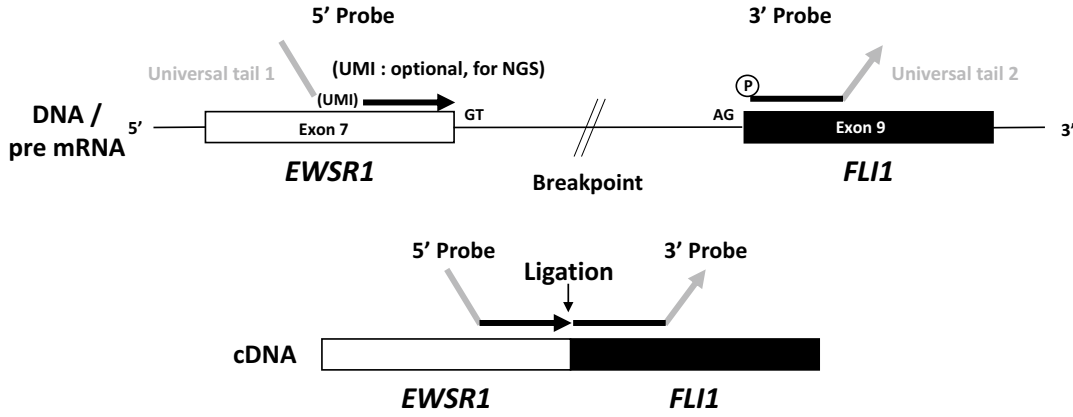


Fig. 2 Design of the LD-RTPCR probes. LD-RTPR probes are custom oligonucleotides. They are chosen exactly at the junction of the exons which are juxtaposed on the fusion transcripts after RNA splicing. Additional tails allow the final PCR amplification step. All 3′ probes are phosphorylated at their 5′ start to allow the ligation reaction

8. For the PCR amplification step, we use the Red'y' Star PCR Mix (Eurogentec).
9. Primers used for the PCR amplification steps are described in Table 1.
10. Thermal cycler with a heated lid.

2.2 Sequencing

1. Pyrosequencing is a rapid and simple sequencing alternative to identify the translocation partners. We use a Qiagen Pyromark® Q24 instrument and the following reagents:
   - Streptavidin Sepharose® Beads, High Performance (GE Healthcare),
   - Qiagen PyroMark Q24 Reagents,
   - Qiagen PyroMark Q24 Wash Buffer,
   - Qiagen PyroMark Q24 Binding Buffer,
   - Qiagen PyroMark Q24 Annealing Buffer,
   - Qiagen PyroMark Q24 Denaturation Buffer.

   The main limit of this approach is that the identification of the partner genes can be challenging when different fusion transcripts are expressed by alternative splicing, of when the level of the background is too high (*see* **Note 2**).
2. Sanger sequencing is more time consuming than pyrosequencing, but can be a method of choice as capillary sequencers are available in many routine diagnosis laboratories. We use a NucleoSpin™ PCR Clean-up Mini kit with columns (Macherey-Nagel) or equivalent, an Applied Biosystem 3130 sequencer and the Applied Biosystems BigDye Terminator V3.1 Cycle sequencing mix.

Table 1
PCR and sequencing primers

Primer name	Sequence
PCR_Pyrosequencing_5	GTGCCAGCAAGATCCAATCTAGA
PCR Pyrosequencing_3	Biotin-GGGTTCCCTAAGGGTTGGA
PCR_Sanger_5	TGTAAAACGACGGCCAGTGTGCCAGCAAGATCCAATCTAGA
PCR_Sanger_3	GGGTTCCCTAAGGGTTGGA
PCR_NGS_5	AATGATACGGCGACCACCGAGATCTACACGTGCCAGCAAGA TCCAATCTAGA
PCR_NGS_3_Barcode_1	CAAGCAGAAGACGGCATACGAGAT*AAATATCG* GGGTTCCCTAAGGGTTGGA
PCR_NGS_3_Barcode_2	CAAGCAGAAGACGGCATACGAGAT*ATGAATTA* GGGTTCCCTAAGGGTTGGA
PCR_NGS_3_Barcode_3	CAAGCAGAAGACGGCATACGAGAT*TATCAAGA* GGGTTCCCTAAGGGTTGGA
PCR_NGS_3_Barcode_4	CAAGCAGAAGACGGCATACGAGAT*GTATCGCG* GGGTTCCCTAAGGGTTGGA
Seq_Pyrosequencing	GTGCCAGCAAGATCCAATCTAGA
Seq_Sanger	TGTAAAACGACGGCCAGT
Seq_NGS	CCGAGATCTACACGTGCCAGCAAGATCCAATCTAGA

Nucleotide sequences in italics represent the Barcode sequence
Different combinations of oligonucleotide primers are provided, which allow the detection of fusion transcripts by pyrosequencing, Sanger sequencing, or next-generation sequencing

3. Next-generation sequencing (NGS) allows an extensive characterization of the final product of the LD-RTPCR reaction. Giving access to the sequence of each individual PCR amplification product, it can allow the detection of fusion transcripts expressed at very low level, or the analysis of poor quality FFPE samples. For these analyses, we use Beckman Coulter® Agencourt AMPure XP beads, Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), and Illumina MiSeq or NextSeq sequencers and their corresponding reagents.
4. EBT buffer: 10 mM Tris–HCl, pH 8.5, 0.1% Tween 20.

3 Methods

3.1 LD-RTPCR Probes Design

1. An extensive and up-to-date review of the literature must be performed to list all the fusion genes that have to be tested in the pathology of interest. Online resources like the Mitelman

Database (https://mitelmandatabase.isb-cgc.org/) can be useful, but are often not sufficient. It is also particularly important to consider that, for each rearrangement, the genomic breakpoint can often occur within different introns (as soon as the functional domains of the two proteins are retained and as the coding frames of the two proteins are conserved) (*see* **Note 3**). A specific attention should thus be paid to dress an exhaustive list of all the exon–exon boundaries that need to be tested.

2. The next step consists in the design of the LD-RTPCR probes. Each probe is made of two parts: a sequence which corresponds to the 3′ or 5′ ends of the exons that will by juxtaposed after mRNA splicing, and an additional tail (Fig. 2). Two categories of probes can be distinguished: the 5′ probes, which correspond to the 3′ ends of the exons of the 5′ gene (e.g., *EWSR1* for the *EWSR1::FLI1* fusion), and the 3′ probes which correspond to the 5′ starts of the exons of the 3′ gene (*FLI1* in this case).
3. For both 5′ and 3′ probes, a sequence which corresponds exactly to the extremity of the targeted exon is designed, ending just before the canonical dinucleotides GT splice donor site for the 5′ probes, and starting immediately after the AG splice acceptor site for the 3′ probes (*see* **Note 4**). These sequences can easily be identified using for example the www.ensembl.org website. The size of these exon-specific sequences is adjusted to obtain a Tm ranging between 70 and 75 °C (which usually correspond to 20–30 bases long sequences).
4. An identical additional tail is next added to the 5′ end of all the 5′ probes (e.g., GTGCCAGCAAGATCCAATCTAGA). Similarly, an additional 3′ tail (different from the tail of the 5′ probes) is added to the 3′ end of all the 3′ probes (e.g., TCCAACCCTTAGGGAACCC).
5. If the final PCR amplification products are sequenced by next-generation sequencing, it can be interesting to insert unique molecular identifier (UMI, NNNNNNN) between gene-specific sequence and the additional tail of the 5′ probes. The analysis of these UMIs will then inform on the actual number of cDNA molecules that are detected, a point that can be particularly important when the quality of the starting tumor sample is poor or when the fusion transcript is expressed at a low level.
6. Finally, all the 3′ probes have to be phosphorylated at their 5′ starts to allow the ligation step.

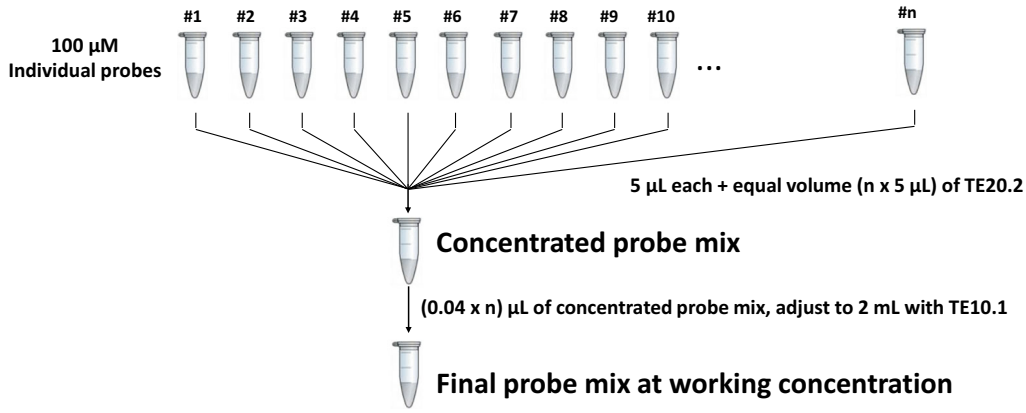


Fig. 3 Production of a LD-RTPCR probe mix. The production of a LD-RTPCR probe mix consists of three steps. Individual probes are first suspended at a concentration of 100 µM. A first concentrated mix is next produced by mixing all the probes and by adding the same volume of TE20.2. The final probe mix is obtained by diluting this concentrated mix in TE10.1

3.2 Production of the LD-RTPCR Probe Mix

1. After receipt, all LD-RTPCR probes in individual tubes are re-suspended in nuclease-free water, at a final concentration of 100 µM. These probes can next be stored at −20 °C.
2. Five µL of each of these probes are next mixed together in a single Eppendorf tube, and an equal volume of TE20.2 solution is added to obtain a concentrated probe mix (Fig. 3). For example, for a mix made of 20 probes, 100 µL (20 × 5 µL) of TE20.2 is added to the 100 µL (20 × 5 µL) of mixed concentrated probes, to obtain a final volume of 200 µL. This concentrated mix can be stored at −20 °C.
3. To obtain the final probe mix, at the working concentration, 0.04 × n µL of the concentrated mix (with n corresponding to the number of probes) is diluted into a final volume of 2 mL with TE10.1 solution. Here, for a mix of 20 probes, 0.8 µL of the concentrated mix would be diluted in 1999.2 µL of TE10.1 solution. The mix which is obtained, at the working concentration, is sufficient to test more than 1200 samples. It can be stored at −20 °C (*see* **Note 5**).

3.3 RNA Extraction and Reverse Transcription

We use standard procedures for RNA extraction. Starting from blood or bone marrow samples for leukemias, we perform RNA extraction using TRIzol™ reagent, following the manufacturer's instructions. Total RNAs are re-suspended at a final concentration of approximately 250 ng/µL in nuclease-free water and stored at −80 °C. When the quantity of tumor material is low or starting from fresh or FFPE biopsies, we use the Maxwell Simply RNA Cells or Tissue kit or the Maxwell RSC RNA FFPE Kits, respectively. RNA samples are eluted in 50 µL of nuclease-free water and stored at −80 °C. The final concentration of these samples varies widely,

according to the quantity and quality of starting material. However, according to our experience, reliable results can be obtained even starting from only few ng of total RNA.

The reverse transcription, hybridization, and ligation reactions are all performed in a thermal cycler with a heated lid, in a pre-PCR environment (*see* **Notes 6** and **7**). For acute leukemia samples, where the percentage of tumor cells is high, we use M-MLV reverse transcriptase reagents (*see* **steps 1–5** in Subheading 3.3). For fresh/frozen or FFPE, we use high performance SuperScript® VILO™ cDNA synthesis kit to increase the sensitivity of the assay (*see* **steps 6–8** in Subheading 3.3).

1. Prepare a Reverse Transcription Mix for n samples, with 1.25 μL 5× first strand buffer, 0.5 μL DTT solution, 1 μL dNTPs solution (10 mM each), and 1 μL random hexamers (100 pmol/μL) per sample.
2. In a 200 μL PCR tube on ice, add 3.75 μL of Reverse Transcription Mix and 2 μL of total RNA (from only few ng to approximately 500 ng, depending on the quality of the RNA sample) (*see* **Note 8**).
3. In a thermal cycler with a heated lid (95 °C), incubate for 2 min at 80 °C and 5 min at 37 °C, and then allow the samples to refrigerate at 4 °C.
4. Keep the samples on ice and add 0.5 μL of M-MLV Reverse Transcriptase.
5. In a thermal cycler with a heated lid, incubate for 15 min at 37 °C and 2 min at 98 °C, and then allow the samples to refrigerate at 4 °C.
6. Prepare a Reverse Transcription Mix for n samples, with 1 μL 5× VILO™ Reaction Mix, 1 μL nuclease-free water and 0.5 μL SuperScript® enzyme mix per sample.
7. In a 200 μL PCR tube on ice, add 2.5 μL of Reverse Transcription Mix and 2.5 μL of total RNA (at varying concentrations).
8. In a thermal cycler with a heated lid (95 °C), incubate for 10 min at 25 °C, 60 min at 42 °C, and 5 min at 85 °C, and then allow the samples to refrigerate at 4 °C.

3.4 Hybridization and Ligation

For the hybridization and ligation steps, we use the SALSA® MLPA® reagent kit components.

1. Prepare a hybridization mix for n samples, with 1.5 μL SALSA MLPA buffer and 1.5 μL LD-RTPCR Probe Mix (at working concentration, *see* **steps 2–3** in Subheading 3.2) per sample.
2. Add 3 μL of the hybridization mix to the cDNA samples (6.25 or 5 μL, depending on the reverse transcription option that has been used), on ice.

3. In a thermal cycler with a lid heated to 95 °C, incubate for 2 min at 95 °C and 60 min at 60 °C (*see* **Note 9**).
4. Prepare a ligation mix for n samples, with 25 µL nuclease-free water, 3 µL SALSA Ligase Buffer A, 3 µL SALSA Ligase Buffer B, and 1 µL SALSA Ligase-65 per sample.
5. Set the temperature of the thermal cycler to 54 °C.
6. In each sample, keeping the 200 µL PCR tube in the thermal cycler block, add 32 µL of the ligation mix (*see* **Note 10**).
7. Incubate for 15 min at 54 °C and 5 min at 98 °C, and then allow the samples to refrigerate at 4 °C.
8. When possible, proceed immediately to PCR amplification. Alternatively, the ligation product should imperatively be frozen at −20 °C as soon as possible. Keeping the ligation product for an extended period at room temperature or even at 4 °C can lead to an important loss of specificity and to a very significant increase of the background (*see* **Note 11**).

3.5 PCR Amplification

Amplification is performed in a second thermal cycler, in a post-PCR environment. Different couples of PCR primers must be used, depending on the sequencing method which has been chosen. Primer sequences are provided in Table 1. All these primers bind to the additional tails of LD-RTPCR probes.

For Pyrosequencing, we use the PCR_Pyrosequencing_5′ biotinylated forward primer in combination with the PCR_Pyrosequencing_3′ reverse primer.

For Sanger sequencing, we use the PCR_Sanger_5′ forward primer in combination with the PCR_Sanger_3′ reverse primer.

For NGS sequencing, we use the PCR_NGS_5′ forward primer in combination with one of the PCR_NGS_3′_Barcode reverse primers. These sequences, with P5 and P7 additional tails, are designed for the Illumina sequencing systems. Different reverse primers must be used for each sample, as all the amplification products are pooled in the sequencing library. The underlined barcode sequences allow the demultiplexing step and the attribution of the sequences to each tumor.

Regardless of the sequencing solution which has been chosen, we use the same PCR amplification conditions.

1. Prepare a PCR amplification mix for n samples, with 12.5 µL of Red'y' Star PCR Mix, 5.5 µL of nuclease-free water per sample.
2. In 200 µL PCR tubes, mix 18 µL of PCR amplification mix, 1 µL of 5′ PCR primer at 10 µM, 1 µL of 3′ PCR primer at 10 µM, and 5 µL of Ligation product.
3. In a thermal cycler with a heated lid (95 °C) use the following PCR program: 6 min at 94 °C, 35 cycles (30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C), 4 min at 72 °C, and allow the samples to refrigerate at 4 °C.

3.6 Sequencing

1. To perform pyrosequencing of PCR products using the PyroMark Q24 system, 18 μL of PCR amplification products are incubated with 2 μL of Streptavidin Sepharose® Beads and 20 μL of PyroMark Q24 Annealing Buffer, without any previous purification. Samples are next processed according to the manufacturer's instructions. Sequences are obtained using the PyroMark™ Q24 sequence analysis (SQA) procedure, with 15 ACTG injections cycles, using the PCR_Pyrosequencing_5′ sequencing primer (Table 1).
2. For Sanger sequencing, PCR amplification products are first purified using NucleoSpin™ PCR Clean-up Mini kit columns or equivalent (the small size of the amplification products, which varies between 100 and 120 bp, should be taken into account to select the appropriate purification method). Sequences are next obtained using the SeqSanger primer (Table 1) and the BigDye Terminator V3.1 Cycle sequencing mix, following the manufacturer's instructions.
3. For next-generation sequencing, 25 μL of the PCR products are incubated with 45 μL of Agencourt AMPure XP beads (ratio 1.8), purified according to the manufacturer's instructions, eluted with 50 μL of TE10.1, and quantified using the Qubit dsDNA HS Assay Kit.
4. Molar concentrations are next calculated, estimating that the PCR amplification products have an average length of 150 bp.
5. All samples are next diluted at an equimolar concentration, for example 4 nM or 2 nM in EBT buffer and pooled to obtain the sequencing library.
6. This library is denatured and loaded on the sequencer following the Illumina Denature and Dilute Libraries Guide and the Illumina MiSeq or NextSeq system guides, using final library concentrations of 8 pM (MiSeq) or 1 pM (NextSeq) and aiming to obtain an average of 10^5 sequencing clusters for each sample. These sequences are generated using the Seq_NGS sequencing primer (Table 1), which is loaded on the cartridge following the MiSeq or NextSeq Custom Primer Guides.
7. To analyze the results, we use a dedicated bio-informatic pipeline. Starting from the FastQ files generated by the sequencer, we first demultiplex the sequencing reads according to the presence of the barcode located between the tail of the 3′ probe and the P7 sequence (*see* **Note 12**). The reads are next aligned with the gene specific sequences to identify the probes that have been ligated, and the UMI are counted to evaluate the number of cDNA molecules which have been detected (*see* **Note 13**).

4 Notes

1. Substitution of the reagents used at the reverse transcription, ligation, or PCR amplification steps is possible, but may impact the quality of the results. Some adjustments of the protocol, and particularly of the annealing temperatures and of PCR enzymes, may then be necessary to avoid unspecific amplifications.
2. In some cases, different fusion transcripts can be expressed from the rearranged locus after alternative RNA splicing over the translocation junction. In such cases, the sequences which are obtained can be difficult to interpret. One solution is to sequence the second strand of the PCR product, using a biotinylated reverse primer and a normal forward primer, or to proceed to NGS sequencing.
3. Difficulties may arise when a gene is involved in multiple translocations with different partners, like for example *RUNX1* implicated in the recurrent *RUNX1::RUNX1T1* and *ETV6::RUNX1* rearrangements in acute leukemia. In such cases, the use of 5′ and 3′ LD-RTPCR probes designed on the same gene can lead to a significant level of background, probably due to the expression of circular transcripts. One solution is to shift some probes from few bases (typically 2–3) over one of the tested junction.
4. Extreme caution should be taken regarding the precise coordinates of the exon boundaries in genomic databases. For example, in the ENSEMBL database, the coordinates of the first exons of the constant genes of the *IGH* locus is shifted from one nucleotide. Checking the actual position of the GT and AG splice sites is thus essential.
5. In our experience, the mixes of LD-RTPCR probes are stable for years at −20 °C when they are diluted in TE10.1. If water is used instead of TE10.1 those mix are by contrast extremely unstable.
6. Due to the small volumes of the reactions, caution should be taken to ensure that the heated lid of the thermal cycler is kept at 95 °C throughout the procedure, to prevent evaporation.
7. Given that all PCR amplifications are performed using identical couples of PCR primers, specific caution should be taken to avoid contaminations. In our laboratory, the reverse transcription, hybridization, and ligation steps are all performed in dedicated pre-PCR areas, while PCR amplifications, PCR purifications, and sequencing reactions are performed in a post-PCR environment.

8. The quantity of 500 ng of total RNA is only indicative. In our experience, fusion transcripts can sometimes be detected from as low as few ng of RNA, even from archival, minute size, FFPE biopsies. The detection limit of the procedure is difficult to evaluate, depending on multiple factors including the size of the tumor sample, the quality of the RNA, the percentage of tumor cells, and the level of expression of each fusion transcript.
9. In our experience, a long hybridization (e.g., overnight) does not lead to any gain of sensitivity, but instead to an important increase of the background. We strongly recommend a 1 h long hybridization.
10. To avoid unspecific hybridization of the LD-RTPCR probes, the tubes should not be removed from the thermocycler, at 54 °C, when the ligation mix is added.
11. The DNA ligase that is used is thermoresistant. Some residual activity can thus lead to the generation of unspecific ligations if the ligation mixture is kept at room temperature for a long period of time at 4 °C after the ligation step. We strongly recommend to proceed immediately to the PCR amplification, or at least to freeze the ligation product at −20 °C.
12. Caution should be taken when multiple samples are analyzed on the same sequencing flow-cell. When highly positive, few positive reads can be erroneously attributed to other samples due to the index hopping. Here again, the analysis of the sequences of the UMI can be important to avoid false positive results.
13. NGS is, by far, the most sensitive sequencing method to detect fusion transcripts by LD-RTPCR. By allowing the characterization of individual PCR amplification products, fusion transcripts expressed at very low levels or from highly degraded RNA samples can be detected. However, this sensitivity comes with some disadvantages regarding the specificity. For example, when the quality of the RNA is poor, specific amplifications can be difficult to differentiate from the background. In our experience, this background is not random but varies according to the pools of LD-RTPCR probes that are used. It should thus be evaluated for each new assay. The usage of UMI molecular marker can be essential in this setting, allowing an accurate evaluation of the number of detected cDNA sequences.

Acknowledgments

This work was supported by "Tom Pouce, Pousse la Moelle" and "La Ligue Contre le Cancer (Comité Seine Maritime)" associations. We thank our colleagues, biologists and pathologists, for their support during the development of this method. We also thank the Biological Resource Center (CRB) of the Centre Henri Becquerel for tumor sample banking and nucleic acid extractions.

Competing Interest Statement Vinciane Rainville, Marie-Delphine Lanic, Fanny Drieux and Philippe Ruminy are named inventor on pending patents describing a method for the detection of fusion transcripts by LD-RTPCR. Other authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Mertens F, Johansson B, Fioretos T, Mitelman F (2015) The emerging complexity of gene fusions in cancer. Nat Rev Cancer 15:371–381. https://doi.org/10.1038/nrc3947
2. Delattre O, Zucman J, Plougastel B et al (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 359:162–165. https://doi.org/10.1038/359162a0
3. Sorokin M, Rabushko E, Rozenberg JM et al (2022) Clinically relevant fusion oncogenes: detection and practical implications. Ther Adv Med Oncol 14:17588359221144108. https://doi.org/10.1177/17588359221144108
4. Soda M, Choi YL, Enomoto M et al (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 448:561–566. https://doi.org/10.1038/nature05945
5. Ruminy P, Marchand V, Buchbinder N et al (2016) Multiplexed targeted sequencing of recurrent fusion genes in acute leukaemia. Leukemia 30:757–760. https://doi.org/10.1038/leu.2015.177
6. Piton N, Ruminy P, Gravet C et al (2018) Ligation-dependent RT-PCR: a new specific and low-cost technique to detect ALK, ROS, and RET rearrangements in lung adenocarcinoma. Lab Investig J Tech Methods Pathol 98:371–379. https://doi.org/10.1038/labinvest.2017.124
7. Mareschal S, Palau A, Lindberg J et al (2021) Challenging conventional karyotyping by next-generation karyotyping in 281 intensively treated patients with AML. Blood Adv 5:1003–1016. https://doi.org/10.1182/bloodadvances.2020002517
8. Drieux F, Ruminy P, Sater V et al (2021) Detection of gene fusion transcripts in peripheral T-cell lymphoma using a multiplexed targeted sequencing assay. J Mol Diagn JMD 23:929–940. https://doi.org/10.1016/j.jmoldx.2021.04.013
9. Lanic M-D, Le Loarer F, Rainville V et al (2022) Detection of sarcoma fusions by a next-generation sequencing based-ligation-dependent multiplex RT-PCR assay. Mod Pathol Off J U S Can Acad Pathol Inc 35:649–663. https://doi.org/10.1038/s41379-021-00980-x
10. Lanic M-D, Guérin R, Wassef M et al (2023) Detection of salivary gland and sinonasal fusions by a next-generation sequencing based, ligation-dependent, multiplex RT-PCR assay. Histopathology 83:685–699. https://doi.org/10.1111/his.14971

Chapter 4

Isolation of a Specific Transcript Using Biotinylated Antisense Probes

Antoine Praité and Laurent Delpy

Abstract

RNA molecules play a central role in the regulation of gene expression, interacting with proteins and undergoing chemical modifications that affect their stability, location, and function. Studying these processes at the level of individual transcripts requires methods that allow selective isolation of RNA with minimal bias. While global RNA enrichment techniques provide a broad overview of the transcriptome, they often lack the resolution required to dissect transcript-specific interactions and modifications. Here we describe a hybridization-based protocol that uses biotinylated antisense DNA probes for selective transcript isolation. This approach can be used as a targeted strategy to enrich for specific RNA molecules, serve as a basis for studies of transcript-associated RNA-binding proteins (RBPome) or RNA modifications, and provide a refined perspective on post-transcriptional regulation. The protocol is outlined in detail, with a focus on the critical steps of probe design, hybridization, and purification, to ensure the highest degree of specificity and reproducibility in RNA capture experiments.

Key words Transcript isolation, RNA pull-down, Biotinylated probe, Transcript enrichment, RNA capture

1 Introduction

In order to understand RNA biology, it is necessary to employ precise methods to study transcript-specific interactions, stability, modifications, and associated protein complexes. Traditionally, transcriptome-wide approaches such as poly(A) selection or ribosomal RNA depletion have been used, but these approaches frequently introduce biases and are not designed to capture specific transcript populations [1]. In contrast, hybridization-based RNA capture techniques permit the targeted isolation of transcripts for downstream analyses, including RNA modification profiling and RNA–protein interaction studies [2].

RNA-binding proteins (RBPs) are key regulators of RNA metabolism, controlling processes such as splicing, stability, translation, and decay. Several approaches, including orthogonal organic

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_4,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

phase separation (OOPS) and phenol-toluol extraction, have been developed to isolate the global RBPome [3, 4]. While these methods provide valuable insights into RNA–protein interactions at a transcriptome-wide scale, they do not distinguish which RBPs associate with specific RNAs. However, a targeted RNA isolation step after OOPS or phenol-toluol extraction enables the characterization of the RBPome specific to a given transcript, offering a more refined view of post-transcriptional regulation. This RNA capture approach, using a biotinylated antisense DNA probe, has been successfully applied to several RNA species in order to identify their interacting RBPs [2, 5].

Beyond the field of RNA–protein interactions, transcript-specific isolation is also valuable for the study of RNA modifications, which influence transcript stability and RBP binding. N6-methyladenosine (m6A) is one of the most prevalent mRNA modifications, regulating translation efficiency, degradation, and alternative polyadenylation [6]. However, it is notable that other RNA modifications, including 5-methylcytosine (m^5C) and pseudouridine (Ψ), also play crucial roles in transcript stability and translation by modulating RNA structure and interactions with RBPs [6]. In this context, mass spectrometry-based approaches have recently emerged as a powerful pipeline for identifying functionally relevant RNA modifications, enabling direct detection of modified nucleotides without requiring antibody-based enrichment (*see* Chapter 14) [7]. The isolation of a specific RNA before mass spectrometry or targeted sequencing allows for a direct assessment of RNA modifications and their functional consequences at the individual transcript level.

The RNA capture approach has been demonstrated to be particularly useful for enriching low-abundance transcripts, which may be difficult to detect in bulk RNA-seq approaches [8]. In addition to rare transcripts, this method is well suited for isolating primary transcripts, thus enabling the study of co-transcriptional regulatory events often too diluted in total RNA populations.

Here, we present a robust and cost-effective method for isolating specific RNA transcripts using biotinylated antisense DNA probes (Fig. 1). This approach facilitates efficient transcript capture without necessitating amplification or extensive sample preparation, rendering it suitable for applications such as RBPome characterization after UV crosslinking, epitranscriptomic modification analysis, and post-transcriptional regulation studies. Through optimizing probe design, hybridization conditions, and capture efficiency, this protocol provides a reproducible method for targeted RNA enrichment, thereby facilitating in-depth functional and structural analyses of specific transcripts.

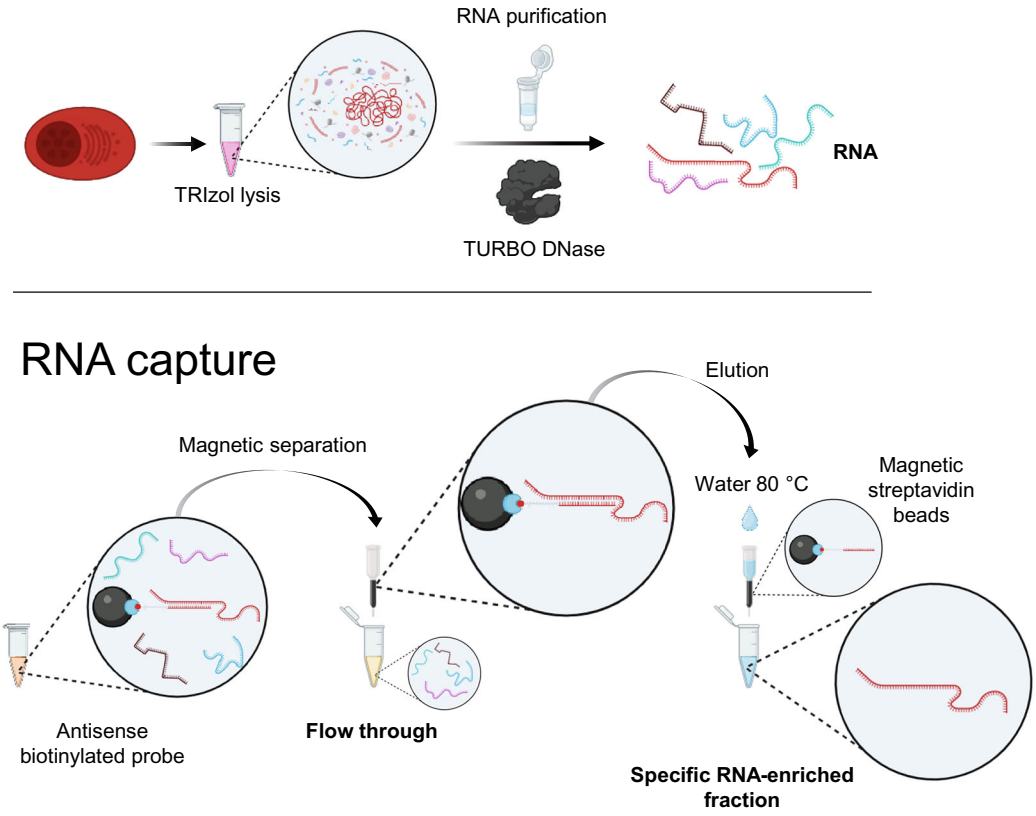


Fig. 1 Method for isolating specific RNA transcripts using biotinylated antisense DNA probes. The following workflow delineates the protocol for the RNA capture method, from the extraction of RNA from cells to the harvesting of the fraction enriched in RNA of interest. The antisense DNA probe is coupled to a spacer (TEG: triethylene glycol) and a biotin molecule (red dot) at its 3′ end. This probe binds to a magnetic bead which is coupled to streptavidin (black bead)

2 Materials

2.1 Antisense DNA Probe

1. Transcript-specific RNase-free HPLC purified antisense DNA probe 3′ modified by a triethylene glycol (TEG) biotin (TS probe) (Integrated DNA Technologies) (*see* **Note 1**). Store 100 μM stock solution and 10 μM aliquots (to minimize freeze-thaw cycles) at −20 °C (*see* **steps 7** and **8** in Subheading 3.1).
2. Irrelevant RNase-free HPLC-purified antisense DNA probe 3′ modified by a triethylene glycol (TEG) biotin (IR probe) (Integrated DNA Technologies) (*see* **Note 2**). Store 100 μM stock solution and 10 μM aliquots (to minimize freeze-thaw cycles) at −20 °C (*see* **steps 7** and **8** in Subheading 3.1).

3. IDTE buffer pH 8.0 (Integrated DNA Technologies). Store at room temperature or in aliquots at −20 °C (*see* **Note 3**).
4. Nuclease-free water.
5. Microcentrifuge.

2.2 RNA Extraction and Purification

1. 1× phosphate-buffered saline (PBS), sterile. Store at 4 °C.
2. 0.5 mL, 1.5 mL, and 5 mL RNase-free tubes.
3. Filtered pipette tips.
4. RNase decontamination reagent, such as RNase AWAY™.
5. TRIzol reagent (Ambion). Store at 4 °C.
6. Absolute ethanol molecular biology grade. Store at room temperature or at 4 °C.
7. Direct-zol™ RNA Microprep (Zymo Research).
8. DNA LoBind 1.5 mL tubes (Eppendorf).
9. Ice and ice bucket.
10. Cooled microcentrifuge.

2.3 DNA Digestion and RNA Isolation

1. TURBO DNA-free™ kit (Invitrogen). Store at −20 °C.
2. Hybridization buffer: IDTE buffer supplemented with 100 mM NaCl (*see* **Note 4**). Store at room temperature or in aliquots at −20 °C.
3. Wash buffer: IDTE buffer supplemented with 50 mM NaCl. Store at room temperature or in aliquots at −20 °C.
4. μMACS streptavidin kit, μMACS separator, and MACS multi-stand (Miltenyi Biotec).
5. Thermomixer.
6. Nanodrop (Thermo Scientific) or Qubit 4 fluorometer with RNA extended range assay kit (Invitrogen).

3 Methods

3.1 Design and Preparation of DNA Probes

1. Copy the reference sequence (RefSeq) into an online RNA secondary structure prediction tool. Identify highly structured regions that interfere with antisense probe hybridization.
2. Copy the sequence into the free online PrimerQuest Tool (https://eu.idtdna.com/pages/tools/primerquest) and select "show custom design parameters", and then assign at least the parameters shown in Table 1 (*see* **Note 5**).
3. Select oligonucleotides outside highly structured regions. Use BLAST to verify the probe's specificity and ensure no off-target

Table 1
Custom design parameters for antisense transcript-specific (TS) DNA probes

Set design parameters for…			
Reverse only—Sequencing			
Reaction conditions			
Monovalent Salt (Na+) (mM)	50		
Primer DNA Conc (nM)	333		
Primer details	**Min**	**Opt**	**Max**
Primer Tm (°C)	65	70	75
Primer GC (%)	40	50	65
Primer size (nt)	25	30	45

binding. Order the best sequence by adding the 3′ TEG-biotin modification (*see* **Note 6**).

4. As a control, design an IR probe such as 5′-GAGACCTTT GACCGCTTCGTGAAAGGG/3BioTEG/ (*see* **Note 2**).
5. Define the probe's melting temperature (T_m) using OligoAnalizerTool (https://eu.idtdna.com/pages/tools/oligoanalyzer). Use the same parameters (oligo and Na^+ concentrations) as on PrimerQuest Tool (Table 1). It is important to specify RNA as the target type.
6. Select an annealing temperature (T_a) 5 °C below the T_m (*see* **Note 7**).
7. All subsequent steps should be carried out in an RNase-free environment. Wear gloves and avoid contact with skin or hair, which can introduce nucleases. Begin by cleaning the workstation with an RNase decontamination solution. Use filtered pipette tips.
8. Briefly centrifuge TS probe and IR probe tubes and resuspend them at 100 μM in nuclease-free water. Vortex gently and invert the tubes a few times to mix. Allow probes to hydrate for at least 10 min on ice.
9. Dilute the 100 μM probe solution to 10 μM in IDTE buffer and store multiple aliquots at −20 °C.

3.2 RNA Extraction and Purification

All subsequent steps should be carried out in a fume hood in an RNase-free environment. Wear gloves and avoid contact with skin or hair, which can introduce nucleases. Begin by cleaning the workstation with an RNase decontamination solution and use filtered pipette tips. The procedure is described for cultured cells.

1. Centrifuge 10^6 cells in a 1.5 mL RNase-free tube at 300 × *g* for 5 min at 4 °C (*see* **Note 8**).
2. Wash the pellet in 1 mL of cold PBS. Centrifuge at 300 × *g* for 5 min at 4 °C.
3. Remove the supernatant and resuspend the pellet in 350 µL of TRIzol. Pipette vigorously and gently vortex until the solution is no longer viscous. Incubate for 5 min at room temperature. At this point, the samples in TRIzol can be stored at −80 °C for long-term preservation.
4. In parallel, allow the centrifuge to reach room temperature before the next steps.
5. Add 350 µL of absolute ethanol. Shake the tubes vigorously for 1 min; do not vortex.
6. Transfer the entire volume to the Direct-zol™ RNA Microprep purification column, following the manufacturer's instructions. At the final step, to recover the total RNA, perform a two-step elution using 60 °C nuclease-free water, yielding a total volume of 55 µL (*see* **Note 9**). Ensure the water makes direct contact with the column filter, and then wait for 1 min before centrifuging. Immediately place the LoBind tubes containing the RNA on ice. At this point, RNA can be stored at −80 °C.

3.3 DNA Digestion

1. Thaw the 10× turbo DNase buffer at 4 °C and then vortex it.
2. Add 5 µl of 10× TURBO DNase Buffer and 1 µL of TURBO DNase enzyme (included in the TURBO DNA-free™ kit) to RNA samples (*see* **Note 10**) and mix gently.
3. Incubate in a thermomixer for 30 min at 37 °C at 300 rpm, protected from light. During the incubation, thaw the DNase inactivation reagent (included in the TURBO DNA-free™ kit) at 4 °C.
4. Vortex DNase inactivation reagent, and then add 5 µL to the tube and mix by pipetting.
5. Incubate for 5 min at room temperature. During incubation, vortex the tube very gently at least 3 times to prevent DNase inactivation reagent sedimentation.
6. Centrifuge tubes at 10,000 × *g* for 90 s. The DNase inactivation reagent forms a pellet that is not very stable; take care not to disturb it. Otherwise, repeat this step.
7. Carefully transfer the supernatant into a new LoBind tube and keep it on ice (*see* **Note 11**).
8. Measuring the quantity of RNA using NanoDrop or Qubit 4 fluorometer. At this point, RNA can be stored at –80 °C.

3.4 RNA Capture Using Antisense Probes

All subsequent steps should be carried out in an RNase-free environment. Wear gloves and avoid contact with skin or hair, which can introduce nucleases. Begin by cleaning the workstation with an RNase decontamination solution and use filtered pipette tips. We recommend working in parallel with two Thermomixers to reduce the time needed to dispense the different solutions, working as closely as possible to the Thermomixers. Each step is carried out in parallel for the TS probe and the IR probe.

1. In a LoBind tube, add 1 μg of RNA to a final volume of 150 μL of nuclease-free water (*see* **Note 12**).
2. Add 150 μL of hybridization buffer and put tubes in a thermomixer. Heat to 75 °C and incubate for 5 min at 300 rpm.
3. Gradually lower the temperature to the T_a. Once at T_a, add 10 μL of TS probe or IR probe.
4. Incubate for 5 min at T_a, 300 rpm. In parallel, put hybridization buffer at T_a.
5. During the incubation time, set up the μMACS separator and the MACS multistand and place μ columns (included in the μMACS streptavidin kit) with collection tubes underneath.
6. Add 100 μL of equilibration buffer for nucleic acid applications (included in the μMACS streptavidin kit) to the μ column, and then wash twice with 200 μL of T_a-preheated hybridization buffer. Take extra care to avoid air bubbles that could disrupt column flow.
7. At the end of the 5-min incubation period (**step 4** in Subheading 3.4), add 100 μL of streptavidin microbeads in tubes. Mix by pipetting and incubate for 2 min at T_a, 300 rpm.
8. Transfer the entire volume to the column and let the solution pass through (*see* **Note 13**).
9. In a thermomixer, heat the wash buffer at 45 °C (*see* **Note 14**).
10. Wash the column 5 times with 200 μL of warm wash buffer. Between each wash, let the tube in the thermomixer at 45 °C.
11. Heat nuclease-free water at 80 °C.
12. Place a LoBind tube under μ columns.
13. Elute by adding 150 μL of 80 °C nuclease-free water to the column (*see* **Note 15**).
14. Place the solution directly on ice. Keep a fraction for enrichment analysis by RT-qPCR (Fig. 2 and *see* **Note 16**). The enriched RNA species is ready for further analysis.

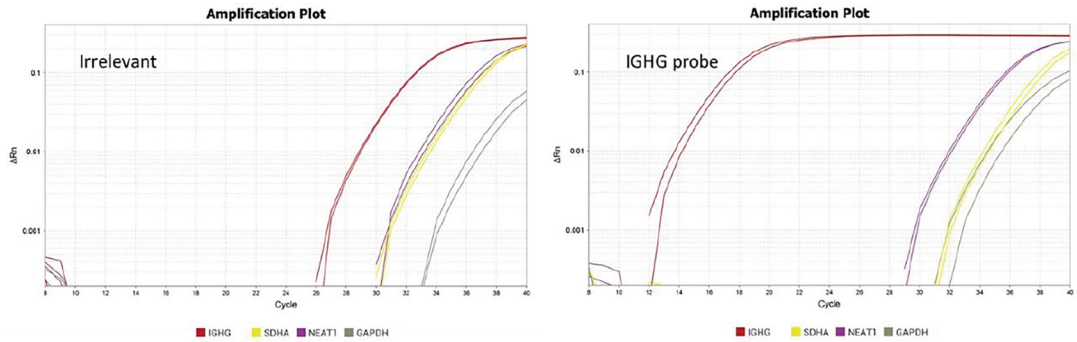


Fig. 2 Assessment of RNA enrichment using antisense biotinylated DNA probe by RT-qPCR. Using RNA extracted from IgG-expressing LP1 myeloma cells, the RNA capture method is applied by specifically targeting *IGHG* (immunoglobulin heavy chain G) transcripts. Other IgG-expressing B lymphoid cell lines (e.g., SUDHL4 and XG6) or even IgG+ primary B cells and plasma cells can be used. Amplification plots showing *IGHG* amplification curves (red) obtained from elution fractions using **a** an irrelevant probe (5′- GAGACCTTT GACCGCTTCGTGAAAGGG/3BioTEG/) or **b** a consensus probe (IGHG probe: 5′-GCTTTGTCTTGGCATTATGCACCTC CAC/3BioTEG/) targeting the RNAs of all IGHG isotypes (i.e., *IGHG1*, *IGHG2*, *IGHG3*, and *IGHG4*). Expression of several endogenous controls is shown as yellow, purple, and grey curves

4 Notes

1. Triethylene glycol is used as a spacer between the nucleotide sequence and biotin to reduce steric hindrance and optimize probe hybridization. The spacer-biotin can also be placed at the 5′-end of the probe. Basically, we recommend designing DNA probes, as they are less expensive. However, DNA/LNA mixmers or 2′-O-methyl probes may improve hybridization efficiency but increase costs.
2. Either a sense, an irrelevant, or a scrambled probe can be used as a negative control. This control probe must be modified in the same way as the transcript-specific probe.
3. IDTE buffer is 0.2 μm filtered: try to keep it as clean as possible.
4. We recommend using commercial RNase-free 5 M NaCl solution to prepare hybridization buffer. Alternatively, prepare a 5 M NaCl solution in nuclease-free water and autoclave it.
5. Other parameters, such as the location of the probe on the transcript, can be added to the design.
6. The rest of the protocol can be carried out using several different probes to compare their effectiveness.
7. T_a is a crucial parameter in the experiment and may be adjusted. A higher T_a reduces the risk of nonspecific hybridization but can drastically reduce yield.

8. If 10^6 cells are not sufficient to obtain a sufficient quantity of RNA, then scale up the number of cells and the volume of TRIzol used according to the manufacturer's instructions. However, if the number of cells exceeds 10^6, then another type of column with a higher loading capacity should be used, or the sample should be separated to load several columns.
9. A small volume of the eluent always remains trapped in the column filter. For instance, eluting with 55 µL results in 50 µL of RNA in the tube.
10. If large amounts of RNA are used, then follow the manufacturer's instructions to adapt the volumes.
11. Be very careful not to pipette DNase inactivation reagent as this could inhibit certain subsequent enzymatic reactions. If some of the compound is pipetted out, return the entire volume to the tube and centrifuge again.
12. The amount of RNA depends on the abundance of the transcript in the model being studied. Use 1 µg of starting material to set up the experiment. Optionally, perform a ribodepletion of the total RNA. In this case, the quantity of starting RNA may be less than 1 µg.
13. Optional: keep the first elution fraction (flow-through) in a LoBind tube to quantify by RT-qPCR the amount of specific transcript retained in the column with the TS probe compared to the IR probe.
14. Temperature and salt concentration of the wash buffer are crucial parameters and may be adjusted. For an initial experiment, apply the conditions given in this protocol [9].
15. Optional: to obtain a more concentrated eluate, do not collect the first drop.
16. We recommend using a high-performance reverse transcriptase for this analysis, such as SuperScript IV or Induro.

Acknowledgments

This work was supported by grants from INCa (PLBIO2022-110, INCA_16734), ANR (2017-CE15-0024-01), Ligue Contre le Cancer (comités Corrèze, Haute-Vienne), Fondation Française pour la Recherche contre le Myélome et les Gammapathies monoclonales (FFRMG), and Région Nouvelle-Aquitaine (AAP ESR 2020 and 2024). AP is funded by the Ministry of Higher Education, Research and Innovation (MESRI).

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Viscardi MJ, Arribere JA (2022) Poly (a) selection introduces bias and undue noise in direct RNA-sequencing. BMC Genomics 23: 530. https://doi.org/10.1186/s12864-022-08762-8
2. Balzarini S, Van Ende R, Voet A, Geuten K (2023) A widely applicable and cost-effective method for specific RNA–protein complex isolation. Sci Rep 13:6898. https://doi.org/10.1038/s41598-023-34157-0
3. Villanueva E, Smith T, Queiroz RML et al (2020) Efficient recovery of the RNA-bound proteome and protein-bound transcriptome using phase separation (OOPS). Nat Protoc 15:2568–2588. https://doi.org/10.1038/s41596-020-0344-2
4. Urdaneta EC, Vieira-Vieira CH, Hick T et al (2019) Purification of cross-linked RNA-protein complexes by phenol-toluol extraction. Nat Commun 10:990. https://doi.org/10.1038/s41467-019-08942-3
5. Wang Y, Zhang S, Kang N et al (2024) Progressive polyadenylation and m6A modification of Ighg1 mRNA maintain IgG1 antibody homeostasis in antibody-secreting cells. Immunity 57:2547–2564.e12. https://doi.org/10.1016/j.immuni.2024.10.004
6. Gilbert WV, Nachtergaele S (2023) mRNA regulation by RNA modifications. Annu Rev Biochem 92:175–198. https://doi.org/10.1146/annurev-biochem-052521-035949
7. Amalric A, Attina A, Bastide A et al (2024) Mass spectrometry-based pipeline for identifying RNA modifications involved in a functional process: application to cancer cell adaptation. Anal Chem 96:1825–1833. https://doi.org/10.1021/acs.analchem.3c02635
8. Wang F, Xu Y, Wang R et al (2023) TEQUILA-seq: a versatile and low-cost method for targeted long-read RNA sequencing. Nat Commun 14: 4760. https://doi.org/10.1038/s41467-023-40083-6
9. Matia-González AM, Iadevaia V, Gerber AP (2017) A versatile tandem RNA isolation procedure to capture in vivo formed mRNA-protein complexes. Methods 118–119:93–100. https://doi.org/10.1016/j.ymeth.2016.10.005

Part II

High-Throughput Sequencing Methods

Chapter 5

Transcriptome-Wide Characterization of Protein:RNA Interactions with UV-Crosslinking Immunoprecipitation and Sequencing

Manuel D. Diaz-Muñoz and Ines C. Osma-Garcia

Abstract

The expansion of the cell genetic code relies on post-transcriptional regulatory mechanisms that shape the expression and translation of different mRNA transcripts into functional proteins. RNA-binding proteins (RBPs) control the splicing, editing, stability, and translation of mRNAs in a timely manner, and constitute an important layer of regulation that is often underestimated in genomic studies. Here we described individual nucleotide resolution UV-crosslinking immunoprecipitation and sequencing (iCLIP), a method that allows precise in vivo annotation of RBP–RNA interactions for identifying not only mRNA targets but also the mechanisms of action by which a given RBP controls the expression of single genes or gene-operons regulating cellular functions.

Key words Crosslinking immunoprecipitation and sequencing, CLIP-seq, Protein:RNA interactions, RNA-binding proteins, RNA splicing, RNA editing, RNA translation

1 Introduction

RNA-binding proteins (RBPs) are essential actors in gene regulation, controlling from the synthesis and maturation of nascent messenger (m)RNAs to their translation into a highly diversify proteome. RBPs are a large group of proteins which includes common enzymes, such as GAPDH or ENO2, with "moonlighting functions" as RNA regulators [1]. RBPs usually contain, but not always, structural motifs, such as RNA recognition motifs (RRM), zinc finger, and dsRNA-binding domain, that recognize RNA regulatory elements (RRE) in a sequence-specific manner [2]. However, in vivo RBP binding to their mRNA targets is hardly predictable. On one hand, RBPs carrying different structural motifs can bind to the same RREs leading to different RNA regulatory outcomes [3]. Additionally, RBP-binding affinity and avidity is

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_5,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

often coupled to sensing of intracellular and external cues that signal the activation or inhibition of post-translational regulatory mechanisms controlling RBP abundance and function [4]. Finally, large mRNA molecules can accommodate dozens of RBPs that contribute to the three-dimensional complexity of ribonucleoprotein (RNP) particles, exposing or hiding RRE for other RBPs or microRNAs [5, 6]. Taken together, RNA target specificity and binding affinity can discreetly change both in a cell-type and context-dependent manner, highlighting the need of experimental approaches for in vivo characterization of RBP:RNA interactions.

RBP:RNA immunoprecipitation (RIP) methods make use of RBP-specific antibodies to identify single or multiple RNA targets, depending on downstream coupling with PCR or high-throughput quantification methods (RIP-CHIP or RIP-seq) [7–9]. Binding enrichment peaks and RNA regulatory motifs (RRMs) can be inferred from RIP-seq data. However, this method lacks resolution for single nucleotide-protein annotation that is often required to precisely uncover functional interactions regulating a given RNA target and can suffer from RBP:RNA re-association during sample processing [10]. Crosslinking immunoprecipitation and sequencing (CLIP-seq) (Fig. 1a) emerged as a major evolution of RIP introducing, first, the use of UV-light (254 nm in CLIP [11] or 365 nm in PAR-CLIP [12]) to specifically crosslink RBP–RNA interactions in living cells and tissues; second, the use of endonucleases to hydrolyze single-strand RNAs; third, the use of highly stringent buffers to remove unspecific binding, and, fourth, strategies for small RNA library generation for sequencing. Later, the realization that enzymatic removal of RBPs during the CLIP procedure leaves an amino acid/small peptide at the UV-crosslink site causing early termination in retro-transcription led to changes in cDNA library preparation for identification of truncated sites marking RBP:RNA physical interaction [13]. More recent advances improve safety by replacing radioactive detection of RBP:RNA immunoprecipitated with the use of infrared dyes, improve sample yield by replacing low efficient enzymatic reactions and sample recovery steps, and improve the unique assignment of RBP:RNA interactions to hundreds of single RNA molecules by using different barcoding strategies or the use of in situ reverse transcription for the investigation of dynamic RBP–RNA interactions [14–18]. The outline of the advantages, challenges, applications, and complementarity of the different CLIP methods as well as methods for analysis of such data have been previously reviewed [19]. Here, we describe in detail one of the latest versions of iCLIP methodology [20] and its application in the annotation of RBP:RNA-binding interactions in living lymphocytes [21–25].

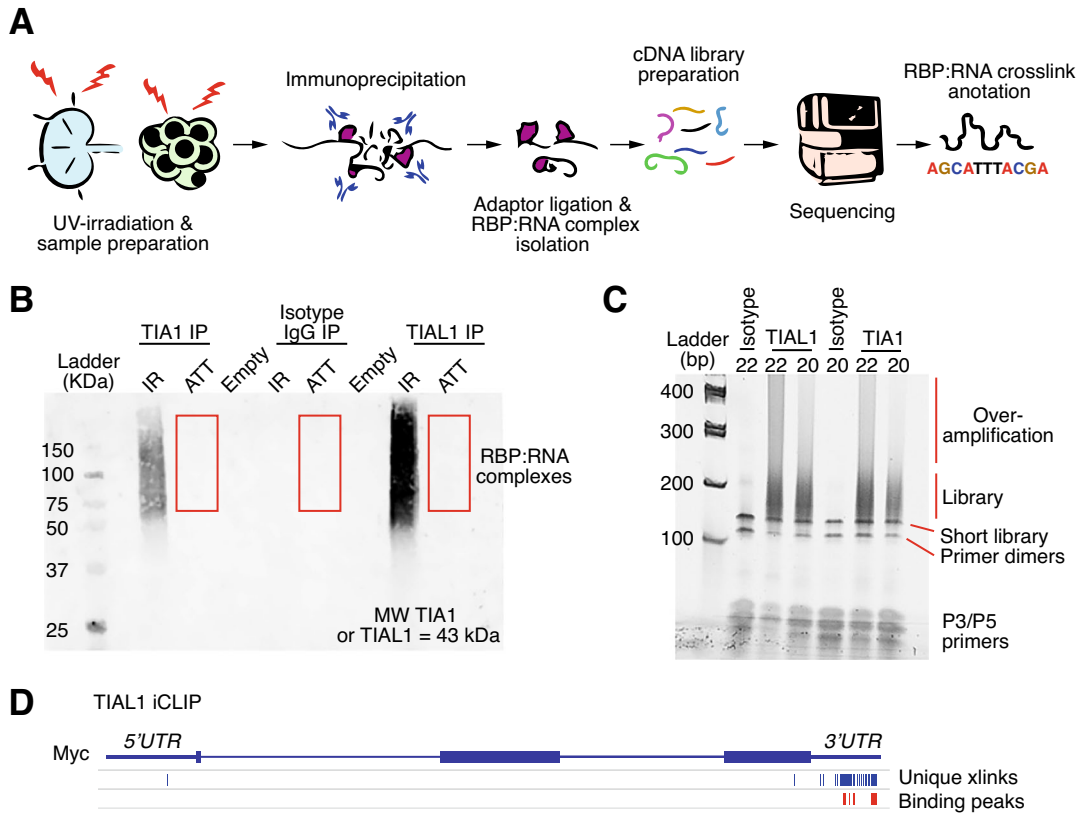


Fig. 1 Analysis of the RNA interactome of TIA1 and TIAL1 in progenitor B cells with iCLIP. **a** Summary of the UV-crosslinking and immunoprecipitation method. **b** Visualization of RBP:RNA complexes labeled with the L3-IR-App adaptor and immunoprecipitated using specific antibodies against TIA1, TIAL1, or an isotype IgG antibody as control. Red boxes mark the location of RNA complexes purified for subsequent cDNA library preparation. **c** Test to determine the number of PCR cycles for cDNA library amplification. **d** Annotation of unique crosslinking sites in the *Myc* transcript. Peak enrichment analysis showing the highest binding of TIAL1 in the 3′UTR is shown in red

2 Materials

Solutions are prepared with ultrapure DNAse/RNase-free water and stored at 4 °C. If not stated otherwise, all steps of the protocol are carried out on ice or at 4 °C.

2.1 Cell Preparation, Lysis, and Immunoprecipitation

1. Dutch-modified RPMI-1640 medium with 2–10% fetal cow serum (FCS), 100 U/mL penicillin, 10 µg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 µM β-mercaptoethanol.
2. 1× phosphate buffer saline (PBS).
3. Lysis buffer: 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1% IGEPAL CA-630, 0.1% SDS, 0.5% sodium deoxycholate. Add

protease inhibitor cocktail, diluted 1 in 200, and 10 units/mL of RNase inhibitor prior cell lysis (*see* **Notes 1** and **2**).

4. High-salt wash buffer: 50 mM Tris–HCl, pH 7.4, 1 M NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 0.1% SDS and 0.3% sodium deoxycholate (*see* **Note 3**).
5. PNK wash buffer: 20 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and 0.2% Tween-20.
6. 5× PNK buffer: 350 mM Tris–HCl, pH 6.5, 50 mM $MgCl_2$, and 5 mM dithiothreitol (DTT).
7. 10× ligation buffer: 500 mM Tris–HCl, pH 7.5, and 100 mM $MgCl_2$.
8. Magnetic beads couple with protein A, protein G, biotin, or other selective molecules for isolation of tagged-proteins.
9. 40 μm cell strainer.
10. 10-cm cell culture plate.
11. UV-crosslinker.
12. Ice and dry ice.
13. High-quality, knock-out validated, antibody against the desired RBP or protein tag.

2.2 RNA and Protein Digestion, Adaptor Ligation

1. RNA degradation mix: lysis buffer containing an amount of RNase I to be determined experimentally and 10 μL of TURBO™ DNase (2 U/mL).
2. Sonicator and thermomixer.
3. Nuclease-free water.
4. 3′ end RNA dephosphorylation mix: 8 μL of 5× PNK buffer, 1 μL of PNK at 10 U/mL, 0.5 μL of FastAP alkaline phosphatase at 1 U/μL, and 0.5 μL of RNase inhibitor at 40 U/mL in a final volume of 40 μL.
5. 3′ adaptor ligation mix: 3 μL of 10× ligation buffer, 0.8 μL of dimethyl sulfoxide (DMSO), 2.5 μL of T4 RNA ligase I at 30 U/μL, 11.25 μL of 40% polyethylene glycol 8000, 0.5 μL of PNK at 10 U/μL, 0.4 μL of RNase inhibitor at 40 U/μL, 0.1 μM of pre-adenylated adaptor L3-IR-App: /5Phos/AG ATC GGA AGA GCG GTT CAG AAA AAA AAA AAA / iAzideN/AA AAA AAA AAA A/3Bio/ or 0.1 μM of pre-adenylated adaptor L3-NNN-App: /5Phos/WN NNN AGA TCG GAA GAG CGG TTC AG/3Bio/ in a final volume of 25 μL [W = A or T; N = any nucleotide; Bio = biotin molecule, iAzide = internal azide molecule].
6. Adaptor removal mix: 2 μL of 10× NEB buffer 2, 0.5 μL of 5′ deadenylase at 50 U/μL, 0.5 μL of RecJf endonuclease at

30 U/μL, 0.5 μL of RNase inhibitor at 40 U/μL, and 5 μL of 40% polyethylene glycol 8000 in a volume of 20 μL.

7. 5′ DNA adenylation kit (New England Biolabs): 10× 5′ DNA adenylation reaction buffer, 1 mM ATP, Mth RNA ligase.
8. IRdye-800CW-DBCO (LICORbio™).
9. 3 M sodium acetate, pH 5.5 and 5 M sodium chloride.
10. SUPERase•In™ RNase inhibitor.
11. PNI buffer and QiaQuick nucleotide removal kit (Qiagen).

2.3 RBP:RNA Complex Isolation and RNA Recovery

1. Gel electrophoresis using 4–12% Novex NuPAGE Bis-Tris polyacrylamide gels, 4× NuPAGE loading buffer with 100 mM DTT, pre-stained protein ladder, and 1× MOPS running buffer.
2. Gel transfer into nitrocellulose membranes (Protran® BA85, Amersham) using 3 M Whatman paper and 1× Tris–glycine transfer buffer containing 10% methanol.
3. Gel electrophoresis and membrane transfer system (Novex™, Invitrogen). Electrophoresis power supply. Imaging system detecting near-infrared fluorescence.
4. Proteinase K buffer: 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, and 0.2% SDS.
5. Protein degradation mix: 10 μL of DNAse- and RNAse-free Proteinase K at 50 U/μL and 190 μL of Proteinase K buffer.
6. Phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA.
7. Phase Lock Gel Heavy tube.
8. Chloroform.
9. Isopropanol.
10. Glycoblue.
11. Ethanol 100%, 85%, and 80%.

2.4 Reverse Transcription, cDNA Isolation, and Library Preparation

1. Reverse transcription mix: 0.25 μL of Superscript IV reverse transcriptase at 200 U/μL, 0.5 μL of 10 mM dNTP mix, 0.25 μL of RNase inhibitor at 40 U/μL, 0.5 μL of 0.1 M DTT, and 2 μL of 5× Superscript IV buffer.
2. 1 pmol/μL of iCLIP_ddRT primer: /5Phos/WWW/5 known nucleotides/NNNNAGATC GGAAGAGCGTCGTGAT/iSp18/GGATCC/iSp18/TACTGAACCGC. Known nucleotides allow library sample multiplexing during sequencing [W = A or T; N = any nucleotide; iSP18 = 18-atom hexaethylene glycol spacer].
3. Agencourt AMPure XP beads.

4. cDNA circularization mix: 0.75 μL of CircLigase II at 100 U/μL, 0.75 μL of 50 mM $MnCl_2$, 3 μL of 5 M betaine, and 1.5 μL of 10× CircLigase Buffer II. Final reaction volume of 10 μL.
5. cDNA library preparation mix: 10 μL of 2× Phusion HF Master mix and 1 μL of 10 μM P3/P5 primer mix (0.5 μL of 10 μM P3 primer: CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT, and 0.5 μL of 10 μM P5 primer: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT).
6. 6% TBE acrylamide gel, 6× TBE loading buffer, 1× TBE electrophoresis buffer, 25 bp DNA ladder, SYBR safe.
7. Crush-Soak Gel buffer: 500 mM NaCl, 1 mM EDTA, and 0.05% SDS.
8. Costar SpinX columns and 1 cm glass pre-filters.
9. 3 M sodium acetate, pH 5.5.
10. TE buffer.
11. 5 M NaCl.
12. 1 M NaOH.
13. 1 M HCl.
14. Thermocycler.
15. BioAnalyzer.

3 Methods

3.1 Sample Collection, UV-Crosslinking, and Cell Lysis

1. Lymphoid organs are extracted and, rapidly, placed on ice. They are smashed using a 40-μm cell strainer and a syringe plunger in a 10-cm cell culture plate containing FCS-supplemented RPMI culture medium with RNase inhibitors (*see* **Note 4**). If primary lymphocytes have been cultured in vitro, transfer cell culture plates on ice for 2–3 min.
2. Plates without the lid are placed inside the UV-crosslinker (*see* **Note 5**) and UV-irradiated in cycles of irradiation (150 mJ) followed by 1 min incubation on ice (*see* **Note 6**).
3. After irradiation, lymphocytes are isolated following standard procedures (e.g., cell separation using magnetic beads or fluorescence-activated cell sorting). After isolation, cells are washed once with ice-cold PBS and resuspended in PBS at a density of $2–3 \times 10^7$ cells/mL. 1 mL is transferred into 1.5 mL centrifuge tubes and centrifugated at $400 \times g$ for 10 min at 4 °C. Remove supernatant and snap-freeze the cell pellet using dry ice. Samples can be stored at −80 °C for several months (*see* **Note 7**).

3.2 Pre-adenylation of L3-IR Adaptor and Conjugation of Infrared Dye

1. Using a 5′ DNA adenylation Kit, a reaction mix is prepared by adding 75 μL of 10× 5′DNA adenylation reaction buffer, 75 μL of 1 mM ATP, 100 μL of Mth RNA ligase, and 300 μL of water to 20 nmoles of L3-IR-phos or L3-NNN-phos adaptors previously resuspended in 200 μL of water.
2. Incubate the mix at 65 °C for 2 h on a thermomixer at 1100 rpm. Then, heat inactivate the mix at 85 °C for 10 min.
3. Precipitate the adaptor by adding 80 μL of 3 M sodium acetate (pH 5.5) and 1 mL of 100% ethanol. Split the sample into two tubes if precipitation and IR-dye labeling are done in 1.5 mL tubes. Leave at −20 °C for 1–18 h. Spin at 21,800 × *g* for 30 min at 4 °C. Remove supernatant and wash the pellet with 500 μL of 80% ethanol.
4. Resuspend pre-adenylated L3-NNN-App adaptor in water to the desired concentration.
5. For the L3-IR-App adaptor, resuspend pellet into 360 μL of PBS and add 40 μL of IRdye-800CW-DBCO (10 mM). Leave at 37 °C for 2 h, and then add 9.6 mL of PNI buffer.
6. Load 40 columns from a QIAquick Nucleotide Removal Kit with 250 μL of the 10 mL mix (*see* **step 5** in Subheading 3.2). Spin for 1 min at 4629 × *g*. Decant the flow-through and wash the column with 750 μL of 80% ethanol. Spin for 1 min at 21,800 × *g*. Remove flow-through and spin again for 2 min to remove any residual ethanol. Transfer columns to new 1.5 mL tubes and elute the adaptor by adding 50 μL of water. Incubate 2 min and spin for 1 min at 21,800 × *g*.
7. Pool samples and quantify the adenylated L3-IR-App adaptor. Dilute L3-IR-App for a final concentration of 1 μM. Aliquot and store at −20 °C (*see* **Note 8**).

3.3 Cell Lysis, Immunoprecipitation, and Adaptor Ligation

1. Place 40 μL of protein A or G magnetic beads on a magnetic stand. Remove buffer and wash beads with 1 mL of iCLIP lysis buffer. Place beads on the magnet, remove buffer, and resuspend in 500 μL of iCLIP lysis buffer containing 2 μg of antibody (*see* **Note 9**). Keep antibody-beads rotating at room temperature for 1 h.
2. Place a frozen cell pellet on ice until thawed. Add 1 mL of iCLIP lysis buffer containing protease inhibitors and 10 U/mL RNase inhibitors. Sonicate avoiding foaming with 10 s bursts at five decibels (three times each sample). Keep samples on ice during sonication.
3. Spin at 15,871 × *g* for 10 min at 4 °C and transfer supernatant into a new 1.5 mL tube.
4. Add RNA degradation mix containing 20 U of TURBO™ DNase. For control samples treated with a high dose of

RNase I, add an RNA degradation mix containing 12 U of RNase I. For samples to be treated with a low dose of RNase I for later RNA isolation and library construction, add an RNA degradation mix containing 0.025 U of RNase I (*see* **Note 10**).

5. Incubate samples at 37 °C for 3 min shaking in a thermomixer at 1100 rpm (*see* **Note 11**).
6. [Optional] Add 40 U of SUPERase•In™ RNase inhibitor to block RNase I activity.
7. Incubate samples on ice for 3 min and then centrifuge them at 15,871 × *g* for 10 min at 4 °C. Transfer supernatant into a new tube.
8. After antibody coupling, place magnetic beads on a magnetic stand and remove the buffer. Add antibody-coupled beads to the RNase-treated samples. Keep samples rotating for 1–18 h at 4 °C (*see* **Note 12**).
9. Place samples on a magnetic stand and remove supernatant. Wash beads twice with iCLIP high-salt wash buffer. Then, wash once with iCLIP PNK wash buffer (*see* **Note 13**).
10. Resuspend beads with 40 μL of 3′ end RNA dephosphorylation mix. Incubate at 37 °C in thermomixer at 1100 rpm for 40 min.
11. Place beads on a magnetic stand and remove supernatant. Wash beads once with 1× ligation buffer. Divide sample in two, keeping one-fifth for RNA labeling with the pre-adenylated L3-IR-App adaptor and the rest to be labeled with a pre-adenylated L3-NNN-App adaptor (*see* **Note 14**). Remove supernatant by placing the samples on a magnetic stand and resuspend beads into 25 μL of 3′ adaptor ligation mix. Incubate 75 min at room temperature. Flick the tubes every 10 min to avoid magnetic bead sedimentation.
12. Place on a magnetic stand to remove the supernatant and wash the beads twice with iCLIP high-salt wash buffer. Then, wash the beads once more using iCLIP PNK wash buffer (*see* **Note 15**).
13. Remove buffer and add 20 μL of the adaptor removal mix to the beads. Incubate at 30 °C for 1 h, and then at 37 °C for 30 min. Keep samples in the thermomixer shaking at 1100 rpm.
14. Place samples on the magnetic stand and wash beads twice with the iCLIP high-salt wash buffer and once with the iCLIP PNK wash buffer. Remove well the supernatant and add 15 μL of 1× NuPAGE loading buffer with DTT, preheated previously at 70 °C. Keep samples shaking at 70 °C for 3 min.
15. Place tubes on a magnetic stand and load the samples into a 4–12% NuPAGE Bis-Tris acrylamide gel (*see* **Note 16**).

3.4 Electrophoresis and RNA Isolation

1. Run the gel for 65 min at 180 V or until dye front has ran to the bottom of gel.
2. Transfer RBP:RNA complexes into a Protan BA85 Nitrocellulose Membrane using a Novex wet transfer system for 2 h at 30 V.
3. After transfer, rinse the membrane with PBS, wrap it with cling film, and place it on a tray for analysis using an imaging system detecting near-infrared fluorescence. Save a picture of the whole membrane at the original dimensions if possible.
4. Using the signal from samples labeled with L3-IR-App adaptors, identify the location of the RBP:RNA complexes (*see* **Note 17**). Cut a wide band of the membrane ~20–60 KDa above the expected molecular weight of the RBP and the L3-NNN-App adaptor (~8 KDa) (*see* **Note 18**) (Fig. 1b).
5. Transfer the nitrocellulose membrane pieces into a 1.5 mL tube and add 200 μL of protein degradation mix. Incubate shaking at 1100 rpm for 60 min at 50 °C.
6. Transfer the solution into a new tube, add 200 μL of phenol: chloroform:isoamyl alcohol, mix well and transfer it into a 2 mL Phase Lock Gel Heavy tube. Incubate at 30 °C shaking at 1100 rpm for 5 min. Spin the tube at room temperature for 5 min at 21,800 × *g*.
7. Add 800 μL of chloroform, mix well by inverting, and centrifuge the tube at 4 °C for 5 min at 21,800 × *g*.
8. Transfer the top aqueous layer to a new 1.5 mL tube containing 0.75 μL of glycoblue and 20 μL of 5 M NaCl. Mix well and add 500 μL of 100% ethanol. Leave at −20 °C for 18 h.
9. Centrifuge at 4 °C for 30 min at 21,800 × *g*. Remove supernatant and wash the pellet with 1 mL of 80% ethanol. Centrifuge at 4 °C for 5 min at 21,800 × *g*. Remove supernatant, let the pellet to get dry. Then, resuspend the RNA in 5.5 μL of water and transfer it into a clean PCR tube.

3.5 Reverse Transcription, cDNA Purification, Circularization, and PCR Amplification

1. Add 1 μL of iCLIP_ddRT primer and 0.5 μL of dNTP. Mix well and incubate at 65 °C for 5 min.
2. Add 3 μL of the reverse transcription mix. Incubate at 25 °C for 5 min, at 50 °C for 5 min, at 55 °C for 5 min, and hold at 4 °C.
3. Add 1.25 μL of 1 M NaOH, mix and incubate at 85 °C for 15 min for alkaline hydrolysis of the RNA. Then, add 1.25 μL of 1 M HCl to neutralize the pH.
4. Purify the cDNA using AMPure XP beads. Briefly, add 37.5 μL AMPure XP beads to each sample and mix. Then, add 21 μL of isopropanol and mix well. Incubate at room temperature for 5 min and place on a magnetic stand for 1 min to remove the

supernatant. Wash with 85% ethanol twice. After the last wash, leave beads to dry and resuspend them in 9 μL of water. Place on a magnetic stand and transfer the supernatant into a new PCR tube.

5. Add cDNA circularization mix and incubate at 60 °C for 2–18 h.
6. Purify cDNA using AMPure XP beads following the procedure detailed above (*see* **step 4** in Subheading 3.5), but scaling up the reaction with 45 μL of AMPure XP beads and 25 μL of isopropanol. Elute samples in 10 μL of water.
7. Add cDNA library preparation mix to 5 μL of each sample. Place on a thermocycler and start the following PCR program: 98 °C for 40 s, 15–25 cycles of 98 °C for 20 s, 65 °C for 30 s, 72 °C for 45 s, incubate 72 °C for 3 min, hold at 25 °C (*see* **Note 19**).
8. (optional) Take 5 μL of the sample after PCR library amplification, add 2 μL of a 6× TBE loading buffer, and load it into a 6% TBE acrylamide gel. Run the gel for 35 min at 200 V in an electrophoresis chamber and visualize the cDNA library by staining the gel with SYBR Safe (*see* **Note 20**). The size of the insert should be larger than 150 bp (*see* **Note 21**) (Fig. 1c).
9. Purify PCR-amplified cDNA library, as described above (*see* **step 4** in Subheading 3.5), by adding 1.8 μL of AMPure XP beads and 1 μL of isopropanol per each μL of PCR product (*see* **Note 22**).
10. Elute iCLIP cDNA library by adding 20–40 μL of TE buffer and proceed to sample quantification and quality check prior sequencing and annotation of protein:RNA-binding sites (Fig. 1d). Various computational pipelines exist to run CLIP analyses on local computing clusters and servers. For example, iMaps: https://imaps.goodwright.com, eCLIP: https://github.com/YeoLab/eclip, PAR-CLIP: https://github.com/ohlerlab/PARpipe, nf-core: https://github.com/nfcore/clipseq) [16, 26, 27].

4 Notes

1. If the tissue contains high RNase A activity, blocking antibodies can be used without affecting downstream sample processing.
2. Isolation of different subcellular fractions is possible at this step using dedicated buffers. For example, analysis of the RBP:RNA interactome in the cytoplasm can be achieved by resuspending sample pellets into cytoplasmic lysis buffer: 10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 2.5 mM MgCl2, 40 μg/mL digitonin,

protease inhibitor cocktail (1:200), and 10 U/mL of RNase inhibitor.

3. Stringency of washing buffer can change depending on the nature of the RBP of interest. For example, high-stringency buffer: 15 mM Tris–HCl (pH 7.4), 120 mM NaCl, 25 mM KCl, 5 mM EDTA, 1% Triton ×100, 0.001% SDS, and 1% sodium deoxycholate; Low-stringency buffer (NT2): 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM MgCl2 and 0.0005% Igepal CA-630. Urea (up to 2 M) can be added into the wash buffer to disrupt protein–protein interactions.
4. The amount of RNase inhibitors needs to be determined empirically dependent on the RNase content of the tissue or cells.
5. Depending on whether iCLIP or PAR-CLIP is performed, the UV-crosslinker should be mounted with a 254-nm or a 365-nm emitting light bulb.
6. The number of cycles might vary dependent on the RBP studied. For RBPs containing RRM domains, such as HuR, TIA1, or PTBP1, two cycles are sufficient for efficient protein:RNA crosslinking.
7. The starting number of cells in iCLIP varies greatly depending on the metabolic status of the cell, the level of protein expression, the RNA-binding capacity of the RBP, and the affinity/avidity of the antibody used. $2–3 \times 10^7$ in vitro activated lymphocytes is normally a good starting point for most RBPs.
8. To monitor the efficiency of pre-adenylation of the L3-IR-App, take an aliquot prior and after adenylation and IR-dye labeling and run a precast 15% TBE acrylamide gel. Correct adenylation and labeling will lead to an upward shift of fluorescence adaptor.
9. Number of beads and antibody varies depending on the RBP.
10. RNase I is inhibited by SDS and should only be diluted in iCLIP lysis buffer prior usage.
11. The optimal RNase I conditions should be determined in initial experiments. Over-digestion will lead to RNAs that are too short to be uniquely mapped to the transcriptome.
12. Time of RBP:RNA immunoprecipitation needs to be optimized.
13. All washes are performed by placing the beads on a magnet, removing the supernatant, adding 1 mL of wash buffer, and leaving on rotation for 1 min in the cold room.
14. Dividing a sample and labeling it with L3-IR-App and L3-NNN-App adaptor allow you to visualize the efficiency of

RNase digestion and purification of RBP:RNA complexes of the right size.

15. If different barcoded pre-adenylated L3 adaptors are used for different samples, these can be multiplexed at this stage.
16. Leave an empty lane between samples to avoid cross-contamination. Samples labeled with L3-IR-App adaptor and L3-NNN-App adaptor should be loaded next to each other as IR signal will guide cutting of RBP:RNA complexes.
17. The L3-IR-App adaptor weights approximately 15 KDa whereas the molecular weight of the L3-NNN-App adaptors is 8 KDa. RBP:RNA complexes digested with a low concentration of RNase I will be visualized as a diffuse band. On the contrary, when digested extensively, RBP:RNA complexes will appear as a discrete band of around the expected molecular weight of the protein plus the L3-IR-App adaptor.
18. Cutting the membrane close to the expected molecular weight of the protein will result on small RNA fragment purification. To optimize RNA annotation cut ~35 kDa above the expected molecular weight.
19. The number of cycles needs to be determined empirically. An initial PCR test is desirable using 1 μL of sample and testing different number of cycles. By default, 17 and 22 cycles are suggested but you should adjust this based on the amount of signal on the membrane and based on your experience.
20. It is possible to visualize the primers (~60 bp) and primer-dimers. Low library fragments (P3/P5 primers + adaptor linker = 131 bp) are often visualized if adaptor degradation with RecJf endonuclease was unsuccessful or not performed.
21. Discard samples where no signal is seen after 25 PCR cycles, if cDNA size is not right, if only primer-dimers are seen, or if equal amount of signal is seen in controls at equal PCR cycle number.
22. If primer-dimers and low fragments are observed in the gel, proceed to cDNA library purification by gel extraction. Briefly, add TBE loading buffer and run a 6% TBE acrylamide gel. After visualization with SYBR safe, cut the band in the range of 150–400 nt. Isolate DNA from the gel pieces by first crushing the gel by piercing 3 holes in the bottom of a 0.5 mL tube using a 21G needle and place this into a 1.5 mL tubes. Place a gel fragment inside the 0.5 mL tube and spin at 21,800 × *g* for 5 min. Then, add 500 μL of Crush-Soak Gel buffer. Incubate shaking at 65 °C for 2 h. Transfer the liquid into a Costar SpinX column with two 1 cm glass pre-filters. Spin at 21,800 × *g* for 1 min. Collect sample and precipitate DNA using 50 μL of 3 M sodium acetate (pH 5.5) and 1.5 mL of 100% ethanol.

Acknowledgments

This work was supported by the Agence Nationale de la Recherche in France (ANR-20-CE15-0007, ANR-22-CE15-0013, and ANR-24-CE15-5291), Fondation pour l'Aide à la Recherche sur la Sclérose En Plaques (ARSEP-1299), Fondation pour la Recherche Médicale (FRM-EQU202303016269), La Ligue Nationale Contre le Cancer (LNCC R21011BB), Institut National du Cancer (INCA-2022-071, INCa-19431), and Cancéropôle Grand Sud-Ouest (R20067BB).

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Hentze MW, Castello A, Schwarzl T, Preiss T (2018) A brave new world of RNA-binding proteins. Nat Rev Mol Cell Biol 19(5): 327–341. https://doi.org/10.1038/nrm.2017.130
2. Corley M, Burns MC, Yeo GW (2020) How RNA-binding proteins interact with RNA: molecules and mechanisms. Mol Cell 78(1): 9–29. https://doi.org/10.1016/j.molcel.2020.03.011
3. Ho JJ, Marsden PA (2014) Competition and collaboration between RNA-binding proteins and microRNAs. Wiley Interdiscip Rev RNA 5(1):69–86. https://doi.org/10.1002/wrna.1197
4. Diaz-Munoz MD, Osma-Garcia IC (2022) The RNA regulatory programs that govern lymphocyte development and function. Wiley Interdiscip Rev RNA 13(1):e1683. https://doi.org/10.1002/wrna.1683
5. Ripin N, Parker R (2023) Formation, function, and pathology of RNP granules. Cell 186(22): 4737–4756. https://doi.org/10.1016/j.cell.2023.09.006
6. Kim S, Kim S, Chang HR, Kim D, Park J, Son N, Park J, Yoon M, Chae G, Kim YK, Kim VN, Kim YK, Nam JW, Shin C, Baek D (2021) The regulatory impact of RNA-binding proteins on microRNA targeting. Nat Commun 12(1):5057. https://doi.org/10.1038/s41467-021-25078-5
7. Keene JD, Komisarow JM, Friedersdorf MB (2006) RIP-Chip: the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts. Nat Protoc 1(1):302–307. https://doi.org/10.1038/nprot.2006.47
8. Penalva LO, Tenenbaum SA, Keene JD (2004) Gene expression analysis of messenger RNP complexes. Methods Mol Biol 257:125–134. https://doi.org/10.1385/1-59259-750-5:125
9. Cloonan N, Forrest AR, Kolle G, Gardiner BB, Faulkner GJ, Brown MK, Taylor DF, Steptoe AL, Wani S, Bethel G, Robertson AJ, Perkins AC, Bruce SJ, Lee CC, Ranade SS, Peckham HE, Manning JM, McKernan KJ, Grimmond SM (2008) Stem cell transcriptome profiling via massive-scale mRNA sequencing. Nat Methods 5(7):613–619. https://doi.org/10.1038/nmeth.1223
10. Mili S, Steitz JA (2004) Evidence for reassociation of RNA-binding proteins after cell lysis: implications for the interpretation of immunoprecipitation analyses. RNA 10(11): 1692–1694. https://doi.org/10.1261/rna.7151404
11. Ule J, Jensen KB, Ruggiu M, Mele A, Ule A, Darnell RB (2003) CLIP identifies Nova-regulated RNA networks in the brain. Science 302(5648):1212–1215. https://doi.org/10.1126/science.1090095
12. Hafner M, Landthaler M, Burger L, Khorshid M, Hausser J, Berninger P, Rothballer A, Ascano M Jr, Jungkamp AC, Munschauer M, Ulrich A, Wardle GS, Dewell S, Zavolan M, Tuschl T (2010) Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell 141(1):129–141. https://doi.org/10.1016/j.cell.2010.03.009

13. Wang Z, Kayikci M, Briese M, Zarnack K, Luscombe NM, Rot G, Zupan B, Curk T, Ule J (2010) iCLIP predicts the dual splicing effects of TIA-RNA interactions. PLoS Biol 8(10): e1000530. https://doi.org/10.1371/journal.pbio.1000530
14. Anastasakis DG, Jacob A, Konstantinidou P, Meguro K, Claypool D, Cekan P, Haase AD, Hafner M (2021) A non-radioactive, improved PAR-CLIP and small RNA cDNA library preparation protocol. Nucleic Acids Res 49(8):e45. https://doi.org/10.1093/nar/gkab011
15. Van Nostrand EL, Nguyen TB, Gelboin-Burkhart C, Wang R, Blue SM, Pratt GA, Louie AL, Yeo GW (2017) Robust, cost-effective profiling of RNA binding protein targets with single-end enhanced crosslinking and immunoprecipitation (seCLIP). Methods Mol Biol 1648:177–200. https://doi.org/10.1007/978-1-4939-7204-3_14
16. Van Nostrand EL, Pratt GA, Shishkin AA, Gelboin-Burkhart C, Fang MY, Sundararaman B, Blue SM, Nguyen TB, Surka C, Elkins K, Stanton R, Rigo F, Guttman M, Yeo GW (2016) Robust transcriptome-wide discovery of RNA-binding protein binding sites with enhanced CLIP (eCLIP). Nat Methods 13(6):508–514. https://doi.org/10.1038/nmeth.3810
17. Lorenz DA, Her HL, Shen KA, Rothamel K, Hutt KR, Nojadera AC, Bruns SC, Manakov SA, Yee BA, Chapman KB, Yeo GW (2023) Multiplexed transcriptome discovery of RNA-binding protein binding sites by antibody-barcode eCLIP. Nat Methods 20(1): 65–69. https://doi.org/10.1038/s41592-022-01708-8
18. Xiao Y, Chen YM, Zou Z, Ye C, Dou X, Wu J, Liu C, Liu S, Yan H, Wang P, Zeng TB, Liu Q, Fei J, Tang W, He C (2024) Profiling of RNA-binding protein binding sites by in situ reverse transcription-based sequencing. Nat Methods 21:247. https://doi.org/10.1038/s41592-023-02146-w
19. Hafner M, Katsantoni M, Köster T, Marks J, Mukherjee J, Staiger D, Ule J, Zavolan M (2021) CLIP and complementary methods. Nat Rev Methods Primers 1(1):20. https://doi.org/10.1038/s43586-021-00018-1
20. Lee FCY, Chakrabarti AM, Hänel H, Monzón-Casanova E, Hallegger M, Militti C, Capraro F, Sadée C, Toolan-Kerr P, Wilkins O, Turner M, König J, Sibley CR, Ule J (2021) An improved iCLIP protocol. bioRxiv:2021.2008.2027.457890. https://doi.org/10.1101/2021.08.27.457890
21. Osma-Garcia IC, Mouysset M, Capitan-Sobrino D, Aubert Y, Turner M, Diaz-Munoz MD (2023) The RNA binding proteins TIA1 and TIAL1 promote Mcl1 mRNA translation to protect germinal center responses from apoptosis. Cell Mol Immunol 20(9):1063–1076. https://doi.org/10.1038/s41423-023-01063-4
22. Osma-Garcia IC, Capitan-Sobrino D, Mouysset M, Aubert Y, Maloudi O, Turner M, Diaz-Munoz MD (2022) The splicing regulators TIA1 and TIAL1 are required for the expression of the DNA damage repair machinery during B cell lymphopoiesis. Cell Rep 41(12):111869. https://doi.org/10.1016/j.celrep.2022.111869
23. Turner M, Diaz-Munoz MD (2018) RNA-binding proteins control gene expression and cell fate in the immune system. Nat Immunol 19(2):120–129. https://doi.org/10.1038/s41590-017-0028-4
24. Matheson LS, Petkau G, Saenz-Narciso B, D'Angeli V, McHugh J, Newman R, Munford H, West J, Chakraborty K, Roberts J, Lukasiak S, Diaz-Munoz MD, Bell SE, Dimeloe S, Turner M (2022) Multiomics analysis couples mRNA turnover and translational control of glutamine metabolism to the differentiation of the activated CD4(+) T cell. Sci Rep 12(1):19657. https://doi.org/10.1038/s41598-022-24132-6
25. Galloway A, Saveliev A, Lukasiak S, Hodson DJ, Bolland D, Balmanno K, Ahlfors H, Monzon-Casanova E, Mannurita SC, Bell LS, Andrews S, Diaz-Munoz MD, Cook SJ, Corcoran A, Turner M (2016) RNA-binding proteins ZFP36L1 and ZFP36L2 promote cell quiescence. Science 352(6284):453–459. https://doi.org/10.1126/science.aad5978
26. Ewels PA, Peltzer A, Fillinger S, Patel H, Alneberg J, Wilm A, Garcia MU, Di Tommaso P, Nahnsen S (2020) The nf-core framework for community-curated bioinformatics pipelines. Nat Biotechnol 38(3): 276–278. https://doi.org/10.1038/s41587-020-0439-x
27. Chakrabarti AM, Capitanchik C, Ule J, Luscombe NM (2023) Clipplotr-a comparative visualization and analysis tool for CLIP data. RNA 29(6):715–723. https://doi.org/10.1261/rna.079326.122

Chapter 6

Targeted 16S rRNA Gene Sequencing for Water Samples

Mélanie Pimenta, Margaux Gaschet, and Sylvain Meyer

Abstract

The choice of variable region to amplify in 16S rRNA-targeted amplicon sequencing has long been a matter of debate. Here, we describe a method for sequencing multiple variable regions with the Ion 16S Metagenomics kit, which amplifies six different amplicons covering seven 16S variable regions. We present the full protocol of water sequencing from sample collection to library preparation.

Key words 16S rRNA sequencing, Variable region, Water samples, Metagenomic, Ion Torrent, Filtration, DNA extraction, Library preparation

1 Introduction

Nowadays, in a *One Health* context, microbiota analysis is a highly informative and frequently used tool for studying microbial composition of various sample origins (environmental, veterinary, or human samples). Bacterial composition can be obtained through targeted amplicon sequencing of the 16S rRNA gene (partial or total amplification) or by shotgun metagenomic sequencing of the total DNA present in the sample [1] (Fig. 1). Even if shotgun metagenomics can inform about other characteristics such as resistance, virulence factors, or the presence of viruses, fungi, and parasites, sequencing depth remains usually low compared to targeted amplicon sequencing. For some samples, the presence of excessive human, plant, or animal DNA is also limiting its use in research.

The 16S rRNA gene exists in all prokaryotes, often in multiple copies, and is one of the most conserved bacterial rRNA genes [2, 3]. It is composed of approximately 1550 bp structured in conserved regions interspaced with nine variable regions. The presence of highly conserved regions between bacterial species is employed to design universal PCR primers, which are used to amplify and sequence variable regions. Amplification and subsequent sequencing of one or several variable regions enable

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_6,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

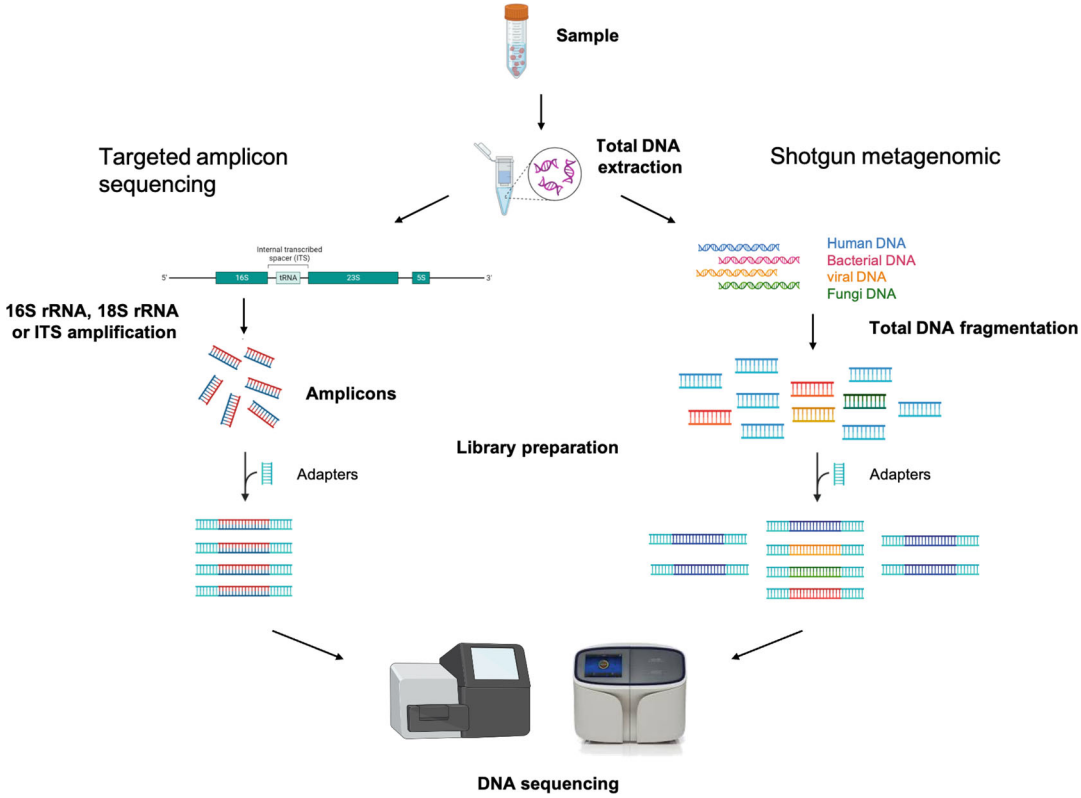


Fig. 1 General comparison between targeted amplicon sequencing and shotgun metagenomic sequencing technologies. (Figure created on BioRender)

bacterial identification after comparison to an appropriate database. Nevertheless, position, length, or structure of each variable region may differ between species, and designed primers may not recognize all species present in the sample.

Even if some studies showed potential bias related to PCR amplification [4], this technology still remains an informative tool to describe microbiota modifications in environmental waters coming from wastewater treatment plants, hospital influents, or rivers [5], but various precautions are required at sampling, storage, processing, and technology levels. The effect of the pre-analytical and analytical process (DNA depletion, DNA extraction, targeted variable region, number of PCR amplification cycles, etc.) may also have an impact on the final results, especially for anaerobes or low-abundance bacteria [6].

The type of variable region to target is still a matter of debate. Some studies pointed out a significant influence on the resulting microbial composition depending on the primer choice [7], and bias was also observed due to primer affinity differences

[4]. Amplification of whole 16S rRNA through long-read sequencing seems to provide more accurate information, but recommended primers have difficulties amplifying 16S rRNA from some specific bacteria [8]. Therefore, 16S short-read sequencing remains an affordable technology, despite its limits.

One fundamental question of 16S short-read sequencing is the choice of the variable region to target. Depending on the selected variable regions, differences in the sequencing results and taxonomic outcome may occur. Amplification of V3-V4 region with Illumina technologies has been the most common sequenced region in literature, but some recent studies revealed that other variable regions may have a better sensitivity and be more informative depending on the sample type and taxa rank analyzed. Several studies tried to compare the best variable regions to target and it appears that there are a lot of factors to take into account. Yang et al. found, for example, that V4-V6 might be the optimal sub-region for the design of universal primers with a superior phylogenetic resolution for bacterial phyla [9], while Bukin et al. showed that V2-V3 has higher resolution for genera and species levels [10]. In recent studies on environmental samples, V4 but also V5-V6 have been used for marine sediment samples [11], whereas V3-V4 regions have been used to analyze microbial communities in water [12]. Onywera and Meiring showed that the generated result between V4 region of Ion Torrent and V3-V4 region with Illumina technologies showed similar results [13].

On the other hand, Winand et al. concluded that selecting regions spanning multiple 16S rRNA gene variable regions and combining information collected substantially improved accuracy [14]. Indeed, some specific primers may not amplify some specific taxon.

To overcome the problem of variable region selection, we decided to follow and to describe here a method for characterizing the microbiome by sequencing several variable regions of the 16S rRNA gene using the Ion S5 technologies and the Ion 16S Metagenomic kit. This kit has the advantage of amplifying six amplicons covering seven variable regions (V2, V3, V4, V6–7, V8, and V9) in a single PCR with six different amplicons (Fig. 2). Bioinformatic analysis can then help to compare results between the different variable regions and select the most performant one or to use a combination of several variable regions [15]. To illustrate the protocol, we will take the example of water filtration samples. Note that this protocol can be adapted to various matrices and sample volumes; for example, we adapted it to human respiratory samples [16].

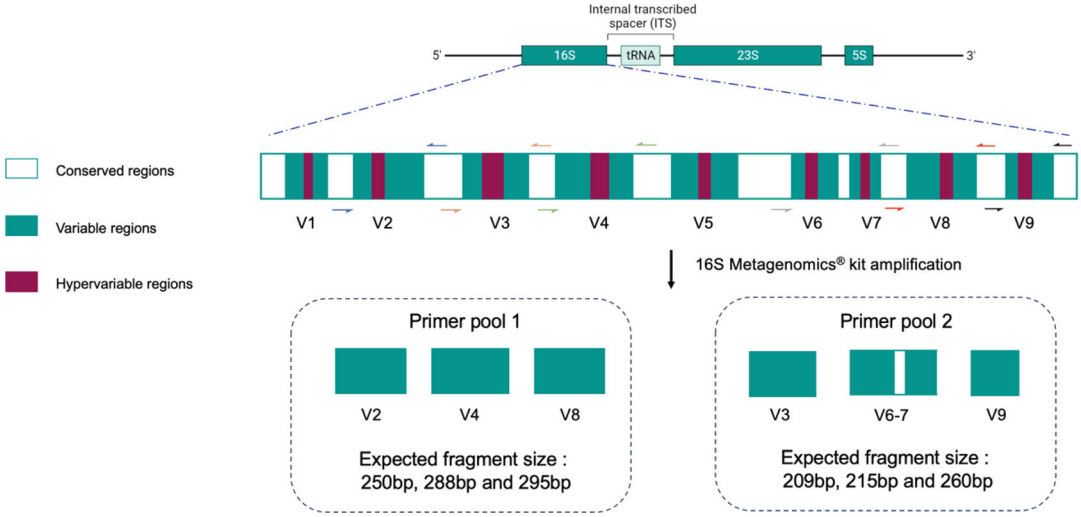


Fig. 2 Expected targeted amplicons of the Ion 16S Metagenomics kit

2 Materials

2.1 Sample Collection and DNA Extraction

1. Filtration ramp (*see* **Note 1**).
2. Sink.
3. 0.45 μm pore size filters, 47 mm diameter.
4. Filter funnels.
5. Sterilized forceps.
6. Tubes suitable for freezing or extracting DNA.
7. Ethanol 70%.
8. DNeasy PowerWater kit (Qiagen), with PW1, PW3, PW4, IRS, and EB solutions.
9. Water bath.
10. Vortex and Vortex adapter.
11. Centrifuge for 15 mL tubes (optional).
12. Microcentrifuge.

2.2 DNA Dosage and Amplification of Variable 16S rRNA Regions

1. Qubit 4 Fluorometer (ThermoFisher Scientific).
2. Qubit dsDNA BR Assay Kit with Buffer, Reagent, and Standards included (ThermoFisher Scientific).
3. Qubit assay tubes (ThermoFisher Scientific).
4. Ion 16S Metagenomic Kit (ThermoFisher Scientific).
5. 0.6 mL PCR tubes or 96-well plate with adhesive seal.
6. Thermocycler.

2.3 Agarose Gel Electrophoresis and Purification of PCR Products

1. Gel trays and tank.
2. Gel imager and conversion screen.
3. 2% agarose gel.
4. 1 kb DNA Ladder.
5. AMPure XP beads: Agencourt AMPure XP Kit (Beckman Coulter).
6. DynaMag-2 magnetic rack for 1.5 mL tubes or 0.6 mL tubes (ThermoFisher Scientific).
7. Nuclease-free sterile water.
8. Centrifuge.

2.4 Preparation of the Libraries

1. Ion Plus Fragment Library Kit for AB Library Builder™ System Kit (ThermoFisher Scientific) with E1 Buffer and End Repair tubes included (*see* **Note 2**).
2. LdN Buffer Module for AB Library Builder™ System (ThermoFisher Scientific).
3. AB Library Builder™ Plastics Module (ThermoFisher Scientific).
4. AB Library Builder™ System.
5. AMPure XP beads: Agencourt AMPure XP Kit (Beckman Coulter).
6. Ion Xpress™ Barcode Adapters 1–16 Kit with Ion Xpress™ P1 Adapter included (ThermoFisher Scientific).
7. Platinum® PCR SuperMix High Fidelity (ThermoFisher Scientific).
8. Library Amplification Primer Mix (ThermoFisher Scientific).
9. 2× Master Mix Environmental TaqMan™ 2.0 included in the kit (ThermoFisher Scientific).
10. LoBind® Tubes (1.5 mL) (Eppendorf).
11. High Sensitivity DNA Kit with DNA Chips, DNA Dye, DNA Gel Matrix, DNA Markers, and DNA Ladder (Agilent Technologies).
12. Chip priming station (Agilent Technologies).
13. 2100 Bioanalyzer Instrument supplied with IKA vortex mixer (Agilent Technologies).
14. Electrode cleaner kit (Agilent Technologies).

2.5 Library Sequencing

1. Ion S5 Chef Supplies (ThermoFisher Scientific).
2. Ion S5 Chef Solution (ThermoFisher Scientific).
3. Ion S5 Sequencing Solution (ThermoFisher Scientific).
4. Ion S5 Sequencing Reagent (ThermoFisher Scientific).

5. Ion 510™ & Ion 520™ & Ion 530™ Chef Reagent (ThermoFisher Scientific).
6. Ion 530™ Chip Kit (ThermoFisher Scientific).

3 Methods

We present here the general protocol for 16S rRNA sequencing of water filtration samples. Note that the advantage of this method is the amplification of several variable regions that can increase the percentage of species-level identification.

External contaminants are nevertheless frequent with the different reagents used for DNA extraction or 16S rRNA amplification, but also cross-contaminations during the different pipetting steps. Therefore, ensure that appropriate negative and positive controls are included in the DNA isolation protocol (*see* **Note 3**).

3.1 Sample Collection

1. Collect water samples with sterile bottles. The volume of water necessary to filter depends on the water quality (*see* **Note 4**).
2. Pour the water sample into the funnels (*see* **Note 5**).
3. Switch on the pump.
4. Open each valve.
5. Wait until all the volume placed in the funnel has passed through each filter before closing the valves (*see* **Note 6**).
6. Repeat the above operations until the filters are saturated (*see* **Note 7**).
7. Switch off the pump.
8. Remove the funnels (*see* **Note 8**).
9. Use sterilized or disinfected forceps to remove each filter and place the filters in the bead tubes provided by the DNeasy PowerWater kit.
10. Store at −80 °C if not used directly.
11. Clean the ramp by pouring ethanol over the ramp adapters.
12. Switch on the pump and open the valves.
13. Close the valves and switch off the pump once the ethanol has been drawn off.

3.2 DNA Extraction

Several commercially available DNA isolation kits, such as the DNeasy PowerWater kit, allow for efficient total DNA extraction from water filtration samples. Other DNA extraction protocols have been shown to be useful when large amounts of DNA are expected in your sample [17].

1. Heat the water bath to 60 °C. When the water bath is hot, put the PW1 and PW3 solutions tubes inside the water bath.
2. Heat the solutions for at least 10 min.
3. When PW1 is at temperature, mix gently.
4. Add 1 mL of PW1 in the tubes containing the filters (*see* **Notes 9** and **10**).
5. Mix by shaking the tubes.
6. Vortex for 10 min on the horizontal vortex.
7. Centrifuge for 1 min at 4000 × *g* at room temperature.
8. Recover the supernatant and place it in a 2 mL tube provided by the kit. Take as much of the supernatant as possible. About 600–650 µL of supernatant should be recovered.
9. Centrifuge for 1 min at 13,000 × *g*.
10. Transfer the supernatant, without taking the pellet, in a new 2 mL tube.
11. Add 200 µL of IRS solution provided by the kit.
12. Vortex lightly and place 5 min at 4 °C (place in fridge).
13. Centrifuge for 1 min at 13,000 × *g*.
14. Transfer the supernatant in a new 2 mL tube.
15. Add 650 µL of PW3 solution provided by the kit and vortex briefly.
16. Load 650 µL of the supernatant in a Spin Column provided by the kit (*see* **Note 11**).
17. Centrifuge for 1 min at 13,000 × *g*. Throw away the flow-through. Repeat until all the supernatant has been processed.
18. When all the samples have passed over the columns, place the Spin Column Filter in a new 2 mL tube provided by the kit.
19. Add 650 µL of PW4 solution provided by the kit; the solution must be shaken before use.
20. Centrifuge at 13,000 × *g* for 1 min and discard the flow-through.
21. Add 650 µL of ethanol provided by the kit and centrifuge at 13,000 × *g* for 1 min.
22. Throw away the flow-through and centrifuge again at 13,000 × *g* for 2 min.
23. Place the Spin Column in a new 2 mL tube provided by the kit.
24. Add 100 µL of EB solution to the center of the filter membrane.
25. Centrifuge at 13,000 × *g* for 1 min.

26. Remove the Spin Column.
27. Store the DNA at −80 °C.

3.3 DNA Dosage

1. Annotate as many Qubit assay tubes as number of samples to dose.
2. Annotate one extra tube for Standard 1 (Tube 1) and one extra tube for Standard 2 (Tube 2).
3. In a 25 mL tube, prepare a working solution with 199 μL of buffer and 1 μL of reagent per sample (multiply by n + 2, where n is the number of samples to dose).
4. Place 198 μL of solution in the assay tubes intended for the samples and 190 μL in the tubes intended for the standards.
5. Add 2 μL of DNA from the samples to the sample tubes and 10 μL of Standard 1 to tube 1 and 10 μL of Standard 2 to tube 2.
6. Vortex and wait 2 min.
7. On the device: Select dsDNA mode > Choose the kit used (dsDNA Broad Range) > Click on "Analyze standards" > Place the tube containing Standard 1 in the device > Analyze the tube > Place the tube containing Standard 2 in the device > Analyze the tube > Select the volume of the initial sample (2 μL) and the concentration unit > Place a sample tube in the device > Analyze the tube and read the displayed value.

3.4 Amplification of 16S Variable Regions

For each PCR, add one positive and one negative control (that are different from positive and negative contamination controls, which need to be added in each DNA extraction/amplification series). Each sample will be divided into two tubes, each containing one primer set V2-V4-V8 (pool1) or V3-V6,7-V9 (pool 2). We recommend using 96-well plates for faster purification of amplicons.

1. Prepare two mixes as indicated in the following preparation mix template depending on your number of samples:
   - Mix Primer Set 1 (for one sample reaction): 15 μL of 2× Master Mix Environmental TaqMan solution (orange cap tube) (*see* **Note 12**), 3 μL of Primer Set 1 (V2-V4-V8) in the first tube of each sample, and 2 μL of sterile water;
   - Mix Primer Set 2 (for one sample reaction): 15 μL of 2× Master Mix Environmental TaqMan solution (orange cap tube), 3 μL of Primer Set 2 (V3–V6, V7–V9) in the second tube and 2 μL sterile water.
2. Distribute in your 96-well plate 20 μL of Mix 1 or Mix 2 (*see* **Note 13**).
3. Add 10 μL of sample DNA in the tube containing Mix 1 and 10 μL in tube containing Mix 2.

4. Dilute the positive control (*E. coli* DNA) with the positive control buffer present in the kit as requested by the manufacturer. Then add 10 µL of diluted positive control to the positive PCR.
5. Add 10 µL of sterile water in the negative control.
6. Seal the PCR plaque with a 96-well plate adhesive seal. Each tube should contain 30 µL of final volume.
7. Carefully centrifuge the plate in a microcentrifuge to drop the liquid at the bottom.
8. Run the following PCR program in a thermocycler: 95 °C for 10 min, 25 cycles (95 °C for 30 s, 58 °C for 30 s, 72 °C for 20 s), cool to 10 °C (until use).
9. Perform gel electrophoresis to control the amplification: pour the 2% agarose gel into the gel tray and place the well comb in the good position. Let the gel at room temperature until it is completely solidified.
10. Remove the well comb and place the gel in the electrophoresis system.
11. Add the 1 kb DNA ladder in the first well.
12. For each PCR reaction, including controls, place 2 µL in a well and proceed to migration.
13. Run the gel by adapting time and voltage to the tank used.
14. Visualize DNA bands (Fig. 3 and *see* **Note 14**).
15. If the samples are well amplified, discard the tubes of the positive and negative PCR controls.

3.5 DNA Purification

The DNA purification step is presented here with the DynaMag-2 magnetic rack for 0.6 mL tubes. Another protocol exists for 1.5 mL tubes, which will not be presented here because it is more time-consuming.

1. Take AMPure XP beads out of the fridge 30 min before use to bring them to room temperature.
2. Homogenize the AMPure XP solution to resuspend the magnetic beads.
3. Prepare fresh 70% ethanol as much as needed; count 1 mL per sample.
4. In a 96-well plate, add the correct volume of AMPure XP beads according to the sample volume, calculated as follows: Volume of AMPure XP beads = 28 µL (remaining sample volume in each well, *see* **steps 6–12** in Subheading 3.4) × 1.8 = 50.4 µL.
5. Homogenize the solution with the pipette.
6. Incubate for 5 min at room temperature.

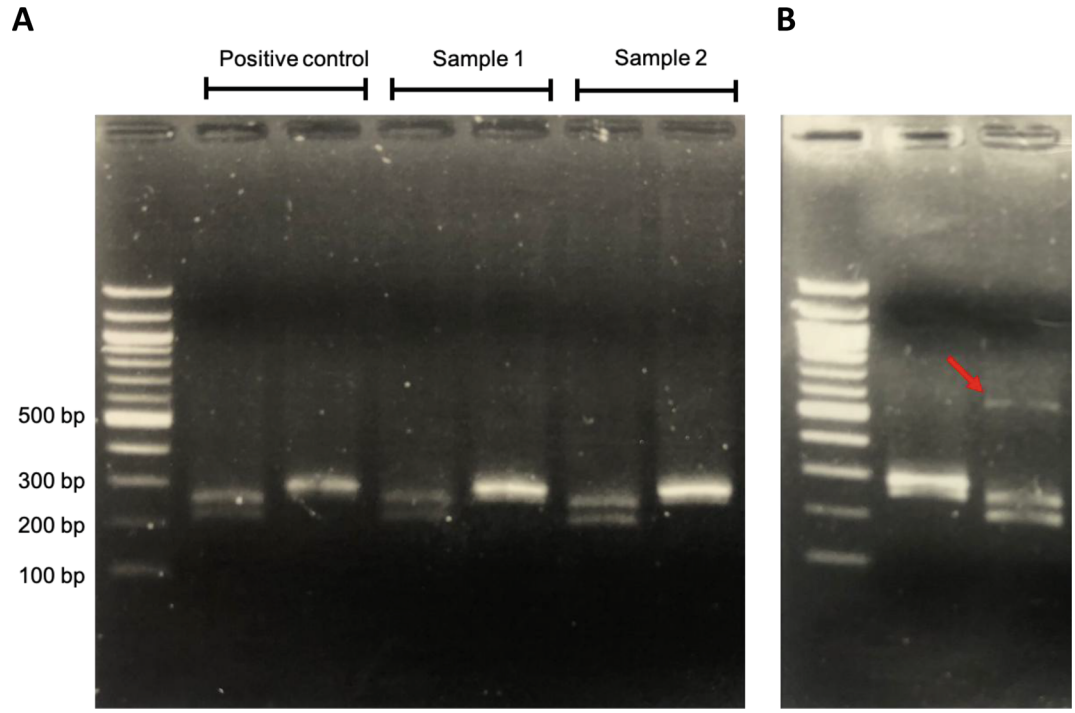


Fig. 3 Amplification profiles of the Ion 16S Metagenomic Kit PCR. **a** Usual amplification profiles obtained using primer pool 1 (lanes 3, 5, and 7) and primer pool 2 (lanes 2, 4, and 6). **b** Possible extra bands detected (red arrow) as exemplified after amplification using primer pool 2 (lane 3)

7. Place the tubes on the DynaMag-2 magnetic rack for 0.6 mL tubes.
8. Incubate for 3 min at room temperature until the solution runs clear.
9. Carefully remove the supernatant without removing the beads.
10. Leave the tube on the magnetic rack and perform an initial wash by adding 500 μL of 70% ethanol.
11. Move the plaque from one rank on the plaque in order to move and clean the beads from any impurity.
12. Carefully remove the supernatant.
13. Carry out a second wash with 70% ethanol, as described in **steps 10** and **11** in Subheading 3.5.
14. Remove the remaining supernatant.
15. Let it dry for 5 min at room temperature.
16. Remove the plate from the magnetic rack and add 15 μL of sterile water on the beads. Pipette vigorously several times.
17. Put the plate on the magnetic rack.
18. Incubate for 1 min at room temperature.

19. Recover the supernatant containing the DNA, and place it in a new, clean LoBind tube labeled. Pool the two mixes from each sample in the same tube.
20. Purified samples can be stored at 4 °C for short-term storage or at −20 °C for long-term storage.
21. For DNA dosage, repeat the same protocol as **steps 1**–7 in Subheading 3.3.

3.6 Preparation of the Libraries

This protocol describes the Ion library preparation steps recommended with the Library Builder automaton. We recommend using this protocol, as it enables to increase the final library yields and highly decreasing the library preparation time. It also strongly prevents the potential cross-contamination. Each Library Builder run is made for 13 samples.

1. Disinfected the inside of the Library Builder machine with appropriate disinfectant in order to prevent any contaminations.
2. Annotated as many Eppendorf screw cap tubes as running samples (maximum 13 per run). Place the sample volume necessary for 100 ng of DNA. Complete with E1 Buffer (provided in the kit) until final volume reaches 50 μL (*see* **Note 15**).
3. Homogenize and centrifuge with a microcentrifuge.
4. Annotated as many LdN buffer tubes as running samples. In these LdN tubes, add 48 μL of E1 buffer, 1 μL of P1 adapter, and 1 μL of selected barcodes.
5. Homogenize and centrifuge the LdN tubes with a microcentrifuge.
6. Open the window of the AB Library Builder™ System and take out the rack for the cartridges.
7. Check that the cartridges have thawed (*see* **Note 16**).
8. Remove the aluminum covering the well number 12 before sliding the cartridges on the support.
9. Vortex the AMPure XP beads.
10. Add 450 μL of AMPure XP beads in well number 10.
11. Place the mixes containing the diluted sample DNA in well number 12.
12. Unscrew the caps from the LdN tubes and place them in well number 11 of the cartridges.
13. Put the rack with the cartridges back into the AB Library Builder™ System and take out the other rack.
14. Centrifuge one End Repair tube per sample (provided in the kit).

15. Remove the caps from the End Repair tubes and place them in row S of the rack.
16. Place filter tips, provided by the kit, in rows T1 and T2.
17. Place the elution tubes in row E. Be careful to remove all the caps before running the machine.
18. Label the caps and place them in the provided space.
19. Put the rack back in the AB Library Builder™ System and close the window.
20. Start the following protocol: AutoSize Selection, AutoShearing, 200 bp shear, and size selection.
21. After 2 h, take back the elution tube, which is containing the libraries in 30 μL (*see* **Note 17**).

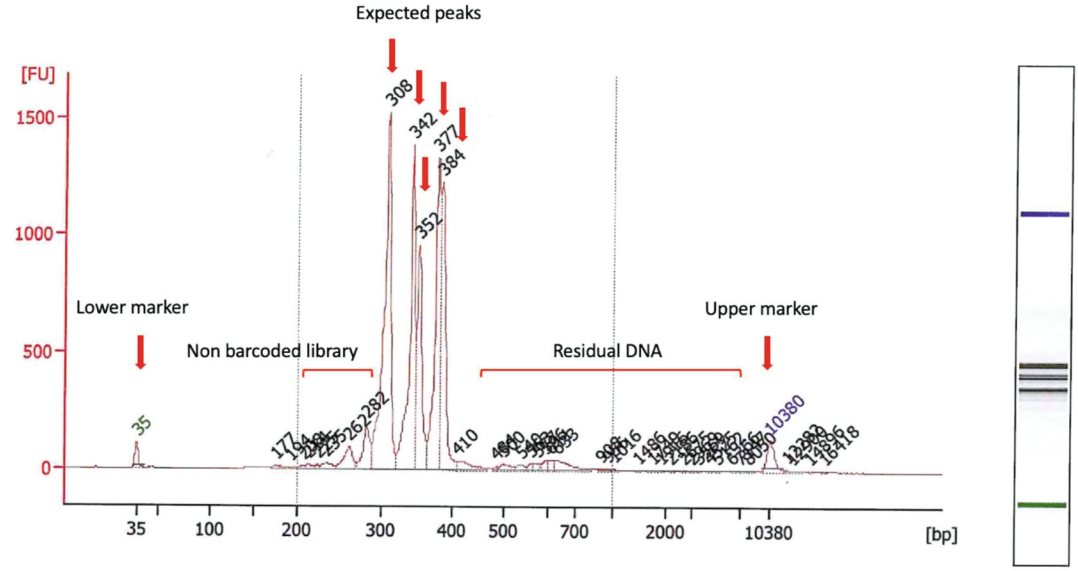
3.7 Amplification of the Libraries and DNA Purification

This step selects the appropriate sequences that have successfully incorporated the adapters and barcodes. This step is not mandatory if you have enough DNA but is usually required to obtain sufficient DNA for the sequencing step.

1. Prepare the following PCR mix (for one sample): 100 μL of Platinum® PCR SuperMix High Fidelity and 5 μL of Library Amplification Primer Mix.
2. Distribute 105 μL of mix in a 96-well plate.
3. Add 25 μL of library (*see* **step 21** in Subheading 3.6) in the tube for a final volume of 130 μL.
4. Separate the 130 μL into two tubes containing approximately 65 μL.
5. Centrifuge the plate and run the following PCR program: 95 °C for 5 min, 8 cycles (95 °C for 15 s, 58 °C for 15 s, 72 °C for 1 min), cool to 10 °C (until use).
6. Repeat the DNA purification protocol (*see* **steps 1**–**15** in Subheading 3.5) except with a volume of AMPure XP beads of $1.5 \times 65 = 98$ μL.
7. For the elution of the magnetic pellet containing the first part of the amplified library (*see* **step 4** in Subheading 3.7), place 20 μL of sterile water and proceed to elution (*see* **steps 16**–**18** in Subheading 3.5).
8. For the elution of the second magnetic pellet containing the second part of the amplified library (*see* **step 4** in Subheading 3.7), place 20 μL of sterile water and proceed to elution (*see* **steps 16**–**18** in Subheading 3.5).
9. Recover the supernatant and combine eluted DNA from the "first primer pool" (*see* **step 7** in Subheading 3.7) and the "second primer pool" (*see* **step 8** in Subheading 3.7) in a 1.5 mL LoBind tube.

3.8 Assessment of the Libraries and Sequencing

1. The Agilent High Sensitivity DNA kit must be taken out of the fridge 30 min before use.
2. Prepare the electrophoresis gel: vortex the blue-capped tube with the high-sensitivity dye for 10 s and centrifuge. Then, transfer 15 μL into the high-sensitivity DNA gel matrix vial (red cap) and vortex for 10 s.
3. Transfer the prepared gel to the column with the filter and centrifuge for 10 min at room temperature at 2240 × *g*.
4. Discard the column and note the date of preparation on the tube of filtered gel. The prepared gel can be kept for 6 weeks.
5. Prepare the chip: place 9 μL of gel dye mix in the well marked "G" (*see* **Note 18**).
6. Place the chip in the chip priming station. Set a timer for 60 s and ensure that the syringe is in the 1 mL position and that the notch is in the lowest position. Close the chip priming station (you will hear a click), press the syringe down to the notch clipped underneath, and trigger the timer. After 1 min, release the syringe from the clip by pressing on it, wait a few seconds for the syringe to rise, and then help it to return to the 1 mL setting. Open the chip priming station and check that the gel has been deposited in all channels.
7. Place 9 μL of gel dye mix into the three wells marked "G."
8. Pipette 5 μL of the high-sensitivity marker (green cap) into the well with the scale symbol and into each of the sample wells. None of the chip's wells must be empty.
9. Place 1 μL of ladder (yellow cap) into the ladder-marked well.
10. Place 1 μL of sample into the well.
11. Vortex the chip for 60 s at 2400 rpm on the IKA vortex mixer.
12. Once the chip has been vortexed, it must be passed through the automaton within 5 min.
13. Chip loading and start-up: open the device and place the chip inside. Start the 2100 Expert Software and choose the type of chip used: dsDNA high sensitivity. Enter sample names (fill sample positions 1 to 11 for a full chip, 1 to n for an unfilled chip) and press START. The procedure takes about 45 min.
14. At the end of the run, discard your chip and rinse with the water chip, provided by the kit for this purpose, by filling it with 350 μL of water (fill in any well, as all the wells are all connected). Place it in the machine and close the lid for 30 s. Collect and empty the water chip into the sink. Close the machine lid.
15. Based on the molarity results on the BioAnalyzer profile (Fig. 4 and *see* **Note 19**), calculate the dilution required to obtain a



Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations	Aligned Migration Time [s]
35	125,00	5 411,3	Lower Marker	43,00
308	1 879,61	9 246,7		70,69
342	1 210,75	5 357,5		73,41
352	747,53	3 218,5	Calculated molarity	74,16
377	1 071,03	4 299,5		76,17
384	885,95	3 496,1		76,69

Fig. 4 Profile of the expected fragments after library preparation on the BioAnalyzer. In this profile, only five peaks are visualized and peak 308 is the fusion of two fragments

final concentration of 35 pmol/L for each of the libraries in a final volume of 50 µL.

16. Pool 5 µL of each library in a single 1.5 mL LowBind tube that will be used for the sequencing step.
17. After appropriate sequencing on the Ion S5 sequencer, fastq raw data can be directly uploaded on the IonReporter software (free online) or implemented in a local pipeline [18].

4 Notes

1. There are also small battery-operated filtration pumps that filter the water directly at the sampling site, eliminating the need to transport bottles of sampled water. But only one sample can be filtered at a time.
2. If no Library Builder system is available, you can follow the protocol used manually, but it increases the technical time with lower library concentration efficiency.

3. We strongly advise adding sequencing negative controls consisting of sterile water that follow each step of the protocol (including the sequencing step) to detect the basal contamination. Use of mock community positive control is also highly recommended to validate the different steps of the protocol, for example, with the ZymoBIOMICS Microbial Community Standard (Zymo Research). We also recommend using a bioinformatic approach to compare negative controls and samples to filter the potential contaminants as described in other journals [19]. Note that Ion Reporter software, which can be used for an easier bioinformatic analysis, uses a 10-read filter that eliminates potential low contaminants. This can be adjusted depending on the bioinformatic pipeline but must be taken into account in 16S rRNA analysis.
4. The volume of water collected depends on the quality of the water. For clean water, such as drinking water, large volumes of water will be needed (several liters), whereas for turbid water, such as wastewater, a smaller volume of water, generally less than 1 L (around 100 mL), will be sufficient.
5. The clearer the water is, the greater the volume to be filtered will be. For cloudy water, it is better to start with 20 mL. Remember to record the different volumes filtered to know the total volume that has been passed through the filter.
6. The time it takes for the water to pass through the filter can be long, especially for turbid water. One way of checking whether the filter is saturated or not is to see if water is running out of the filtration ramp pipe. If there is still water, the filter is not yet saturated.
7. Filters are saturated once the water no longer passes through the filter. If there is still water in the funnel, then the excess can be removed using a pipette. Try not to scrape the filter with the tip of the pipette to avoid removing the bacteria from the filter.
8. Funnels can be kept if the same water needs to be filtered several times; just replace the filter with a new one.
9. Lysis is more efficient when the PW1 solution is hot; consequently, this step should be performed quickly. Reheat the solution if it has cooled down.
10. If in the sample there are organisms difficult to lyse, add solution PW1 and heat the sample for 10 min at 65 °C.
11. Spin Columns are the limiting components of the kit.
12. The 2× Master Mix Environmental TaqMan solution is clearly the limiting reagent of the kit. Be aware that it is not commercially available alone. Therefore, carefully pipet the correct amount of mix to be able to amplify 50 samples (100 reactions) with one kit.

13. We strongly recommend preparing the plate plan with either all Mix 1 on one side of the plate and Mix 2 on the other side or alternating between Mix 1 and Mix 2. Be aware that pipetting contaminations are critical in this step.
14. For each mix, three bands between 200 and 300 bp are expected, as visualized for the positive control (Fig. 3). The expected fragment sizes are as follows:
    - For Primer Set 1: three fragments at 250 pb (V2), 288 pb (V4), and 295 pb (V8). As the last two amplicons are close in size, they cannot be separated, but the band grouping them together should have a higher intensity.
    - For Primer Set 2: three fragments at 215 pb (V3), 260 pb (V6–7), and 209 pb (V9). As before, V3 and V9 cannot be separated, and the band grouping them together should have a higher intensity.

      No amplification should be detected for the PCR negative control, but bands can sometimes be detected for the sequencing negative controls depending on the basal contamination levels.

      Extra bands above 300 bp can exceptionally occur. For example, we detected an extra band around 600 bp (Fig. 3b, red arrow), which corresponded to an amplicon chimera that was not detected in the final result (too high for the sequencing step). We did not notice lower size bands. If a smear is present, it might be residual DNA from human or environmental origin. A DNA depletion method such as the MolYsis™ Basic 5 kit (Molzym) should be performed before the DNA extraction step to deplete this residual DNA.
15. DNA quantity can be lowered until 30 ng. We do not recommend going below.
16. It is really important that the first two wells of the reagent card are thawed before running the machine; otherwise the automaton could rapidly be in error.
    1. In case of critical error: (i) take out all the filter tips and replace them, (ii) discard all the AMPure XP beads present in the different wells, and (iii) provide new AMPure XP beads in well 10. Running the machine this way enables your experiment to not be lost!
17. After carefully taking out your elution samples, verify that no residual AMPure XP beads are present in the eluate. If so, use the magnetic rack to transfer the eluate into a new elution tube and discard the residual beads.
18. No bubbles should stay in the well before using the syringe; otherwise, the migration might fail.

19. A typical example of what is expected after the BioAnalyzer step is shown in Fig. 4. Six amplicons of around 209–295 bp were amplified, and library preparation added 96 bp on each fragment (adapters + barcode). In the final profile, six peaks are expected around 305–391 bp. Note that the expected sizes are really close, so three to six peaks can be visualized (fusion of the close peaks). To calculate the final molarity of the combined peaks, you should add the molarity found for each peak.

Acknowledgments

This work was supported by grants from ANR/BMBF second French-German call for projects on antimicrobial resistance 2020 and by funding from INSERM.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Durazzi F, Sala C, Castellani G et al (2021) Comparison between 16S rRNA and shotgun sequencing data for the taxonomic characterization of the gut microbiota. Sci Rep 11:3030. https://doi.org/10.1038/s41598-021-82726-y
2. Větrovský T, Baldrian P (2013) The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PLoS One 8:e57923. https://doi.org/10.1371/journal.pone.0057923
3. Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271. https://doi.org/10.1128/mr.51.2.221-271.1987
4. Qin Y, Wu L, Zhang Q et al (2023) Effects of error, chimera, bias, and GC content on the accuracy of amplicon sequencing. mSystems 8:e0102523. https://doi.org/10.1128/msystems.01025-23
5. Buelow E, Rico A, Gaschet M et al (2020) Hospital discharges in urban sanitation systems: long-term monitoring of wastewater resistome and microbiota in relationship to their eco-exposome. Water Res X 7:100045. https://doi.org/10.1016/j.wroa.2020.100045
6. Conrads G, Abdelbary MMH (2019) Challenges of next-generation sequencing targeting anaerobes. Anaerobe 58:47–52. https://doi.org/10.1016/j.anaerobe.2019.02.006
7. Abellan-Schneyder I, Matchado MS, Reitmeier S et al (2021) Primer, pipelines, parameters: issues in 16S rRNA gene sequencing. mSphere 6:e01202–e01220. https://doi.org/10.1128/mSphere.01202-20
8. Matsuo Y, Komiya S, Yasumizu Y et al (2021) Full-length 16S rRNA gene amplicon analysis of human gut microbiota using MinION™ nanopore sequencing confers species-level resolution. BMC Microbiol 21:35. https://doi.org/10.1186/s12866-021-02094-5
9. Yang B, Wang Y, Qian P-Y (2016) Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC Bioinform 17:135. https://doi.org/10.1186/s12859-016-0992-y
10. Bukin YS, Galachyants YP, Morozov IV et al (2019) The effect of 16S rRNA region choice on bacterial community metabarcoding results. Sci Data 6:190007. https://doi.org/10.1038/sdata.2019.7
11. Rajeev M, Sushmitha TJ, Toleti SR, Pandian SK (2020) Sediment-associated bacterial community and predictive functionalities are influenced by choice of 16S ribosomal RNA hypervariable region(s): an amplicon-based diversity study. Genomics 112:4968–4979. https://doi.org/10.1016/j.ygeno.2020.09.006
12. Chan S, Pullerits K, Keucken A et al (2019) Bacterial release from pipe biofilm in a full-scale

drinking water distribution system. NPJ Biofilms Microbiomes 5:9. https://doi.org/10.1038/s41522-019-0082-9

13. Onywera H, Meiring TL (2020) Comparative analyses of Ion Torrent V4 and Illumina V3-V4 16S rRNA gene metabarcoding methods for characterization of cervical microbiota: taxonomic and functional profiling. Sci Afr 7: e00278. https://doi.org/10.1016/j.sciaf.2020.e00278
14. Winand R, Bogaerts B, Hoffman S et al (2019) Targeting the 16S rRNA gene for bacterial identification in complex mixed samples: comparative evaluation of second (Illumina) and third (Oxford Nanopore Technologies) generation sequencing technologies. Int J Mol Sci 21:298. https://doi.org/10.3390/ijms21010298
15. Malczynski M, Zhu A, Zembower T, Qi C (2021) Diagnostic performance of Ion 16S metagenomics kit and ion reporter metagenomics workflow for bacterial pathogen detection in culture-negative clinical specimens from sterile sources. Diagn Microbiol Infect Dis 101:115451. https://doi.org/10.1016/j.diagmicrobio.2021.115451
16. Meyer S, Gaïa N, Lazarevic V et al (2023) Could daily changes in respiratory microbiota help predicting early Staphylococcus aureus ventilator-associated pneumonia? Intensive Care Med Exp 11:34. https://doi.org/10.1186/s40635-023-00521-7
17. Vickery TW, Kofonow JM, Ramakrishnan VR (2017) Characterization of sinus microbiota by 16S sequencing from swabs. Methods Mol Biol 1616:23–38. https://doi.org/10.1007/978-1-4939-7037-7_2
18. Jones CB, White JR, Ernst SE et al (2022) Incorporation of data from multiple hypervariable regions when analyzing bacterial 16S rRNA gene sequencing data. Front Genet 13: 799615. https://doi.org/10.3389/fgene.2022.799615
19. Cornet L, Baurain D (2022) Contamination detection in genomic data: more is not enough. Genome Biol 23:60. https://doi.org/10.1186/s13059-022-02619-9

Chapter 7

RNA-Based High-Throughput Sequencing of the Human Immunoglobulin Repertoire

Séléna Teillaud, Sébastien Bender, and Virginie Pascal

Abstract

Lymphocytes use somatic diversification processes to express a wide variability of antigen receptors, generating a highly diversified repertoire that is unique to each individual. The study of these repertoires is now possible with the advent of next-generation sequencing (NGS) techniques. Here we describe the "RACE Rep-Seq" methodology for high-throughput sequencing of immunoglobulin (Ig) repertoires using RNA templates. The preparation of libraries is based on the conversion of RNA samples into cDNA using the 5′RACE (Rapid Amplification of cDNA Ends) technique and a set of primers specific for the constant domains of the heavy (Cμ, Cγ, Cα) and light (Cκ, Cλ) chains, thus targeting almost the entire Ig repertoire. During this reverse transcription step, unique molecular identifiers (UMIs) are incorporated at the 5′ ends to correct PCR amplification biases and sequencing errors. An initial amplification step is then performed using nested primers specific for the heavy and light constant domains to increase the specificity of the amplification of Ig transcripts. This is followed by two sequential PCRs required for paired-end sequencing using asymmetric paired-end Illumina® sequencing to obtain sequences of up to 750 base pairs (bp). This length covers the entire Ig variable domain and part of the constant region required for isotype determination. Finally, the raw sequencing data is processed and analyzed to provide a complete Ig repertoire, enabling significant advances in both basic immunology and various clinical applications.

Key words Immunoglobulin repertoire, Next-generation sequencing, 5′RACE, RepSeq, mRNA, Asymmetric paired-end sequencing, Unique molecular identifiers (UMI)

1 Introduction

The humoral adaptive immune system involves immunoglobulins (Ig) expressed by B lymphocytes and plasma cells, which provide a diverse repertoire of high-affinity receptors for specific antigens. This diversity is achieved by molecular diversification processes that modify the B lymphocyte genome during maturation, resulting in a highly diversified immune profile described as a polyclonal repertoire. However, certain physiological or pathological events can modulate this repertoire, causing it to evolve toward an

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_7,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

oligoclonal or even monoclonal immune profile. The study of these repertoires is of great interest for a variety of clinical applications, including the detection of monoclonal components [1, 2] and the identification of "signature repertoires" specific to particular immune contexts [3–5].

Advances in next-generation sequencing (NGS) have greatly facilitated the study of antigen receptor repertoires, and in particular Illumina® short reads sequencing technology offers a good compromise between the depth of analysis and the length of the sequences obtained. In this context, our team has adapted a technique for studying the Ig repertoire using high-throughput sequencing on an RNA template, called "Race RepSeq" [6]. The use of an RNA template gives particularly high sensitivity for the detection of Ig transcripts, which are naturally synthesized at high levels in B lymphocytes and especially in plasma cells. Regions of interest are amplified using cDNA obtained by 5′RACE (Rapid Amplification of cDNA Ends) on RNA extracted from blood samples, bone marrow, or lymph node aspirates collected in RNA preservation tubes containing a cell lysis and RNA stabilization reagent. To generate single-stranded cDNA products covering almost the entire Ig repertoire, the reverse transcription step is performed using a pool of five antisense primers hybridizing specifically to the constant region of Ig heavy (Cμ, Cγ, Cα) and light (Cκ or Cλ) chains. When it reaches the 5′ end of the RNA strand, the reverse transcriptase (RT), which also has terminal transferase activity, adds a series of identical deoxynucleotides (4 dC in our case) to the 5′ end of the newly synthesized cDNA strand. The use of a universal sense primer (Template Switch Oligo: TSO), containing 4 G-type ribonucleotides and a given sequence of deoxynucleotides at the 3′ and 5′ ends, respectively, allows the RT enzyme to continue its extension by incorporating the TSO-defined sequence at the 5′ end of the newly synthesized cDNA. This sequence is then used as an anchor point for the subsequent PCR (Fig. 1a).

Random deoxynucleotide sequences are also incorporated within the TSOs, as unique molecular identifiers (UMIs) to allow specific identification of each of the newly formed cDNA molecules. The use of UMIs is essential to ensure the quality and reliability of the Ig sequences obtained [7–9]. The cDNA is then amplified by semi-nested PCR (PCR1) using a sense primer (primer 5′_PCR1) specific to the TSO anchor region and a set of antisense primers (primer C(n) 3′_PCR1) specific to the first exon of the constant domain (CH1) for Ig heavy chains, or near the 5′ end the constant domain for Ig light chains (Cκ and Cλ). A sufficient portion of the constant region is retained to allow subsequent identification of the Ig isotype (Fig. 1b).

The amplified sequence, including the 5′UTR and the sequences encoding the leader peptide and the variable region (i.e., with rearranged V—Variable, D—Diversity: restricted to Ig

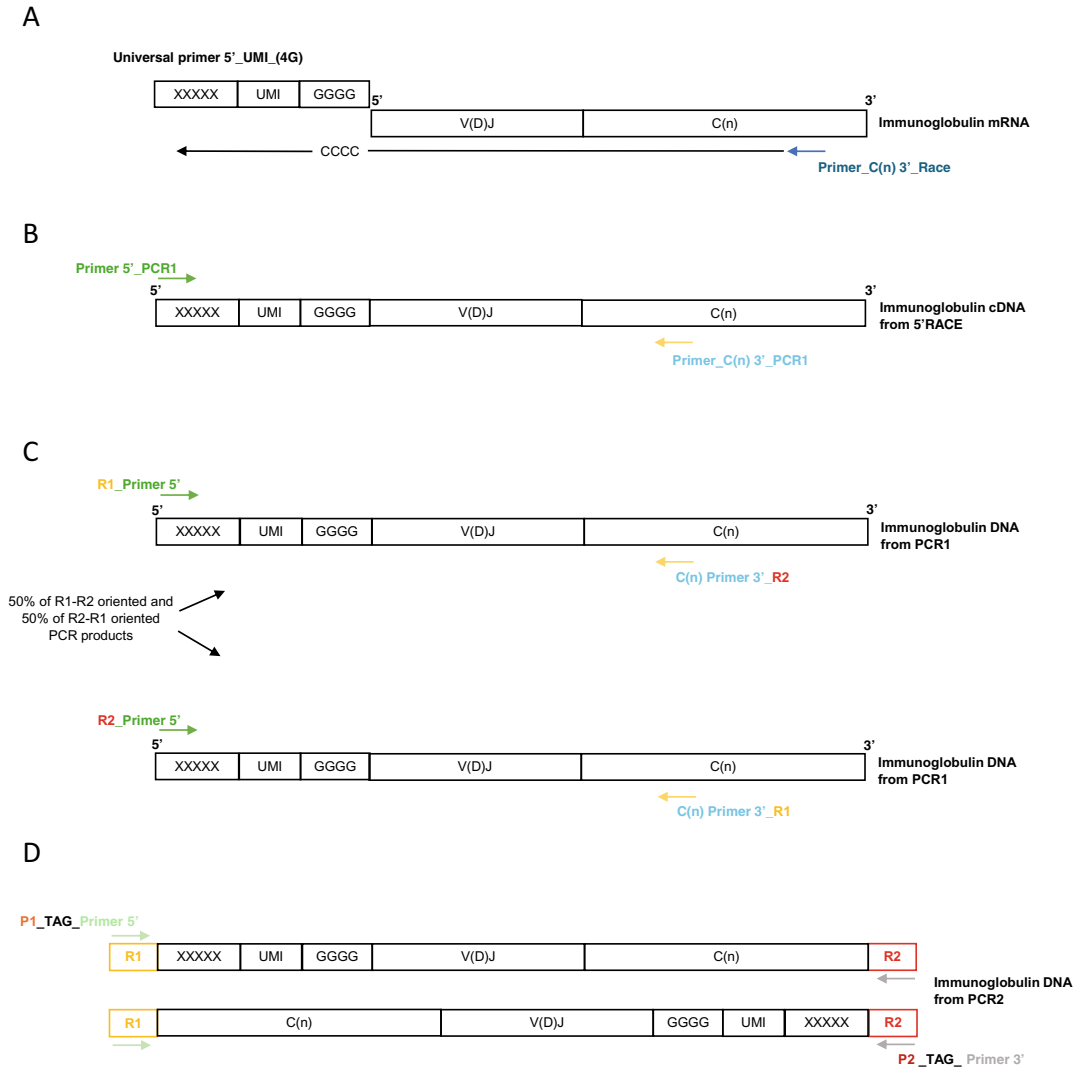


Fig. 1 Experimental Workflow for 5′RACE-RepSeq. **a** Reverse transcription by 5′RACE (Rapid Amplification of cDNA Ends). C(n)3'_RACE is a reverse primer specific for one of the constant regions (Cμ3', Cγ3', Cα3', Cκ3', and Cλ3') of Ig heavy and light chains. The primer forward ("Universal primer 5'_UMI_(4G)") which contains the Unique Molecular Identifier (UMI) sequence and four G ribonucleotides at its 3' end, allows the reverse transcriptase to switch template and continue elongation by integrating a UMI sequence and a defined sequence (XXXXX) at 5′ end of the newly synthesized cDNA, the latter will serve as an anchor for the subsequent PCR. The forward primer "universal primer 5'_UMI_(4A)_(4G)" used as a equimolar mixture with "Universal primer 5'_UMI_(4G)" is not depicted. **b** PCR1 is a semi-nested PCR. C(n)3'_PCR1 is a reverse primer specific for the 5' part of the constant regions of the Ig heavy (CH1) and light chains. The conserved part of the constant region will allow subsequent identification of the Ig isotype. The forward primer (5'_PCR1 primer) anchors in the defined sequence (XXXXX) at the 5' end of the cDNA. **c** PCR2 generates R1-R2 and R2-R1 oriented libraries for asymmetric sequencing. This amplification step allows the addition of R1 and R2 Illumina® linkers (for Read 1 and Read 2, respectively) to PCR1 amplification products. To generate oriented libraries in a 50/50 ratio for each sample, "R1_primer 5' and C(n) Primer 3'_R2" are used as primer pairs on half of the PCR1 amplification products and "R2_primer 5′ and C(n) Primer 3′_R1" for the other half. **d** PCR3 ensures the addition of P1 and P2 of Illumina® adapters in a 50/50 ratio as well as patient-specific indexing. This is done by inserting P1 and P2 adapters at the 5′ and 3′ ends, respectively, on half of the PCR2 products, and an opposite P2-P1 insertion on the other half

heavy chains—and J—Joining segments), has an average length of approximately 600–640 bp on the mRNA. If we add the few nucleotides (nt) used to identify the constant region, most of this length could theoretically be covered by paired-end sequencing using the Illumina® V3 MiSeq kit, which reads 2 × 300 bp. However, for the analytical assembly of the two R1 and R2 reads generated during Illumina sequencing and corresponding to the 5′ and the 3′ part of the amplicon, an overlap of 50 nt is required, reducing the number of complete Ig sequences by 30–40% after bioinformatic analysis.

To address this issue, we adopted a method largely inspired by Turchaninova et al. [10], based on "asymmetric" sequencing reads. Using an Illumina® V2 MiSeq kit and asymmetric 400 + 100 nt paired-end sequencing, this protocol provides sequence lengths up to 750 nt with minimal error. To achieve this, we generate libraries in which each starting RNA molecule is sequenced in both orientations (50/50). A second PCR (PCR2) is therefore carried out to introduce Illumina® linkers (referred to as R1 and R2) on either side of each DNA fragment generated in PCR1. This produces a library containing R1-R2 and R2-R1 oriented sequences (Fig. 1c). In a third PCR (PCR3), Illumina® adapters (P1 and P2) and a patient-specific index (NEXTERA XT index) are annealed to both ends of the library. This process produces a library in which half of the sequences carry the P1 adapter at the 5′ end and the P2 adapter at the 3′ end, while the remaining sequences have the opposite orientation (Fig. 1d). This means that, during sequencing, approximately 50% of the 400-bp read1 (with P1-R1) will cover the cDNA molecules from the 5′ end, while the other 50% will cover the 3′ end.

After sequencing, a bioinformatic analysis of the reads is performed. This involves data pre-processing to convert the raw reads into corrected and annotated Ig sequences. A minimum UMI consensus of five sequences (Molecular Identification Groups, MIG = 5) is applied to the 400 bp 5′-3′ and 3′-5′ aligned sequences, retaining only sequences read at least five times with the same UMI.

Following this pre-processing step, high-quality 400 bp sequences are generated, covering either the 3′ or 5′ end of the cDNA separately. When sequencing the 5′ end of the cDNA, the UMI is located in read1, while for sequences corresponding to the 3′ end, the UMI is extracted from read2 (100 bp). In the next analysis phase, the 400 nt sequences spanning the 5′-3′ direction are merged with the reverse complement of the 3′-5′ sequences, using a common UMI as a reference. This merging process, which includes a minimum 50 bp overlap, results in high-quality 400 + 400 nt paired-end sequences, accurately reconstructing the original cDNA molecules (Fig. 2) [10]. These merged sequences are aligned with the IMGT (international ImMunoGeneTics

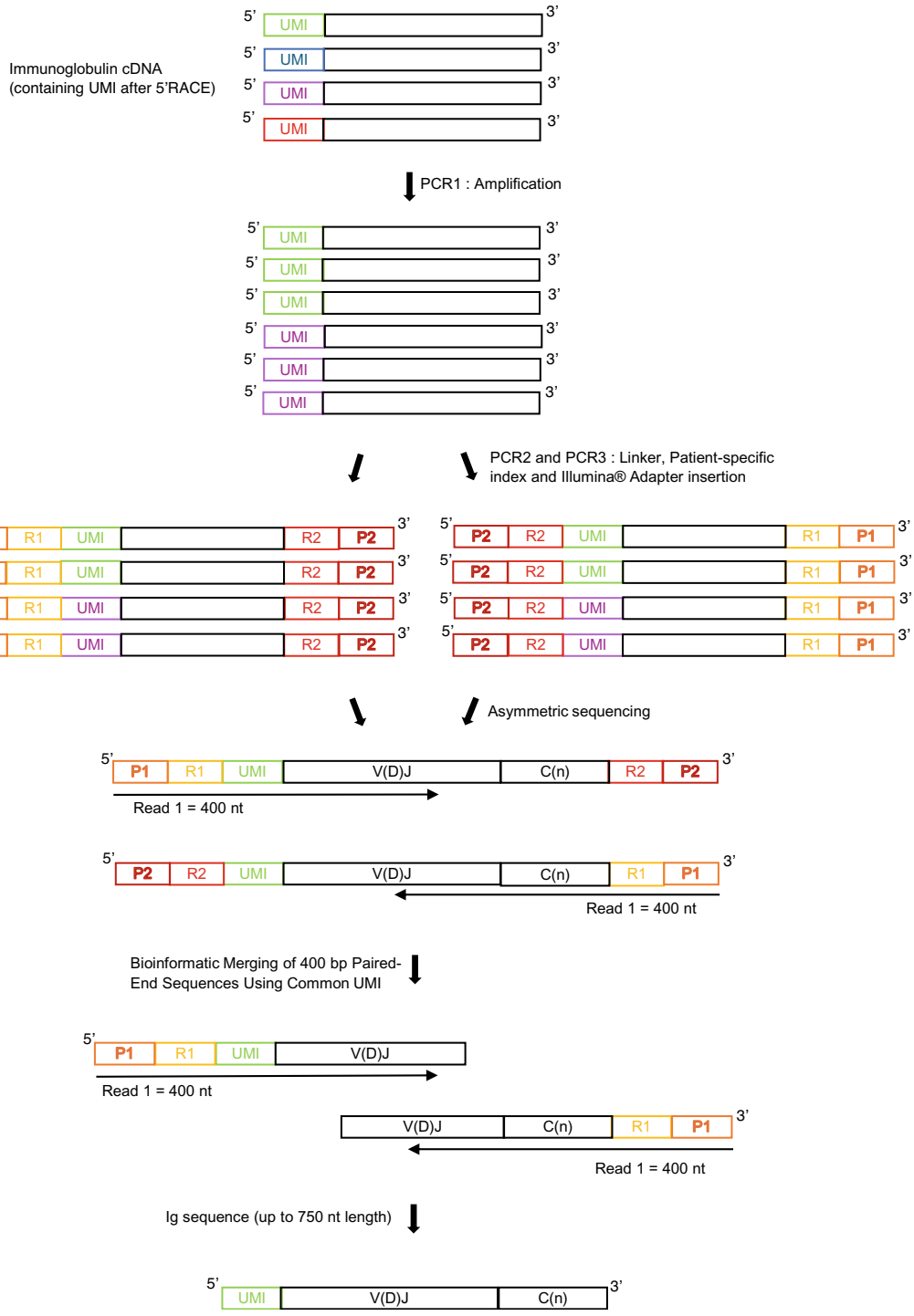


Fig. 2 Bioinformatic Merging of 400 nt Paired-End Sequences Using Common UMI. The generation of a 50/50 oriented library allows for "asymmetric" sequencing. To achieve this, the sequencer is programmed to read 400 nt on Read 1 and 100 nt on Read 2. The bioinformatic assembly of Read 1 based on the UMI generates 400 nt sequences covering the 5′ or 3′ ends of the cDNA separately. In the next analysis step, the 5′-3′ 400 bp sequences are merged with the reverse complement of the 3′-5′ sequences based on a common UMI, with a 50 bp overlap, resulting in paired high-quality 400 + 400 nt sequences of the original cDNA molecules

information system) [11] or IgBLAST database [12] to identify V(D)J segments of the Ig variable regions and to assess the qualitative and quantitative characteristics of the Ig repertoire.

The technical steps required to obtain a human Ig repertoire from an RNA template by high-throughput sequencing are detailed below.

2 Materials

2.1 Sample Collection, RNA Extraction, and 5′ RACE

1. Tempus™ Blood RNA Tubes (ThermoFisher®) to stabilize RNA and lyse cells in one step.
2. Tempus Spin RNA Isolation Reagent Kit (ThermoFisher®).
3. PCR tubes.
4. Nuclease-free water.
5. 10× Dithiothreitol (DTT): 0.1 M.
6. dNTP: mixture of deoxynucleotides in solution at 10 mM each.
7. Protoscript II Reverse Transcriptase (RT): 200,000 U/mL (BioLabs®).
8. 5× Protoscript II RT Reaction Buffer (BioLabs®).
9. Uracil-DNA Glycosylase (UDG): 5000 U/mL (BioLabs®).
10. For reverse transcription of Ig heavy chain mRNAs by 5′RACE (Fig. 1a): forward primers (equimolar mixture of universal primer 5′_UMI_(4A)_(4G) and universal primer 5′_UMI_(4G); *see* Table 1) at a final concentration of 2 μM, and reverse primers (equimolar mixture of IgA heavy chain primer 3′_RACE, IgM heavy chain primer 3′_RACE, and IgG heavy chain primer 3′_RACE; *see* Table 1) at a final concentration of 10 μM (*see* **Notes 1** and **2**).
11. For reverse transcription of Ig light chain mRNAs by 5′RACE (Fig. 1a): forward primers (equimolar mixture of universal primer 5′_UMI_(4A)_(4G) and universal primer 5′_UMI_(4G); *see* Table 1) at a final concentration of 1 μM, and reverse primers (equimolar mixture of Kappa light chain primer 3′_RACE, and Lambda light chain primer 3′_RACE; *see* Table 1) at a final concentration of 10 μM (*see* **Notes 1** and **2**).
12. Nucleospin®, Gel and PCR Clean-up purification Kit (Macherey-Nagel®).
13. Nanodrop spectrophotometer instrument.
14. Microcentrifuge.

Table 1
Primers used for reverse transcription and 5′RACE

Primer name	Sequence
Universal primer 5′_**UMI**_(4A)_(4G)	AGGCAGUGGTGUGACCGCAGAGU**NNNNUNNNNU NNNNUCTT**AAAA(GGGG)
Universal primer 5′_**UMI**_(4G)	AGGCAGUGGTGUGACCGCAGAGU**NNNNU NNNNUNNNNUCTT**(GGGG)
IgA heavy chain primer 3′_RACE	GGAAGTTTCTGGCGGTCACG
IgM heavy chain primer 3′_RACE	GTGATGGAGTCGGGAAGGAAG [10]
IgG heavy chain primer 3′_RACE	GTGTTGCTGGGCTTGTGAT
Kappa light chain primer 3′_RACE	GCGTTATCCACCTTCC [10]
Lambda light chain primer 3′_RACE	CTGGCCGCRTACTTGTTGTT

With R = A or G
List of forward (5′) and reverse (3′) primers. The nucleotide sequences in bold correspond to the Unique Molecular Identifier (UMI), which enables individual molecules to be tracked and amplification bias to be corrected. The 4G ribonucleotides are shown in brackets

Table 2
Primers used for PCR1

Primer name	Sequence
Primer 5′_PCR1	AGGCAGTGGTGTGACCGCAGAGT
IgA heavy chain primer 3′_PCR1	GCGAYGACCACGTTCCCATCT
IgM heavy chain primer 3′_PCR1	CGGGTRCTGCTGATGTCAGA
IgG heavy chain primer 3′_PCR1	GAAGTAGTCCTTGACCAGGCA
Kappa light chain primer 3′_PCR1	GCGGGAAGATGAAGACAGAT
Lambda light chain primer 3′_PCR1	GTTGGCTTGRAGCTCCTCAG

With R = A or G; Y = C or T

2.2 PCR 1, 2, and 3

1. Thermocycler.
2. DMSO.
3. 5× Phusion® HF Reaction Buffer (BioLabs®).
4. 2000 U/mL Phusion® HF DNA Polymerase (BioLabs®).
5. 10 μM forward and 10 μM reverse PCR1 primers (Table 2, *see* **Note 2**).
6. 10 μM forward and 10 μM reverse PCR2 primers (Tables 3a and b, *see* **Notes 2** and **3**).
7. 10 μM forward and 10 μM reverse PCR3 primers (Table 4, *see* **Note 2**).

Table 3
Primers used for PCR2 and the generation of oriented libraries

Primer name	Sequence
(a) Oriented R1-R2 primers	
R1_primer 5′ long	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGG**ACTAGGCAG TGGTGTGACCGCAGAGT
R1_primer 5′ short	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGG**ACT
*R2*_ IgA heavy chain primer 3′	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GCGA YGACCACGTTCCCATCT
*R2*_ IgM heavy chain primer 3′	*GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAG*AGGGGGAAAAGGGTTG
*R2*_ IgG heavy chain primer 3′	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GAAGTAGTCC TTGACCAGGCA
*R2*_ Kappa light chain primer 3′	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GCGGGAAGA TGAAGACAGAT
*R2*_ Lambda light chain primer 3′	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GTTGGCTTG RAGCTCCTCAG
(b) Oriented R2-R1 primers	
*R2*_Primer 5′ long	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG*ACTAGGCAG TGGTGTGACCGCAGAGT
*R2*_Primer 5′ short	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG*ACT
R1_ Ig A heavy chain primer 3′	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GCGA YGACCACGTTCCCATCT
R1_ Ig M heavy chain primer 3′	**TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAG**AGGGGGAAAAGGGTTG
R1_ Ig G heavy chain primer 3′	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GAAGTAGTCC TTGACCAGGCA
R1_Kappa light chain primer 3′	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GCGGGAAGA TGAAGACAGAT
R1_Lambda light chain primer 3′	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTTGGCTTG RAGCTCCTCAG

With R = A or G; Y = C or T
Nucleotide sequences in bold and italics correspond to the Illumina® sequences associated with Read 1 and Read 2, respectively. These sequences ensure correct priming and sequencing start

2.3 Purification Using Magnetic Beads

1. AMPure XP Beads (Beckman Coulter®).
2. 80% Ethanol: 8 mL of 100% ethanol +2 mL of nuclease-free water.
3. Elution buffer: Buffer EB (Quiagen®).
4. 96-well plates.

Table 4
Primers used for PCR3 and sequencing index addition

Primer name	Sequence
*P1*_**Tag**_Primer 5′	
Fw1	*AATGATACGGCGACCACCGAGATCTACAC***TCGACTAG**TCGTCGGCAGCGTC
Fw2	*AATGATACGGCGACCACCGAGATCTACAC***TTCTAGCT**TCGTCGGCAGCGTC
Fw3	*AATGATACGGCGACCACCGAGATCTACAC***CCTAGAGTT**CGTCGGCAGCGTC
Fw4	*AATGATACGGCGACCACCGAGATCTACAC***GCGTAAGAT**CGTCGGCAGCGTC
Fw5	*AATGATACGGCGACCACCGAGATCTACAC***CTATTAAGT**CGTCGGCAGCGTC
Fw6	*AATGATACGGCGACCACCGAGATCTACAC***AAGGCTATT**CGTCGGCAGCGTC
Fw7	*AATGATACGGCGACCACCGAGATCTACAC***GAGCCTTAT**CGTCGGCAGCGTC
Fw8	*AATGATACGGCGACCACCGAGATCTACAC***TTATGCGAT**CGTCGGCAGCGTC
*P2*_**Tag** _Primer 3′	
Rv1	*CAAGCAGAAGACGGCATACGAGAT***TAGCGAGT**GTCTCGTGGGCTCGG
Rv2	*CAAGCAGAAGACGGCATACGAGAT***GTAGCTCC**GTCTCGTGGGCTCGG
Rv3	*CAAGCAGAAGACGGCATACGAGAT***TACTACGC**GTCTCGTGGGCTCGG
Rv4	*CAAGCAGAAGACGGCATACGAGAT***AGGCTCCG**GTCTCGTGGGCTCGG
Rv5	*CAAGCAGAAGACGGCATACGAGAT***GCAGCGTA**GTCTCGTGGGCTCGG
Rv6	*CAAGCAGAAGACGGCATACGAGAT***CTGCGCAT**GTCTCGTGGGCTCGG
Rv7	*CAAGCAGAAGACGGCATACGAGAT***GAGCGCTA**GTCTCGTGGGCTCGG
Rv8	*CAAGCAGAAGACGGCATACGAGAT***CGCTCAGT**GTCTCGTGGGCTCGG

Nucleotide sequences in bold correspond to the patient barcode sequences used for sample identification. Nucleotide sequences in italics represent the Illumina® adapters associated with Read 1 for Primer 5′ (forward) and Read 2 for Primer 3′ (reverse), ensuring compatibility with the sequencing workflow

5. Micro SSP™ PCR Tray Seals (One Lambda®).
6. Magnetic 96-Well Separator: magnetic stand for 96-well plates.
7. Microplate shaker.

2.4 Agarose Gel Migration

1. 0.5× TBE (Tris, Borate, EDTA): add 950 mL of distilled water to 50 mL of ready-to-use 10× TBE buffer solution.
2. 1–2% agarose gel: 1–2 g of agarose in 100 mL of 0.5× TBE, heated three times to boiling point in a microwave followed by addition of Midori Green (10 µL per 100 mL of gel).
3. 6× Gel Loading Dye Orange (BioLabs®).
4. 0.5 µg/µL 100 bp Plus DNA Ladder (ThermoFisher®).
5. Horizontal agarose gel electrophoresis system.

2.5 Quantification, Denaturation, and Sequencing of the Libraries

1. NEBNext Library Quant Kit for Illumina (BioLabs®).
2. MicroAmp® Optical 96 Well Reaction Plate (BioSystems®).
3. MicroAmp® Optical Adhesive Film (BioSystems®).
4. 1 M sodium hydroxide (NaOH) solution.

5. 1.5 mL DNA LoBind Tubes (Eppendorf®).
6. MiSeq® Illumina Sequencer.
7. Miseq Reagent Kit V2 (500 cycles) (Illumina®).
8. PhiX Control V3 (Illumina®).

3 Methods

3.1 Sample Collection, RNA Extraction, and 5′ RACE

1. Total RNA is extracted from samples in Tempus tubes according to the manufacturer's recommendations.
2. RNA concentrations are determined using a Nanodrop Spectrophotometer instrument. Store RNA at −80 °C until use.
3. Thaw all required reagents and RNA on ice. Keep on ice throughout the preparation.
4. Prepare the 5′RACE mix in a 1.5 mL micro tube as described in Table 5 (*see* **Note 4**).
5. Dispense 500 ng/μL (for Ig heavy chain) or 250 ng/μL (for Ig light chain) of RNA into the PCR tubes (1 sample/tube) and adjust the volume to 10 μL with nuclease-free water (*see* **Notes 4**–**6**).
6. Add 1 μL of dNTP and 1 μL of the appropriate reverse primer mix (*see* **Note 2**) to each PCR tube and mix well (final volume per tube = 12 μL). Prepare two separate tubes for reverse transcription of Ig heavy and light chain mRNAs (*see* Subheading 2.1, **items 10** and **11**).
7. Centrifuge briefly and put the tubes in the thermocycler. Then, run the first two steps of the 5′RACE program (*see* Table 6).
8. Stop the thermocycler after 2 min at 42 °C.
9. Centrifuge briefly and immediately place tubes on ice for 2 min.
10. Add 8 μL of the 5′RACE mix (*see* Subheading 3.1, **step 4**) to each tube and mix gently with a pipette (final volume/tube = 20 μL).

Table 5
5′RACE reaction mix (for one sample)

Reagents	Volume
5× Protoscript II RT Buffer	4 μL
10× DTT (0.1 M)	2 μL
Mixture of universal 5′_UMI primers (2 μM for Ig heavy chains or 1 μM for Ig light chains)	1 μL
Protoscript II RT (200,000 U/mL)	1 μL

Table 6
Thermocycler programs

5′RACE	PCR1	PCR2	PCR3
70 °C—3 min	98 °C—2 min	98 °C—2 min	98 °C—2 min
42 °C—2 min	98 °C—10 s	98 °C—10 s	98 °C—10 s
42 °C—60 min	62 °C—30 s } 18 cycles	62 °C—30 s } 16 cycles	62 °C—30 s } 13 cycles
65 °C—20 min	72 °C—45 s	72 °C—45 s	72 °C—45 s
37 °C—40 min	72 °C—5 min	72 °C—5 min	72 °C—5 min

Table 7
PCR1 reaction mix (for one sample)

Reagents	Volume
Nuclease-free water	28.3 µL
5× Phusion® HF Reaction Buffer	10 µL
dNTP (10 mM)	1 µL
DMSO	1.2 µL
Primer 5′_PCR1 (10 µM)	2 µL
Appropriate mixture of reverse Primer_3′ (10 µM)	2 µL
Phusion® HF DNA Polymerase (2000 U/mL)	0.5 µL

11. Centrifuge briefly and the next two steps of the 5′RACE program (*see* Table 6).
12. Stop the thermocycler after 20 min at 65 °C.
13. Place the tubes on ice and add 1 µL of UDG to each tube (*see* **Note 7**).
14. Return the tubes to the thermal cycler and complete the 5′ RACE program (*see* Table 6).
15. Add 20 µL of nuclease-free water to each tube.
16. Purify the cDNA using the "Nucleospin®, Gel and PCR Clean-up" kit, according to the manufacturer's protocol.

    Stop point: at this stage, cDNA can be stored overnight at 4 °C or frozen at −20 °C for long-term storage (up to 1 month).

3.2 Semi-nested PCR1

1. Thaw all required mixes on ice except DMSO, which thaws at room temperature.
2. Prepare the reaction mix as described in Table 7 and add 45 µL to each PCR tube. Prepare two separate tubes for the

amplification of Ig heavy and light chains. Ensure that each tube contains the appropriate reverse primers, and then mix briefly.

3. Add 5 µL of purified cDNA (*see* Subheading 3.1, **step 16**).
4. Centrifuge briefly and run the program for PCR1 as described in Table 6 (*see* **Note 8**).
5. For the purification of the PCR products with AMPure XP Beads (beads/sample volume ratio = 0.6) (*see* **Note 9**), vortex the vial containing the beads (>20 s) and dispense 30 µL into a 96-well plate (multiply by the number of samples).
6. Transfer the PCR products (50 µL) into the wells already containing the beads and mix well by repetitive pipetting.
7. Seal the plate and incubate for 5 min at room temperature on a shaker at 1000 rpm.
8. Meanwhile, prepare an 80% ethanol solution (~600 µL per reaction).
9. Place the plate on a magnetic 96-well separator, remove the seal, and wait for 5 min (*see* **Note 10**).
10. Carefully remove the supernatant with a pipette, avoiding disturbing the ring of separated magnetic beads.
11. Without removing the 96-well plate from the magnetic stand, add 200 µL of 80% ethanol and incubate for 30 s.
12. Gently remove the supernatant with a pipette.
13. Repeat the washing steps twice (*see* Subheading 3.2, **steps 11–13**).
14. Remove all the residual ethanol with a 10 µL pipette and incubate for 4 min at room temperature to ensure that all traces of ethanol are removed (*see* **Note 11**).
15. Remove the plate from the magnet, add 20 µL of elution buffer, and resuspend the beads by vigorous pipetting.
16. Seal the plate and incubate for 2 min at room temperature on a shaker at 1000 rpm.
17. Place the plate on the magnetic sand, remove the seal, and wait for 5 min (*see* **Notes 10** and **12**).
18. At the end of the purification, collect 14 µL of each purified PCR product for the next step (*see* **Note 13**).

    Stop point: at this stage, the PCR products can be stored frozen at −20 °C for a few days.

3.3 PCR2: Illumina® Linker Addition for R1-R2 and R2-R1 Oriented Libraries

For asymmetric reading, this step is performed in duplicate for each sample. Two PCR mixes (R1-R2 and R2-R1) are prepared by changing only the forward and reverse primers. This results in PCR products in which 50% of the fragments have an R1-R2 linker

Table 8
PCR2 reaction mix (for one sample)

Reagents	Volume
Nuclease-free water	11.65 µL
5× Phusion® HF Reaction Buffer	5 µL
dNTP (10 mM)	0.5 µL
DMSO	0.6 µL
Appropriate mixture of (forward) primer 5′ (10 µM)	1 µL
Appropriate mixture of (reverse) primer 3′ (10 µM)	1 µL
Phusion® HF DNA Polymerase (2000 U/mL)	0.25 µL

orientation (i.e., mix with forward R1 primers and R2 reverse primers, *see* Table 3a) and 50% have a R2-R1 orientation (i.e., mix with forward R2 primers and R1 reverse primers, *see* Table 3b).

1. Thaw the reagents on ice except DMSO, which thaws at room temperature.
2. Prepare a reaction mix for PCR2 as described in Table 8.
3. Add 20 µL of the appropriate primer mix (R1-R2 and R2-R1) to a PCR tube and mix briefly.
4. Add 5 µL of the purified PCR1 products (*see* Subheading 3.2, **step 19**) to the corresponding PCR tubes.
5. Run the PCR2 program as described in Table 6 (*see* **Note 8**).
6. Prepare a 2% agarose gel during the PCR run time.
7. When PCR is complete, mix 3 µL of the PCR2 product to 2 µL of 6× Gel Loading Dye Orange solution, and 7 µL of nuclease-free water for each sample in separate tubes and run on a 2% agarose gel for approximately 20 min at 180 V (*see* **Note 14**) (Fig. 3).
8. During migration, purify the remaining PCR products with AMPure XP beads as described in **steps 6–19** in Subheading 3.2, except using 24 µL of the bead solution per 40 µL of sample (pool of R1-R2 and R2-R1–oriented PCR2 products: combine 20 µL of R1-R2 and 20 µL R2-R1 PCR products).
9. At the end of purification, in addition to collecting 14 µL of each purified PCR product for the next step, collect 3 µL of each purified product for quantification on the Nanodrop Spectrophotometer (*see* **Notes 13** and **15**).

   Stop point: at this stage, the purified PCR2 products can be stored frozen at −20 °C for a few days

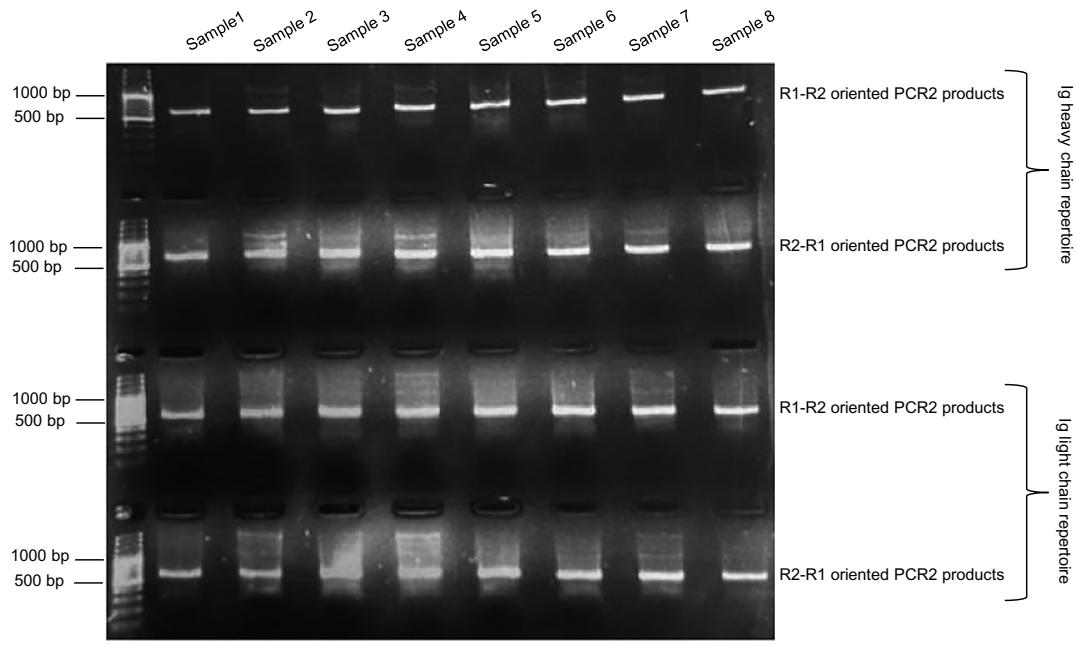


Fig. 3 Electrophoresis gel profile obtained after PCR2. Gel electrophoresis analysis of the PCR2 products from eight samples. The oriented R1-R2 and R2-R1 amplification products obtained for Ig heavy (~850 bp; top lines) and light (~650 bp; bottom lines) chains are represented

Table 9
PCR3 reaction mix (for one sample)

Reagents	Volume
Nuclease-free water	24.5 µL
5× Phusion® HF Reaction Buffer	10 µL
dNTP (10 mM)	1 µL
Phusion® HF DNA Polymerase (2000 U/mL)	0.5 µL

3.4 PCR 3: Insertion of Patient-Specific Index and Illumina® Adapter

1. Thaw the reagents on ice except DMSO, which thaws at room temperature.
2. Prepare a reaction mix for PCR3 as described in Table 9.
3. In a PCR tube, add the volume of PCR2 product required for a DNA amount of 150 ng (maximum 10 µL) and adjust to 10 µL with nuclease-free water if necessary.
4. To create a unique tag combination for each sample, add 2 µL of one of the Illumina forward primers (Fw1-Fw8) and 2 µL of one of the Illumina reverse primers (Rv1-Rv8) to the PCR tubes (Table 4), and mix well.

5. Add 36 µL of the mix (*see* Subheading 3.4, **step 2**) to each PCR tube and mix briefly.
6. Run the PCR3 program as described in Table 6 (*see* **Note 8**).
7. Purify the PCR3 products with AMPure XP beads as described in **steps 6–19** in Subheading 3.2.
8. At the end of the purification, in addition to collecting 14 µL of each purified PCR product for the next step, collect 3 µL of each purified PCR product for quantification on the Nanodrop Spectrophotometer (*see* **Notes 13** and **15**).

   Stop point: at this stage, the purified PCR3 products can be stored at −20 °C (until the library preparation).

3.5 Purification, Denaturation, and Sequencing of the Libraries

To optimize library sequencing, it is essential to perform a final fragment size purification step to eliminate abnormally short PCR products.

1. Pool all the purified and indexed PCR3 products (*see* Subheading 3.4, **step 8**) in a stoichiometric ratio (*see* **Note 16**). At this stage, PCR3 products from Ig heavy and light chain are mixed.
2. Prepare a 1% agarose gel with wide wells to allow loading of a maximum of 150 µL per sample (*see* **Note 17**).
3. Mix the entire pool with 25 µL of Gel Loading Dye Orange (to a maximum of 150 µL of final product) and run the electrophoresis.
4. After migration (Fig. 4), cut a wide band of around 500–1000 bp on the agarose gel to ensure that both Ig heavy and light PCR3 products are harvested. Clean the library using

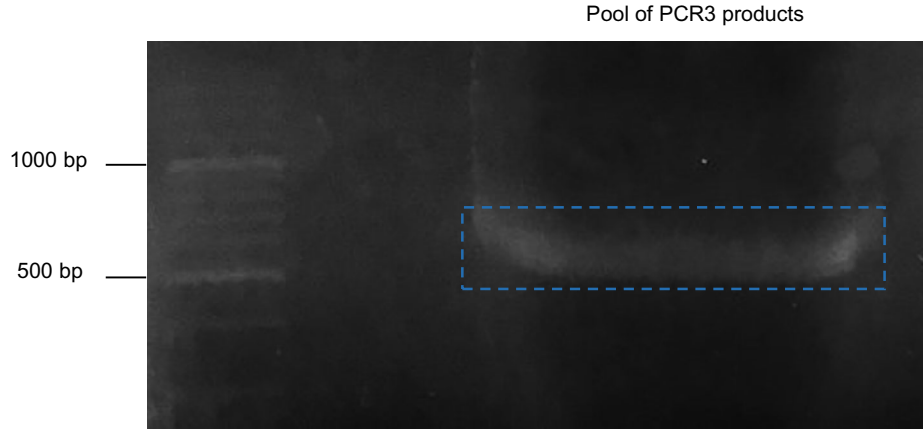


Fig. 4 Electrophoresis gel profile of pooled libraries. After PCR3, the amplification products of Ig heavy and light chain repertoires from the eight samples are pooled. After migration, this pool is cut (blue square) for the next steps of the protocol and high-throughput sequencing of libraries

the "Nucleospin®, Gel and PCR Clean-up Kit" according to the manufacturer's protocol.

5. To determine the library concentration by qPCR, dilute the purified library to 1/10,000 and 1/100,000 to obtain concentrations within the standard curve.
6. Perform qPCR according to the manufacturer's protocol provided in the "NEBNext Library Quant Kit."
7. Dilute the library with nuclease-free water to obtain a concentration of 4 nM in a final volume of 20 μL.
8. Denature the diluted library according to the Illumina "Denature and Dilute Libraries Guide" for the MiSeq System.
9. Prepare a fresh 0.2 M NaOH solution: add 100 μL of 1 M NaOH to 400 μL of nuclease-free water.
10. Mix 5 μL of the library (4 nM solution) with 5 μL of 0.2 M NaOH into a DNA LoBind tube.
11. Vortex and briefly centrifuge the tube, and then incubate for 5 min at room temperature (*see* **Note 18**).
12. Stop denaturation by adding 990 μL of HT1 buffer (included in the MiSeq Reagent Kit V2) to the tube. Mix thoroughly to homogenize. This gives a library concentration of 20 pM in a final volume of 1 mL.
13. To prepare the Illumina sequencing flow cell, thaw the MiSeq V2 Flow cell (500 cycles) (included in the MiSeq Reagent Kit V2) according to the manufacturer's recommendations.
14. Load 600 μL of the library at a concentration of 10 pM. To achieve this, dilute the 20 pM solution obtained in **step 12** with HT1 Hybridization Buffer (included in the MiSeq Reagent Kit V2) to reach a final concentration of 10 pM in a total volume of 600 μL, ensuring that the final mix contains 10% PhiX Control V3.
15. Program the MiSeq Illumina® sequencer to read 401 nt on Read 1 (R1) and 101 nt on Read 2 (R2). Insert the flow cell and start the run. Then, proceed to the analysis (*see* **Note 19**).

4 Notes

1. The forward primer solution used in the 5′RACE step consists of an equimolar mixture of two primers that differ by the presence or absence of 4A between the UMI sequence and the 4G ribonucleotides. To achieve this, add 5 μL of each primer solution (100 μM) to 90 μL of nuclease-free water. This design creates a read offset during sequencing, preventing

simultaneous reading of the same base across the entire flow cell. This approach avoids potential sequencing anomalies, or even complete stoppage of the sequencing process.

2. To optimize analysis time and reduce costs, we use a multiplex PCR approach to simultaneously amplify the repertoires of the different Ig heavy chain isotypes (except IgE) on one hand and the repertoires of the kappa and lambda light chains on the other hand. Therefore, for each step of this protocol, we prepare a mix containing all the Ig heavy chain-specific reverse primers at a concentration of 10 μM each by adding 10 μL of each primer solution (100 μM) in a final volume of 100 μL adjusted with nuclease-free water. Follow the same procedure for the mix of kappa and lambda light chain reverse primers.
3. Prepare a mixture of long and short forward primers by adding 2 μL of long primer and 8 μL short primer to 90 μL of nuclease-free water. This will increase amplification efficiency.
4. In the bioinformatics step, in order to effectively correct PCR and sequencing errors, it is necessary to group at least five reads that share the same UMI (MIG = 5) [7], originating from the same template cDNA molecule. However, within a repertoire, the number of reads sharing the same UMI depends on a balance between several parameters: the amount of cDNA, the number of samples grouped on the same flow cell, and the capacity of the latter in terms of pass-filter fragments. In this context, homogenization of the library preparation and determination of the minimum number of reads required for each repertoire are essential for optimal use of the UMI tool. An excess of cDNA molecules (or UMIs) to be analyzed will result in insufficient coverage of each molecule and an inability to efficiently correct Ig sequences. Conversely, an insufficient number of cDNAs can reduce the depth of analysis. Use an excess of 500 ng template RNA and a limited amount of UMI-containing 5′RACE primer at 2 μM (dilution of the previously prepared pool to 10 μM) for the heavy chain repertoire. These amounts of template RNA and primer are halved for the light chain repertoire. If the amount of RNA is too low, increase the amount of UMI-containing 5′RACE primer to achieve better coverage of the repertoire.
5. When the RNA concentration exceeds 500 ng/μL for the Ig heavy chain repertoire or 250 ng/μL for the light chain repertoire, the volume required to obtain an adequate amount of RNA is less than 1 μL. These RNAs are first diluted to one-tenth of their original volume to avoid working with such a small amount.

6. Separate experiments must be performed for the Ig heavy and light chain repertoires. Therefore, it is necessary to prepare the tubes containing the heavy chain primer and the light chain primer independently of each other.
7. Uracil DNA Glycosylase (UDG) is used to eliminate the uracil residues in the universal forward primer.
8. It is recommended to bring AMPure XP beads to room temperature 30 min before use. To avoid forgetting this step, remove them immediately after starting the PCR program.
9. A bead to sample ratio of 0.6 allows elimination of small DNA fragments.
10. To avoid contamination, the seal must be carefully removed as small drops of sample may be deposited on it during shaking.
11. To avoid DNA loss, it is essential that the evaporation time does not exceed 4 min.
12. To promote bead fixation and facilitate pipetting due to the low volume, it is recommended to use a system that raises the 96-well plate by a few millimeters above the magnetic plate during this step.
13. This step is delicate due to the risk of sample contamination by the beads during pipetting. To minimize this risk, pipette the 14 μL in two steps of 7 μL each. This reduces the risk of contamination and ensures sample quality.
14. The expected sizes for Ig heavy and light chain PCR2 products are ~850 bp and ~650 bp, respectively. This gel also allows verification that the amplification has been effective and consistent between the two orientations, R1-R2 and R2-R1 PCR2 products.
15. At this stage, the concentration of the samples must be higher than 15 ng/μL to obtain a high-quality depth repertoire.
16. To maintain the relative abundance of each product, be sure to pool the PCR3 products in a stoichiometric ratio. This can be done by estimating the concentration of each of the PCR products using a Nanodrop Spectrophotometer and combining them in a ratio that reflects their original amounts. The different fragment sizes between the Ig heavy and light chain PCR3 products must be considered. Accurate stoichiometric mixing is critical to maintaining the representation of each sample in the final library.
17. If you don't have a wide-tooth comb that can hold 150 μL of product, you can use tape to attach several teeth of the comb together to create a large well.

18. Do not exceed the time indicated to avoid DNA degradation.
19. For accurate Ig repertoire analysis, we expect 500,000 to one million raw reads per sample. A MiSeq V2 Flow cell (500 cycles) will yield approximately 15 million reads. To achieve repertoires of good depth, we have decided to limit the number of samples to 16 per library. Each sample includes three Ig heavy chain isotypes (IgM, IgG, and IgA) and the two Ig light chains (Kappa and Lambda).

Acknowledgments

This work was supported by grants from the Fondation Française pour la Recherche contre le Myélome et les Gammapathies monoclonales (FFRMG) and the Ligue Contre le Cancer. We also thank Pr. Sirac Christophe and Pr. Cogné Michel for their guidance during the initial development of this method in our laboratory.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Calis JJA, Rosenberg BR (2014) Characterizing immune repertoires by high throughput sequencing: strategies and applications. Trends Immunol 35:581–590. https://doi.org/10.1016/j.it.2014.09.004
2. Agathangelidis A, Darzentas N, Hadzidimitriou A et al (2012) Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. Blood 119:4467–4475. https://doi.org/10.1182/blood-2011-11-393694
3. Parameswaran P, Liu Y, Roskin KM et al (2013) Convergent antibody signatures in human dengue. Cell Host Microbe 13:691–700. https://doi.org/10.1016/j.chom.2013.05.008
4. Tipton CM, Fucile CF, Darce J et al (2015) Diversity, cellular origin and autoreactivity of antibody-secreting cell population expansions in acute systemic lupus erythematosus. Nat Immunol 16:755–765. https://doi.org/10.1038/ni.3175
5. Georgiou G, Ippolito GC, Beausang J et al (2014) The promise and challenge of high-throughput sequencing of the antibody repertoire. Nat Biotechnol 32:158–168. https://doi.org/10.1038/nbt.2782
6. Bender S, Javaugue V, Saintamand A et al (2020) Immunoglobulin variable domain high-throughput sequencing reveals specific novel mutational patterns in POEMS syndrome. Blood 135:1750–1758. https://doi.org/10.1182/blood.2019004197
7. Egorov ES, Merzlyak EM, Shelenkov AA et al (2015) Quantitative profiling of immune repertoires for minor lymphocyte counts using unique molecular identifiers. J Immunol 194:6155–6163. https://doi.org/10.4049/jimmunol.1500215
8. Khan TA, Friedensohn S, Gorter de Vries AR et al (2016) Accurate and predictive antibody repertoire profiling by molecular amplification fingerprinting. Sci Adv 2:e1501371. https://doi.org/10.1126/sciadv.1501371
9. Friedensohn S, Khan TA, Reddy ST (2017) Advanced methodologies in high-throughput sequencing of immune repertoires. Trends Biotechnol 35:203–214. https://doi.org/10.1016/j.tibtech.2016.09.010
10. Turchaninova MA, Davydov A, Britanova OV et al (2016) High-quality full-length immunoglobulin profiling with unique molecular barcoding. Nat Protoc 11:1599–1616. https://doi.org/10.1038/nprot.2016.093

11. Alamyar E, Duroux P, Lefranc M-P, Giudicelli V (2012) IMGT(®) tools for the nucleotide analysis of immunoglobulin (IG) and T cell receptor (TR) V-(D)-J repertoires, polymorphisms, and IG mutations: IMGT/V-QUEST and IMGT/HighV-QUEST for NGS. Methods Mol Biol 882:569–604. https://doi.org/10.1007/978-1-61779-842-9_32

12. Ye J, Ma N, Madden TL, Ostell JM (2013) IgBLAST: an immunoglobulin variable domain sequence analysis tool. Nucleic Acids Res 41:W34–W40. https://doi.org/10.1093/nar/gkt382

Chapter 8

5′RACE Method for RNA-Based Sequencing of the Mouse Immunoglobulin Repertoire

Sandy Al Hayek, Antoine Praité, Jean-Marie Lambert, Sébastien Bender, and Laurent Delpy

Abstract

This chapter outlines a detailed methodology for high-throughput sequencing of the immunoglobulin (Ig) repertoire from mouse RNA samples. We describe RNA extraction from splenic B cells, followed by the conversion into cDNA using the 5′RACE (Rapid Amplification of cDNA Ends) technique and PCR amplification of the Ig heavy and light chain repertoire. The amplified products are prepared using specific primers for Illumina next-generation sequencing (NGS) and unique molecular identifiers (UMI) as molecular barcodes. The protocol includes stringent quality control steps to ensure the integrity of RNA and the accuracy of amplification. We describe the amplification of *Ighm* and all *Ighg* subclasses, for Ig heavy chains and for the predominant Igκ light chain isotype. RNA-based 5′RACE-RepSeq proves very useful to analyze the diversity and clonality of Ig repertoire, as well as somatic hypermutations, providing insights into the adaptive immune response. This method enables the high-throughput characterization of the mouse Ig repertoire in immunological contexts.

Key words Immunoglobulin repertoire, RNA, 5′RACE, Next-generation sequencing, Paired-end sequencing, Unique molecular identifiers (UMI)

1 Introduction

Immunoglobulins (Ig) can be expressed as B cell receptors (BCRs) on the surface of B lymphocytes or secreted by plasma cells in the form of antibodies. Ig is at the heart of the adaptive immune response, enabling the body to accurately recognize, neutralize, and remember foreign antigens [1].

The process of V(D)J recombination ensures that each B cell clone expresses a unique Ig capable of binding to a specific antigen. In bone marrow B cell progenitors, the joining of VDJ (variable-diversity-joining) segments starts at the Ig heavy chain locus (IgH), followed by VJ-joining at the Ig light chain locus [1, 2]. In the mouse, the vast majority (~95%) of B cells express Igκ light chains,

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_8,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

while Igλ-expressing cells are far less frequent [3]. Random selection of V(D)J segments and imprecise junctions create highly diverse variable domains with unique CDR3 (complement determining region 3) regions involved in antigen binding.

Once positively selected, mature B cells migrate to secondary lymphoid organs and may undergo a further series of Ig modifications via somatic hypermutations (SHM) in variable exons to increase Ig affinity for antigen, and class switch recombination (CSR) replacing constant Cμ exons with a constant downstream gene [4, 5]. Instead of IgM and IgD expressed on naive B cells, class-switched B cells express various Ig isotypes (IgG, IgA, IgE) with distinct effector functions.

Analysis of the Ig repertoire and the preferential expansion of clones enables us to interpret the humoral immune response in different physiological and pathological contexts. High-throughput sequencing of Ig repertoire offers a powerful approach for examining antibody diversity [6]. This is critical for applications such as evaluating vaccine efficacy, understanding immune responses to infections, and investigating autoimmune or monoclonal disorders.

This chapter describes RNA-based amplification of the mouse Ig repertoire using 5′ RACE (Rapid Amplification of cDNA-ends) combined with semi-nested PCR strategy [7, 8]. Reverse transcription (RT) is performed using antisense primers targeting constant exons of Ig heavy (Cμ and all Cγ subclasses) and light (Cκ) chains. The reverse transcriptase enzyme incorporates a series of deoxynucleotides (dC) at the 5′-end of the cDNA, and the addition of a Template Switch Oligo (TSO) containing ribonucleotide (4G) allows the continuation of cDNA synthesis [7–9]. This TSO serves as the anchor point for the subsequent PCR amplification, enabling the specific detection of complete variable exons across a broad range of Ig isotypes. The resulting amplicons are then sequenced on the Illumina® platform to generate high-quality reads that represent the diverse Ig repertoire (Fig. 1).

By incorporating unique molecular identifiers (UMIs) during the amplification process, we ensure the accurate identification of each individual cDNA molecule, thus minimizing sequencing errors and amplification biases [10]. This methodology allows us to capture both abundant and rare Ig clones, providing a comprehensive view of the immune response. The use of paired-end sequencing with overlapping reads, along with advanced bioinformatic tools, enables the reconstruction of full-length Ig sequences, which are then aligned with reference databases such as IMGT [11] or IgBLAST [12] for detailed analysis of the variable regions, clonality, and isotype distribution of the Ig repertoire.

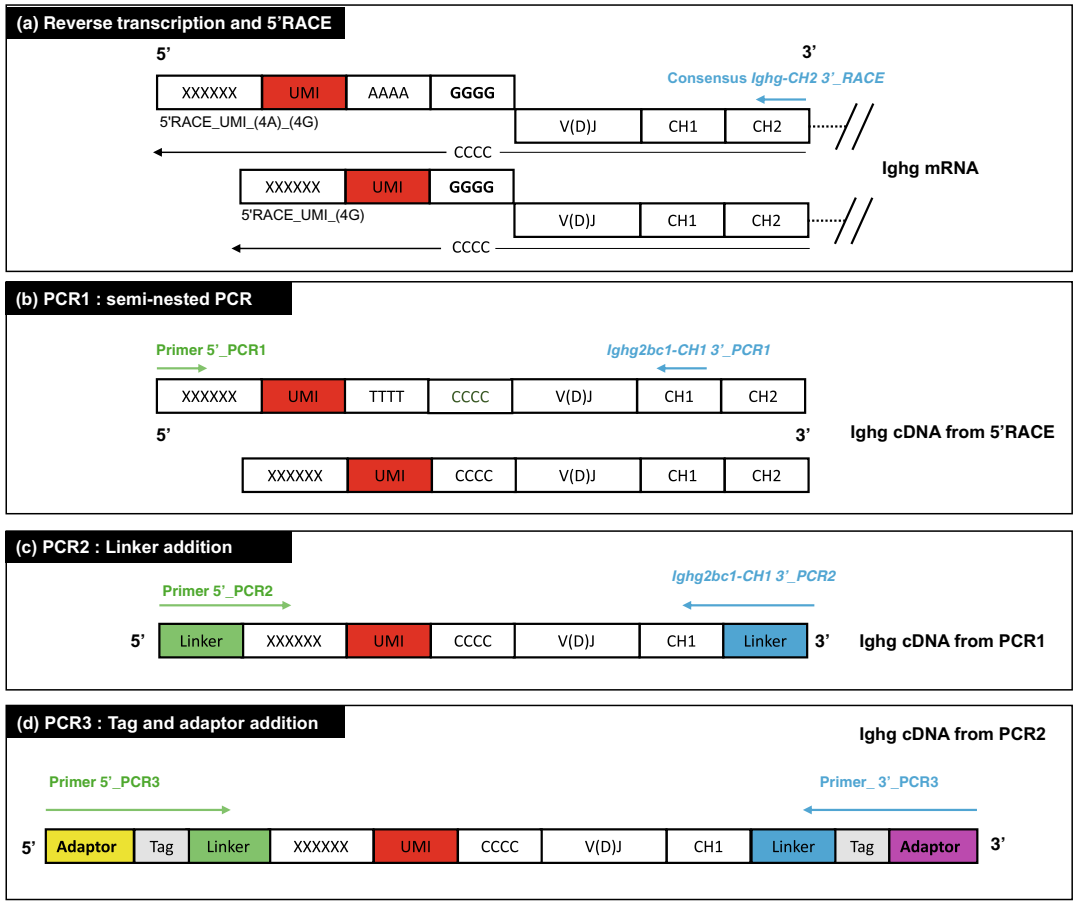


Fig. 1 Experimental Workflow for 5′RACE-based cDNA Amplification of Mouse *Ighg*. (**a**) 5′RACE is performed using the reverse primer *Primer_3′_RACE*, which specifically anneals to the constant region of Ig heavy or light chains ($C\gamma$, $C\mu$, $C\kappa$ in this study). For clarity, we illustrate the workflow using a consensus primer targeting a conserved region in constant *CH2* exon, common to *Ighg1, Ighg2b, Ighg2c*, and *Ighg3* isotypes. Two forward primers, that differ only by the presence of 4A, are used in equimolar amounts: *5′RACE_UMI_(4G)* and *5′ RACE_UMI_(4A)_(4G)*. Both primers contain a unique molecular identifier (UMI) sequence, followed by four G ribonucleotides (bold) at their 3′ end. These ribonucleotides act as template-switching signals, allowing the reverse transcriptase to extend the cDNA while incorporating the UMI and a defined sequence (*XXXXX*) at the 5′ end. This sequence serves as an anchor for subsequent PCR amplifications. (**b**) PCR1: a semi-nested PCR1 is performed to amplify the cDNA. The nested reverse primer (*Primer_3′_PCR1* targets the 5′ part of the constant region (CH1) while the forward primer *Primer_5′_PCR1* binds to the defined sequence (*XXXXX*) at the 5′ end of the cDNA, ensuring selective amplification of full-length transcripts containing the UMI. (**c**) PCR2: linker addition to shape the amplicons for the addition of sequencing tags and adaptors in the next step. (**d**) PCR3: the final PCR incorporates sequencing adaptors and sample-specific Tags on the libraries for paired-end sequencing. The expected size of the final amplicons is approximately 600–700 bp

2 Materials

2.1 Isolation of Spleen Cells

1. C57BL/6 mice.
2. Surgery equipment (scissors, tweezers).
3. 6-well plates.
4. 1× phosphate-buffered saline (PBS), pH 7.4.
5. 10 mL syringe.
6. 15 mL and 50 mL sterile conical tubes.
7. 1× ammonium-chloride-potassium (ACK) lysis buffer to lyse red blood cells.
8. Centrifuge.
9. 70 μm cell strainer.
10. Trypan blue.
11. Cell counting machine.

2.2 RNA Extraction and Column-Based Purification

1. Pipettes and tips.
2. TRIzol™ reagent.
3. Absolute, 95% and 70% ethanol molecular biology grade.
4. Direct-zol RNA Micropreps Kits (Zymo Research): prepare Direct-zol™ RNA PreWash and RNA wash buffer in 95% ethanol, according to the manufacturer's recommendation.
5. Zymo-Spin™ IC Columns and collection tubes.
6. RNase-free 1.5 mL tubes.
7. Microcentrifuge.
8. Nuclease-free water.
9. NanoDrop spectrophotometer or Qubit fluorometer with appropriate quantification assay kits.
10. RNase surface decontamination solution.

2.3 Reverse Transcription and PCR Amplification: 5′ RACE, PCR1, PCR2, and PCR3

1. 25–500 ng of RNA samples (*see* **Note 1**).
2. 4 μM Forward RACE primers (Template Switch Oligo): equimolar mixture of 2 μM universal primer 5′_UMI_(4A)_(4G) and 2 μM universal primer 5′_UMI_(4G) (*see* Table 1).
3. 10 μM Reverse RACE primers (*see* Ig isotype-specific Primer_3′_RACE in Table 1).
4. 10 μM Forward Primer 5′_PCR1 and 10 μM Reverse PCR1 primers (Table 2).
5. 10 μM Forward Primer 5′_PCR2 (mixture of 8 μM UnivShortFw+linker primer and 2 μM of UnivLongFw+linker primer) and 10 μM Reverse PCR2 primers (Table 3).

Table 1
Primers used for Reverse Transcription and 5′RACE on mouse Ig mRNAs

Primer Name	Sequence
Universal primer 5′_**UMI**_(4A) _(4G)	AGGCAGUGGTGUGACCGCAGAGU**NNNNUNNNNU NNNNUCTT**AAAA(GGGG)
Universal primer 5′_**UMI**_(4G)	AGGCAGUGGTGUGACCGCAGAGU**NNNNUNNNNU NNNNUCTT**(GGGG)
Consensus *Ighg-CH2 3′_RACE*	TGACCYTGCATTTGAACTCC
Ighm-CH1 3′_RACE	CAGGTGAAGGAAATGGTGCT
Igκ 3′_RACE	GAGGCACCTCCAGATGTTAACTG

With Y = C or T
The universal forward (5′) primers contain unique molecular identifiers (UMI) (bold) and 4G ribonucleotides (brackets). The consensus reverse (3′) *Ighg* primer targets a conserved sequence in the constant CH2 exon of all Ighg isotypes (Ighg3, Ighg1, Ighg2b, and Ighg2c). The *Ighm* and *Igκ* reverse primers anneal to the constant CH1 and Cκ exons, respectively

Table 2
Primers used for PCR1

Primer name	Sequence
Primer 5′_PCR1	AGGCAGTGGTGTGACCGCAGAGT
Ighg2bc1-CH1 3′_PCR1	CAGGGGCCAGTGGATAGAC
Ighg3-CH1 3′_PCR1	CCAGGGACCAAGGGATAGAC
Ighm-CH1 3′_PCR1	GGGAAGACATTTGGGAAGGA
Igκ-3′_PCR1	GTGGGAAGATGGATACAGTTGG

Sequence of forward (Primer 5′_PCR1) primer correspond to the 5′-end of Universal primer 5′ used for 5′RACE. Nested reverse (3′) primers hybridize in the 5′-end of CH1 and Cκ exons. Ighg2bc1-CH1 3′_PCR1 primer is specific for Ighg2b, Ighg2c, and Ighg1 isotypes

6. 10 μM Forward and Reverse PCR3 primers (Table 4).
7. 10 mM dNTP mix.
8. Thermocycler.
9. Microcentrifuge.
10. Ice.
11. 5× Protoscript II Buffer.
12. 200,000 U/mL Protoscript II Reverse transcriptase (RT).
13. 10× DTT: 0.1 M.
14. 5000 U/mL Uracil-DNA glycosylase.
15. 2000 U/mL Phusion® High-Fidelity (HF) DNA Polymerase.
16. 5× Phusion Reaction buffer.

Table 3
Primers used for PCR2 and linker addition

Primer name	Sequence
Primer 5′_PCR2	
UnivShortFw+linker (4×)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGACT**
UnivLongFw+linker (1×)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGACT**AGGCAG TGGTGTGACCGCAGAGT
Ighg2bc1-CH1 3′ _PCR2	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CAGGGGCCAG TGGATAGAC
Ighg3-CH1 3′ _PCR2	**GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAG**CCAGGGACCAAGGGATAGAC
Ighm-CH1 3′ _PCR2	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGGAAGACA TTTGGGAAGGA
Igκ-3′_PCR1	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GTGGGAAGA TGGATACAGTTG

The linker (bold) sequences included in forward and reverse primers allow the subsequent addition of Illumina sequencing adapters. The 3′-end sequence of UnivLongFw+linker is identical to Primer 5′_PCR1 forward primer. 4× indicates a four-fold higher concentration of the UnivShortFw+linker primer, exhibiting only the linker (bold) sequence, than the UnivLongFw+linker primer in the mixture of forward primers used in PCR2

Table 4
Primers Used for PCR3 and Sequencing Index Addition

Primer Name	Sequence
P1_**Tag**_Primer 5’	
Fw1	**AATGATACGGCGACCACCGAGATCTACAC**TCGACTAGTCGTCGGCAGCGTC
Fw2	**AATGATACGGCGACCACCGAGATCTACAC**TTCTAGCTTCGTCGGCAGCGTC
Fw3	**AATGATACGGCGACCACCGAGATCTACAC**CCTAGAGTTCGTCGGCAGCGTC
Fw4	**AATGATACGGCGACCACCGAGATCTACAC**GCGTAAGATCGTCGGCAGCGTC
Fw5	**AATGATACGGCGACCACCGAGATCTACAC**CTATTAAGTCGTCGGCAGCGTC
Fw6	**AATGATACGGCGACCACCGAGATCTACAC**AAGGCTATTCGTCGGCAGCGTC
Fw7	**AATGATACGGCGACCACCGAGATCTACAC**GAGCCTTATCGTCGGCAGCGTC
Fw8	**AATGATACGGCGACCACCGAGATCTACAC**TTATGCGATCGTCGGCAGCGTC
P2_**Tag** _Primer 3’	
Rw1	**CAAGCAGAAGACGGCATACGAGAT**TAGCGAGTGTCTCGTGGGCTCGG
Rw2	**CAAGCAGAAGACGGCATACGAGAT**GTAGCTCCGTCTCGTGGGCTCGG
Rw3	**CAAGCAGAAGACGGCATACGAGAT**TACTACGCGTCTCGTGGGCTCGG
Rw4	**CAAGCAGAAGACGGCATACGAGAT**AGGCTCCGGTCTCGTGGGCTCGG
Rw5	**CAAGCAGAAGACGGCATACGAGAT**GCAGCGTAGTCTCGTGGGCTCGG
Rw6	**CAAGCAGAAGACGGCATACGAGAT**CTGCGCATGTCTCGTGGGCTCGG
Rw7	**CAAGCAGAAGACGGCATACGAGAT**GAGCGCTAGTCTCGTGGGCTCGG
Rw8	**CAAGCAGAAGACGGCATACGAGAT**CGCTCAGTGTCTCGTGGGCTCGG
Rw9	**CAAGCAGAAGACGGCATACGAGAT**GTCTTAGGGTCTCGTGGGCTCGG
Rw10	**CAAGCAGAAGACGGCATACGAGAT**ACTGATCGGTCTCGTGGGCTCGG
Rw11	**CAAGCAGAAGACGGCATACGAGAT**TAGCTGCAGTCTCGTGGGCTCGG
Rw12	C**AAGCAGAAGACGGCATACGAGAT**GACGTCGAGTCTCGTGGGCTCGG

The P1_Tag_Primer 5′ (Fw1 to Fw8) and P2_Tag_Primer 3′ (Rw1 to Rw12) primers include the P1 (bold) and P2 (bold) adapters, respectively, which are required for Illumina sequencing. These primers also contain unique tag sequences for indexing (red), which help identify different samples during sequencing. Each primer set is designed to add specific sequences for efficient amplification and indexing. The 3′-end sequence of these primers is identical to the linker sequence added during PCR2

2.4 Agarose Gel Migration

1. 0.5× TBE (Tris, Borate, EDTA): add 950 mL of distilled water to 50 mL of ready-to-use 10× TBE buffer solution.
2. 1–2% agarose gel: 1–2 g of agarose in 100 mL of 0.5× TBE, heated three times to boiling point in a microwave followed by addition of Midori Green (10 μL per 100 mL of gel).
3. 6× Gel Loading Dye Orange.
4. 0.5 μg/μL 100 bp Plus DNA Ladder.
5. Horizontal Agarose Gel Electrophoresis System.
6. Scalpel.
7. UV lamp.

2.5 Purification of PCR Products Using Columns and Magnetic Beads

The column-based purification kit is useful for both gel extraction or PCR clean up.

1. Nucleospin, Gel and PCR Clean-up purification kit.
2. Binding buffer.
3. Wash buffer (prepared according to manufacturer's recommendations).
4. Elution buffer.
5. Extraction columns.
6. Collection tubes.
7. Thermomixer.
8. Vortex.
9. DNA LoBind 1.5 mL tubes.
10. AMPure XP Beads (Beckman Coulter®).
11. Magnetic Rack.
12. 80% Ethanol solution.

2.6 Library Quantification and Sequencing

1. NEBNext® Library Quant Kit for Illumina® (New England BioLabs®).
2. MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems®).
3. MicroAmp® Optical Adhesive Film (Applied Biosystems®).
4. Real-time PCR machine.
5. DNA LoBing tubes.
6. 10 mM sodium hydroxide solution.
7. 200 mM Tris–HCl, pH 7.0.
8. MiSeq Reagent Kit v3 (600 cycles) (Illumina®).
9. PhiX Control v3 (Illumina®).
10. Illumina MiSeq sequencing platform.

3 Methods

3.1 Isolation of Mouse Spleen Cells

1. Collect the spleen and place the organ in one well of a 6-well plate.
2. Add 2 mL of 1× PBS and dissociate the spleen with the plunger of a 10 mL syringe.
3. Transfer into 15 mL conical tubes.
4. Add 5 mL of 1× ACK lysis buffer to lyse the red blood cells.
5. Incubate for 5 min at room temperature. Shake lightly and regularly to thoroughly lyse red cells.
6. Add 10 mL of 1× PBS to dilute 1× ACK and stop cell lysis.
7. Centrifuge the tubes at 300× *g* for 7 min at room temperature.
8. Resuspend the cell pellet by gently pipetting up and down in 1 mL of 1× PBS.
9. Pass the cell suspension through a 70 μm cell strainer to remove debris.
10. Place the tubes on ice and take a small aliquot for cell counting (optional: use Trypan blue to assess viability).
11. Take the cell volume required for the next steps ($1–2 \times 10^6$ cells) and centrifuge at 300× *g* for 7 min.
12. Resuspend the pellet in 350 μL of TRIzol™ (1 mL TRIzol per $5–10 \times 10^6$ cells).
13. Pipette vigorously and gently vortex. Incubate for 5 min at room temperature.
14. Store the samples at −80 °C for long-term storage or proceed to the next steps for RNA extraction.

3.2 RNA Extraction and Column-Based RNA Purification

Clean the workstation with an RNase surface decontamination solution.

1. Thaw RNA at room temperature (if necessary). Add 350 μL of absolute ethanol (equal volume to the TRIzol), in each sample tube.
2. Shake the tubes vigorously for 1 min. Do not vortex.
3. Transfer the 700 μL into a Zymo-Spin™ IC Column placed in a Collection tube.
4. Centrifuge at 12,000× *g* for 30 s and discard the flow-through.
5. Place the column in a new collection tube.
6. Add 400 μL of Direct-zol™ RNA PreWash to the column.
7. Centrifuge at 12,000× *g* for 30 s and discard the flow-through.
8. Repeat **steps 6** and 7, and then add 700 μL of RNA Wash Buffer to the column.

9. Centrifuge at 12,000× *g* for 1 min to ensure thorough removal of the wash buffer.
10. Transfer the column carefully into a 1.5 mL RNase-free tube.
11. To elute RNA, carefully add 15 μL of DNase/RNase-free water directly to the column matrix in contact with the column filter.
12. Incubate for 1 min and centrifuge at 12,000× *g* for 1 min.
13. Place the RNA samples on ice.
14. Measure RNA concentration and purity using a NanoDrop spectrophotometer.
15. Store RNA samples at −80 °C or process immediately.

3.3 5′RACE (Rapid Amplification of cDNA-Ends)

The 5′RACE method uses a gene-specific reverse primer for RT and a Template Switch Oligonucleotide to extend the 5′ region of the cDNA. The 5′RACE technique is useful for characterizing 5′ untranslated regions (5′ UTRs) and alternative transcription start sites. This method is also well suited to amplifying highly diverse 5′-ends of Ig transcripts (Fig. 1a).

1. Preparation of Mix 1: ~500 ng of total RNA, 1 μL of Ig isotype-specific reverse primer (Primer_3′_RACE, *see* Table 1), 1 μL of dNTP, and nuclease-free water to a final volume of 12 μL (*see* **Note 2**).
2. Incubate the mix for 3 min at 72 °C and 2 min at 42 °C (*see* Table 5).
3. Perform a short spin and place on ice for 2 min.
4. Meanwhile, prepare Mix 2: 4 μL of 5× Protoscript II Buffer, 2 μL of DTT, 1 μL of Protoscript II RT, and 1 μL of RACE forward primers (equimolar mixture of Universal primer 5′_UMI_(4A)_(4G) and Universal primer 5′_UMI_(4G); *see* Table 1).

Table 5
Thermocycler programs for each step

5′RACE	PCR1	PCR2	PCR3
72 °C—3 min	98 °C – 2 min	98 °C – 2 min	98 °C – 2 min
42°C—2 min 42°C—60 min 65°C—20 min	98°C—10 s 62°C—30 s 72°C—45 s } 18 cycles	98°C—10 s 62°C—30 s 72°C—30 s } 17 cycles	98°C—10 s 62°C—30 s 72°C—45 s } 14 cycles
37 °C—40 min	72 °C – 5 min	72 °C – 5 min	72 °C – 5 min

5. Add 8 μL of Mix 2 to the tube containing 12 μL of Mix 1.
6. Incubate for 60 min at 42 °C and then for 20 min at 65 °C (*see* Table 5).
7. Add 1 μL of uracil-DNA glycosylase to eliminate the uracil residues in the Universal 5′ primers.
8. Incubate for 40 min at 37 °C (*see* Table 5).
9. Add 19 μL of nuclease-free water to the samples ("5′RACE products") and proceed with column purification using Nucleospin PCR Clean-up Kit.
10. Heat the elution buffer for 10 min at 50 °C before starting (*see* **Note 3**).
11. Add 80 μL of binding buffer to 40 μL of the 5′RACE products.
12. Load the mixture onto the column in a collection tube.
13. Centrifuge at 12,000× *g* for 30 s at room temperature.
14. Wash the column with 700 μL of wash buffer.
15. Centrifuge at 12,000× *g* for 30 s at room temperature.
16. Repeat the wash step: add 700 μL of wash buffer and centrifuge at 12,000× *g* for 30 s.
17. Dry the column by centrifuging at 12,000× *g* for 30 s at room temperature.
18. Add 25 μL of preheated elution buffer to the column.
19. Centrifuge at 12,000× *g* for 30 s at room temperature to elute the product.
20. Reload the eluate onto the column and centrifuge at 12,000× *g* for 30 s at room temperature.
21. Store the 5′RACE products at −20 °C or proceed to the next steps.

3.4 Semi-nested PCR1

The aim of this step is to perform a semi-nested PCR pre-amplification (PCR1) to enrich 5′RACE products generated in the previous step and improve the specificity by using a nested reverse primer located upstream the primer used for reverse transcription (Fig. 1b).

For Ig heavy (Ighm and all Ighg subclasses: Ighg3, Ighg1, Ighg2b, and Ighg2c) and light (Igκ) chain isotypes, nested primers are located in the 5′-part of the constant CH1 and Cκ exons, respectively. The forward primer (Primer 5′_PCR1) used in this step is located upstream the UMI sequence (*see* Table 2). This strategy enhances the amplification of the desired Ig transcripts while minimizing nonspecific products.

1. Prepare a PCR1 mix as described in Table 6. Make one tube per Ig isotype (i.e., Ighm, Igκ, Ighg2b-2c-1, or Ighg3) using the appropriate reverse primer (*see* Table 2) and add 2.5 μL of the

Table 6
PCR1,2,3 reaction mix (for one sample)

Reagents	Volume
Nuclease-free water	14.75 µL
5× Phusion® HF Reaction Buffer	5 µL
10 mM dNTP	0.5 µL
10 µM Forward Primer 5′_PCR1, 2 or 3	1 µL
10 µM Reverse Primer_3′_ PCR1, 2 or 3	1 µL
2000 U/mL Phusion® HF DNA Polymerase	0.25 µL

corresponding 5′RACE PCR products (*see* **Notes 2** and **4**). Prepare four replicates per sample for a total volume of 10 µL (4 × 2.5 µL) of 5′RACE PCR products in this step (*see* **Note 5**).

2. Run the PCR1 program as described in Table 5.
3. Purify the PCR1 products using magnetic beads (*see* **Note 6**).
4. Pool the four replicates of each sample in one 1.5 mL tube.
5. Vortex the beads for 30 s and add 60 µL of bead suspension per sample.
6. Incubate for 5 min at room temperature at 1000 rpm.
7. Place tubes on magnetic rack and wait for 5 min.
8. Gently remove the supernatant. Then, wash with 300 µL of 80% ethanol and wait for 30 s.
9. Repeat the washing step three times.
10. Gently remove the supernatant and eliminate the residual ethanol.
11. Wait for 4 min for complete ethanol evaporation (*see* **Note 7**).
12. Remove the tubes from magnetic rack.
13. Add 25 µL of elution buffer.
14. Resuspend the beads by energetic pipetting.
15. Incubate for 2 min at room temperature under stirring at 1000 rpm.
16. Replace the tubes on the magnetic rack and wait for 5 min.
17. Carefully collect ~20 µL of eluate. Do not pipette up any beads. If beads are accidentally drawn into the pipette, replace the tube on the magnetic rack and allow the beads to separate again.

3.5 PCR2 Step: Addition of Linker Sequences

The aim of this step is to perform a second round of amplification while incorporating linker sequences for subsequent addition of Illumina sequencing adapters (Fig. 1c, *see* **Note 8**).

1. Prepare a PCR2 mix as described in Table 6. Make one tube per isotype using the appropriate reverse primer (*see* Table 3) and add 2.5 μL of the corresponding PCR1 products (*see* **Notes 2** and **4**). Prepare four replicates per sample for a total volume of 10 μL of PCR1 products (*see* **Note 5**).
2. Run the PCR2 program as described in Table 5.
3. Pool the four replicates of each sample in one tube.
4. Add 20 μL of 6× loading dye.
5. Migrate the PCR2 product on a 2% agarose gel at 120 V for ~30 min (*see* Note **9**).
6. Cut the band at 600–700 bp under UV lamp (Fig. 2a).
7. Proceed with gel extraction and purification of PCR2 products using Nucleospin Gel clean-up kit, according to the manufacturer's instructions.
8. Elute purified PCR2 products in 25 μL of elution buffer.
9. Store the PCR2 products at −20 °C or proceed to the next steps.

3.6 PCR3 Step: Tag and Adapters Addition

The goal of the last PCR3 step is to add sequencing adapters and tags to the amplified PCR2 products (Fig. 1d), ensuring compatibility with high-throughput sequencing platforms like Illumina. This is achieved by incorporating Illumina-specific primers, which attach necessary adapter and index sequences to the PCR2 products. These adapters are essential for the efficient processing and proper attachment of the amplicons to the sequencing flow cell, enabling the subsequent sequencing process.

1. Prepare a PCR3 mix as described in Table 6. Use distinct forward and reverse primer pairs for each sample (Table 4, *see* **Note 10**). Add 2.5 μL of the purified PCR2 products and prepare four replicates per sample, so that a total volume of 10 μL of PCR2 products is used for this step.
2. Run the PCR3 program as described in Table 5.
3. Pool the four replicates of each sample in one tube.
4. Add 20 μL of 6× loading dye and migrate the PCR3 product on a 2% agarose gel at 120 V for ~30 min (*see* Note **9**).
5. Cut the band at 600–700 bp under UV lamp (Fig. 2b).
6. Proceed with gel extraction and purification of indexed PCR3 products using Nucleospin Gel clean-up kit, according to the manufacturer's instructions.
7. Elute purified PCR3 products in 20 μL of elution buffer.

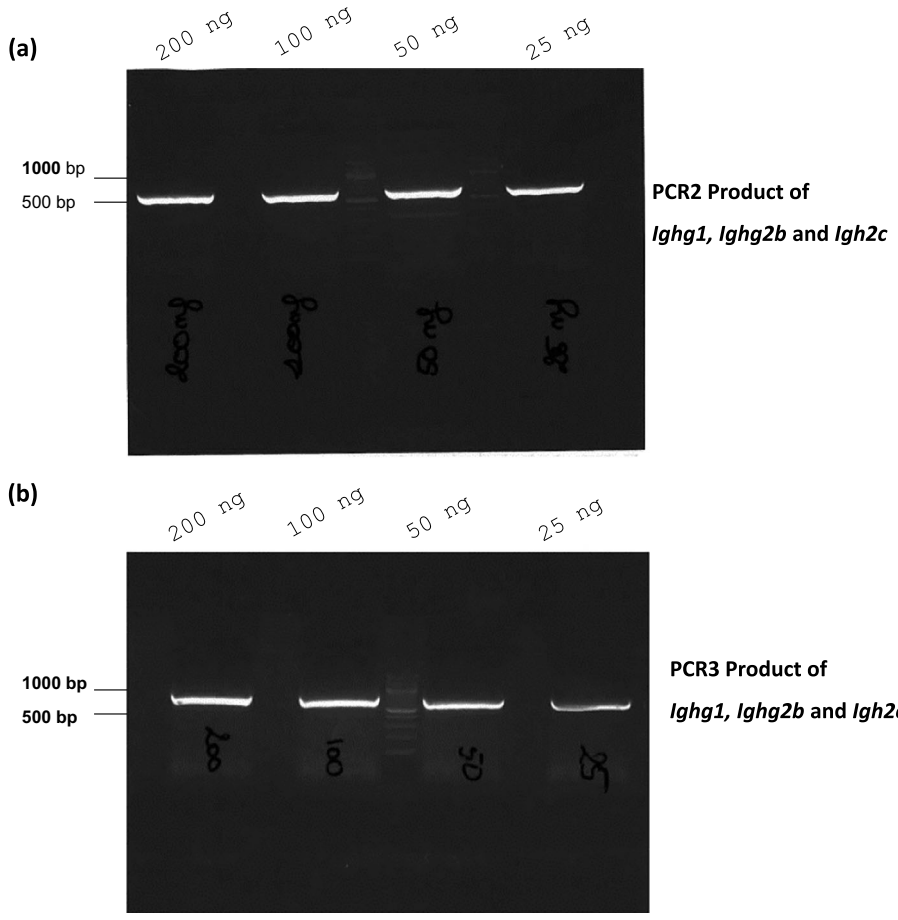


Fig. 2 Electrophoresis gel profile obtained after PCR2 and PCR3. Gel electrophoresis analysis of the PCR2 (**a**) and PCR3 (**b**) products of *Ighg2b, Ighg2c*, and *Ighg1* (~650 bp) heavy chain are represented. Four initial concentrations of total RNA are shown (200 ng, 100 ng, 50 ng, and 25 ng) to indicate the sensitivity of this method

8. Quantify the samples using Qubit Fluorometer and appropriate quantification assay kits.
9. Pool 30 ng of each sample, regardless of the volume (*see* Note **11**).
10. Quantify the pooled library using Qubit Fluorometer.
11. Migrate a small aliquot (~1 μL) of the pooled library on 2% agarose gel at 120 V for ~30 min (*see* Note **12**).
12. Store the indexed libraries at −20 °C until sequencing.

3.7 Sequencing of the Libraries and Bioinformatics Analysis

The following steps are carried out according to Illumina's "Denature and Dilute Libraries" guide.

1. Dilute the library 1/10,000 and 1/100,000 to ensure concentrations fall within the standard curve range.

2. Perform qPCR according to the manufacturer's protocol for the NeBNext Library Quant Kit.
3. Dilute the library in nuclease-free water to a final concentration of 4 nM in a total volume of 20 μL.
4. Prepare a fresh 0.2 M NaOH solution by diluting 100 μL of 1 M NaOH in 400 μL of nuclease-free water.
5. To denature the diluted library: mix 5 μL of 4 nM library with 5 μL of 0.2 M NaOH in a DNA LoBind tube.
6. Vortex and briefly centrifuge the tube, and then incubate for 5 min at room temperature.
7. Stop the denaturation: add 990 μL of HT1 buffer (included in the MiSeq Reagent Kit v3) to obtain a library concentration of 20 pM in a final volume of 1 mL.
8. Mix thoroughly to ensure homogeneity.
9. Prepare the Flow Cell for Illumina Sequencing: thaw the MiSeq v3 (600 cycles) Flow Cell according to the manufacturer's instructions.
10. Load 600 μL of the diluted library in HT1 buffer, ensuring a final concentration between 9 and 10 pM, including 10% PhiX control.
11. Program the Illumina MiSeq platform for a 301-cycle read 1 and 301-cycle read 2.
12. Insert the flow cell and start sequencing.
13. Paired-end sequencing is performed to capture both forward and reverse reads, which are then merged to generate a single consensus sequence for each amplicon.
14. To ensure accurate identification and quantification of individual cDNA molecules, unique molecular identifiers (UMIs) are incorporated into the primers during PCR amplification. The UMIs were treated and processed using the MIGEC (Molecular Identifier-based Gene Expression Cloning) software, which helps to correct the amplification biases and sequencing errors by tracking and collapsing duplicate sequences that originated from the same cDNA molecule (*see* **Note 13**).
15. After sequencing, the merged reads are subjected to repertoire analysis using the IMGT/HIGHV-QUEST online tool (http://imgt.org/). This tool provides a comprehensive analysis of Ig sequences, including the identification of V(D)J gene usage, junctional diversity, and somatic hypermutations (*see* **Note 14**).

4 Notes

1. A wide range of RNA amounts can be used (*see* Fig. 2 for 25–200 ng assays). However, we recommend using >100 ng of total RNA (ideally ~500 ng) because low RNA inputs may impede detection of small clones**,** likely due to a lower number of unique template molecules being reverse transcribed and amplified. Thus, although the method is robust over a wide range of RNA inputs, the use of higher amounts (i.e. 200–500 ng) can maximize repertoire diversity and minimize amplification bias.
2. Although a mixture with reverse RACE (Primer_3′_RACE) primers for all Ig isotypes can be used, we recommend to make one separate tube for each Ig isotype (e.g. Ighm, consensus Ighg, and Igκ light chain).
3. We recommend to preheat the elution buffer to 50 °C to improve the yield of column purification.
4. As mentioned in **Note 2**, a mixture of reverse primers (3′_PCR1) for all Ig isotypes can be used. However, we recommend to make one separate tube for each Ig isotype (e.g. Ighm or Igκ light chain). For Ighg isotypes, we designed two distinct reverse primers: one specific for the Ighg3 isotype and one specific for Ighg1, Igh2b, and Igh2c (Ighg2bc1-CH1 3′_PCR1) isotypes. There is no major difference in the nt sequences of these primers and cross-hybridization is possible. We recommend to use Ighg2bc1-CH1 3′_PCR1 for preliminary analysis of Ighg repertoire and Ighg3-CH1 3′_PCR1 only if the aim is to study this isotype specifically.
5. The number of PCR1 replicates can be increased when the multiplex strategy, amplifying all Ig isotypes in the same tube, is performed (*see* **Notes 2** and **4**).
6. Ensure that beads are at room temperature for at least 30 min before starting the purification to ensure optimal binding efficiency.
7. Do not exceed the 4-min waiting time for ethanol evaporation to avoid over-drying the beads, which may affect their efficiency.
8. The linker sequences are often added to both forward and reverse primers. These linkers help make the amplified DNA compatible with sequencing technologies by providing binding sites for sequencing adapters (used by Illumina or other next-generation sequencing platforms).
9. To ensure sufficient space for the migration of the PCR products, tape the wells in advance. This will allow enough room to load a total volume of 120 μL (100 μL of PCR product and

20 μL of loading dye) into each well without overflow, ensuring optimal separation of the PCR fragments on the 2% agarose gel. Leave at least two empty wells between samples to prevent any interference during migration.

10. When preparing the PCR mixes, ensure to use a distinct set of reverse and forward primers for each sample to avoid cross-contamination. If amplifying different antibody isotypes from the same RNA sample, you may use the same set of primers for all isotypes, as long as they are derived from the same sample. This will maintain the integrity of your sequencing adapters and tags, ensuring compatibility with high-throughput sequencing platforms like Illumina.
11. To ensure balanced sequencing results, adjust sample volumes so that each contributes equally to the final pool. This prevents any single sample from being over- or under-represented during sequencing.
12. Agarose gel migration is a way to check the quality of the libraries. This makes sure that the DNA is not damaged and that only 600–700 bp indexed PCR3 products are present.
13. The integration of UMI-based error correction with high-throughput sequencing data provides a highly accurate representation of the Ig repertoire, enabling the identification of both abundant and rare clones in a given sample.
14. IMGT/HIGHV-QUEST aligns the sequenced reads to a reference database of Ig gene segments, allowing for precise characterization of the Ig repertoire. This tool also facilitates the analysis of the CDR3 region, as well as the identification of clonal abundance.

Acknowledgments

This work was supported by grants from INCa (PLBIO2022-110, INCA_16734), ANR (2017-CE15-0024-01), Ligue Contre le Cancer (comités Corrèze, Haute-Vienne), Fondation Française pour la Recherche contre le Myélome et les Gammapathies monoclonales (FFRMG), and Région Nouvelle-Aquitaine (AAP ESR 2020 and 2024). The authors would like to thank Dr. Saintamand Alexis and Pr. Cogné Michel for their contribution during the initial development of this method in our laboratory.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Rajewsky K (1996) Clonal selection and learning in the antibody system. Nature 381: 751–758. https://doi.org/10.1038/381751a0
2. Jung D, Giallourakis C, Mostoslavsky R, Alt FW (2006) Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. Annu Rev Immunol 24:541–570. https://doi.org/10.1146/annurev.immunol.23.021704.115830
3. Popov AV, Zou X, Xian J et al (1999) A human immunoglobulin λ locus is similarly well expressed in mice and humans. J Exp Med 189:1611–1620
4. Honjo T, Kinoshita K, Muramatsu M (2002) Molecular mechanism of class switch recombination: linkage with somatic hypermutation. Annu Rev Immunol 20:165–196. https://doi.org/10.1146/annurev.immunol.20.090501.112049
5. Dudley DD, Chaudhuri J, Bassing CH, Alt FW (2005) Mechanism and control of V(D)J recombination versus class switch recombination: similarities and differences. Adv Immunol 86:43–112. https://doi.org/10.1016/S0065-2776(04)86002-4
6. Georgiou G, Ippolito GC, Beausang J et al (2014) The promise and challenge of high-throughput sequencing of the antibody repertoire. Nat Biotechnol 32:158–168. https://doi.org/10.1038/nbt.2782
7. Ouk C, Roland L, Gachard N et al (2021) Continuous MYD88 activation is associated with expansion and then transformation of IgM differentiating plasma cells. Front Immunol 12:641692. https://doi.org/10.3389/fimmu.2021.641692
8. Thomas M, Bruzeau C, Martin OA et al (2023) A dual function for the chromatin organizer special A-T rich binding protein 1 in B-lineage cells. Cell Mol Immunol 20:1114–1126. https://doi.org/10.1038/s41423-023-01069-y
9. Bender S, Javaugue V, Saintamand A et al (2020) Immunoglobulin variable domain high-throughput sequencing reveals specific novel mutational patterns in POEMS syndrome. Blood 135:1750–1758. https://doi.org/10.1182/blood.2019004197
10. Turchaninova MA, Davydov A, Britanova OV et al (2016) High-quality full-length immunoglobulin profiling with unique molecular barcoding. Nat Protoc 11:1599–1616. https://doi.org/10.1038/nprot.2016.093
11. Alamyar E, Duroux P, Lefranc M-P, Giudicelli V (2012) IMGT® tools for the nucleotide analysis of immunoglobulin (IG) and T cell receptor (TR) V-(D)-J repertoires, polymorphisms, and IG mutations: IMGT/V-QUEST and IMGT/HighV-QUEST for NGS. In: Christiansen FT, Tait BD (eds) Immunogenetics: methods and applications in clinical practice. Humana Press, Totowa, pp 569–604
12. Ye J, Ma N, Madden TL, Ostell JM (2013) IgBLAST: an immunoglobulin variable domain sequence analysis tool. Nucleic Acids Res 41:W34–W40. https://doi.org/10.1093/nar/gkt382

Chapter 9

A Wet Lab Procedure for Gene Fusion Analysis by Targeted RNA Next-Generation Sequencing in Lung Cancer

Sylvain Lacorre, Maéva Chaumet, Murat Sahin, Emilie Guérin, Paco Derouault, Léa Darnaud, Alexandre Perani, and Karine Durand

Abstract

RNA sequencing (RNA-Seq) is a next-generation sequencing method, routinely used in laboratories to detect abnormal transcripts with oncogenic activity after RNA extraction from formalin-fixed-paraffin-embedded (FFPE) samples of non-small-cell-lung cancers (NSCLC), then allowing targeting of the corresponding fusion kinase with adapted pharmacologic compounds in the clinical practice. Here, we provide a detailed protocol for total nucleic acid (i.e., RNA and DNA) extraction from FFPE samples and targeted RNA-Seq, by the FusionPlex® method which allows the detection of the most current fusion transcripts found in NSCLC.

Key words RNA-Seq, NSCLC, Fusion transcript, FFPE, RNA extraction, Wet lab process

1 Introduction

Gene fusions, resulting from chromosomal rearrangements, are common molecular alterations in many cancers. Their transcription into fusion transcripts, then translation into chimeric proteins originally coded by one or two different genes, result in acquisition of new abilities such as transcription factor or protein kinase functions [1]. They play a central role in oncogenic processes and cancer propagation mechanisms, generally referred to as the cancer hallmarks [2].

In lung cancers, particularly non-small-cell lung cancers (NSCLC), many fusion transcripts are reported as oncogenic addictions on which the tumor depends for its propagation. They complete the list of potentially actionable oncogenic addictions, i.e., *EGFR, BRAF, KRAS, MET,* or *HER2* mutations, and can lead to

Authors Alexandre Perani and Karine Durand have equally contributed to this chapter.

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_9,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

prescription of specific pharmacological inhibitors capable of targeting the fusion protein implementing the essential field of current personalized medicine for NSCLC [3, 4]. The most common and best-known fusion transcripts concern, in order of frequency, the ALK, ROS-1, RET, and NTRK oncogenes, all generating fusion kinase with abnormal activity and cellular location [5, 6]. Since their analysis is not as easy as the detection of DNA point mutations and short deletion/insertions, fusion transcripts detection created new challenges for molecular biology laboratories.

Different approaches can be used to detect fusion transcripts and/or encoded fusion proteins in routine clinical diagnosis [7]. First Immunohistochemistry (IHC) can be performed as a rapid screening method to detect fusion protein at the phenotypic level, followed by fusion transcript detection by fluorescent in situ hybridization (FISH). If this last method is considered as the gold standard, it can only detect one rearrangement, limiting the possibility of detecting a fusion partner other than the one targeted by the designed probe. IHC and FISH have the undeniable advantage of being able to analyze alterations in situ, enabling correlation with the tumor histology, but the inconvenient to be time and tumor material consuming in regard to the increasing number of biomarkers to be routinely analyzed. Molecular biology solutions on bulk extracted-DNA or RNA also exist, like multiplex digital polymerase chain reaction (PCR) [8] or real-time quantitative PCR [9, 10]. Although highly sensitive, these targeted methods allow the detection of a limited number of targets.

As a consequence, the last few years have seen the advent of next-generation massive parallel sequencing (NGS) as an efficient strategy allowing simultaneously sequencing of many genetic or genomic targets [7, 11]. Today, it's often routinely used in laboratories for cancer diagnosis and therapeutic management, its application for RNA sequencing (RNA-Seq) allows, simultaneously and in a short time, the search for several oncogenic events. Its efficacy to detect point mutation, exon skipping, as well as fusion transcripts and simultaneously identify the fusion partners leads an increasing number of laboratories to adopt a "RNA-first" strategy, particularly adapted to low tumor cellularity samples.

This chapter is aimed to describe the commercial targeted RNA-Seq with the Invitae FusionPlex® (previously called Archer FusionPlex®) method to search current NSCLC transcript fusions after total nucleic acid (TNA) extraction, containing both DNA and RNA, from formalin-fixed-paraffin-embedded (FFPE) tumor samples, the type of sample most commonly available to date in pathology departments for analysis of this type of cancer. This method also allows the simultaneous detection of point mutations and exon skipping.

2 Materials

2.1 Safety Precautions, Common Reagents, and Consumables

All molecular biology procedures involving sample preparation, nucleic acid extraction, and amplification prior to dry lab analyses require strict adherence to good laboratory practices (*see* **Note 1**).

1. Nuclease-free or ultra-pure water.
2. RNase AWAY (Thermo Fisher Scientific).
3. Sterile and nuclease-free microtubes: 0.2 mL, 0.5 mL, 1.5 mL, and 2 mL.
4. Microtube racks.
5. Vortex agitator.
6. Dry-heating bath.
7. Benchtop centrifuge.
8. Isothermal container with ice.
9. Aerosol-barrier pipette tips (low retention tips when recommended).

2.2 Preparation of FFPE Tissues

Special precautions must be taken when preparing FFPE samples (*see* **Note 2**).

1. Powder-free nitrile gloves.
2. 70% ethanol.
3. Absorbent paper.
4. Microtome.
5. Microtome blades.
6. Slides and scalpel blade (to be changed between each sample).

2.3 TNA (Total Nucleic Acid) Extraction and Quantification (See Note 3)

1. Maxwell® RSC RNA FFPE Kit (Promega) including mineral oil, lysis buffer, proteinase K solution, and blue dye.
2. Automatic extraction system Maxwell 16 (Promega).
3. Hybridization oven with stirring plate.
4. QuantiFluor® RNA System kit (Promega).
5. 20× TE Buffer: 200 mM Tris–HCl, 20 mM EDTA, pH 7.5, included in the QuantiFluor® RNA System kit.
6. Fluorescent RNA-binding dye: QuantiFluor RNA Dye, included in the QuantiFluor® RNA System kit.
7. RNA standard: 100 μg/μL, included in the QuantiFluor® RNA System kit.
8. Quantus fluorimeter (Promega).
9. QuantiFluor® RNA Dye working solution: prepare 1× TE buffer by adding 10 μL of 20× TE buffer and 190 μL of nuclease-free water, then add QuantiFluor RNA Dye to 1×

TE buffer in a 1:400 (vol:vol) dilution. This solution must be prepared extemporaneously, before each manipulation. Before quantification, allow all reagents to return to room temperature for 15–30 min in the dark.

2.4 RNA Sequencing from TNA

We use the Invitae FusionPlex® AMP (Anchored Multiplex PCR) technology, according to the FusionPlex® protocol for Illumina (*see* **Note 4**). The Invitae FusionPlex™ Lung panel allows identification of known and novel fusions, splicing, exon-skipping, insertions, deletions, and single nucleotide variants with targeted NGS of 17 genes relevant for NSCLC: *ALK*, *BRAF*, *EGFR*, *FGFR1*, *FGFR2*, *FGFR3*, *KRAS*, *MET*, *NRG1*, *NTRK1*, *NTRK2*, *NTRK3*, *RET*, and *ROS1*. Method must be performed with ≥10 ng TNA, obtained from fresh, FFPE, or frozen tissues in a hands-on time estimated to 3.5 h, and a total library preparation time to 1.5 days.

1. Invitae reagents supplied in lyophilisated form in individual 0.2 mL tubes (*see* **Note 5**).
2. Random-priming 2.0: green PCR tube strips included in the kit Invitae FusionPlex™.
3. Thermocycler with a heating lid (*see* **Note 6**).
4. First-strand cDNA synthesis: purple PCR tube strips included in the kit Invitae FusionPlex™.
5. QuantStudio™ 5 (QS5) Real-Time PCR System (Thermo Fisher Scientific).
6. 96-well plates or strips of eight tubes for QS5 real-time PCR system: MicroAmp™. EnduraPlate™ Optical 96-Well Fast Clear Reaction Plates with Barcode or Strips of 8 tubes 0.2 mL specific of QS5 (Life Technologies).
7. Plastic films for optical plate wrapping MicroAmp™ (Life Technologies).
8. iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories).
9. 10× VCP primer mix, included in the Invitae FusionPlex™ Lung v2 panel.
10. Second Strand cDNA Synthesis: yellow PCR tube strips included in the kit Invitae FusionPlex™.
11. cDNA end-repair: blue PCR tube strips included in the Invitae FusionPlex™ Lung v2 panel.
12. Invitae Molecular Bare Code (MBC) Adapters for Illumina: transparent PCR tube strips included in the kit Invitae FusionPlex™ (numbered from 1 to 96, the exact sequence must be entered in the sample sheet before sequencing).
13. Ligation STEP1: green PCR tube strips included in the kit Invitae FusionPlex™.

14. Ligation STEP2: pink PCR tube strips included in the kit Invitae FusionPlex™.
15. Ligation Cleanup Beads (Invitae FusionPlex™ Lung v2 panel).
16. Ligation Cleanup Buffer (Invitae FusionPlex™ Lung v2 panel).
17. Magnetic beads for PCR clean-up: HighPrep™ PCR (MAG-BIO). Allow to stand at room temperature for at least 30 min before use and vortex to homogenize before each use. Always pipette slowly.
18. 70% ethanol (prepared extemporaneously).
19. Magnetic plate: DynaMag™-96 Side Magnet (ThermoFisher Scientific).
20. Tris solution: 10 mM Tris–HCl, pH 8.0.
21. 5 mM NaOH.
22. Low binding PCR tubes.
23. First PCR: transparent PCR tube strips included in Invitae FusionPlex™ Lung v2 panel.
24. Second PCR: transparent PCR tube strips included in Invitae FusionPlex™ Lung v2 panel, with Index 1 (P7) (*see* **Note 7**).
25. FusionPlex panel GSP1 included in Invitae FusionPlex™ Lung v2 panel.
26. FusionPlex panel GSP2 included in Invitae FusionPlex™ Lung v2 panel.
27. KAPA Library Quantification Kit (specifically designed for qPCR-based quantification of Illumina libraries, flanked by the P5 and P7 flow cell oligo sequences): Library Quantification DNA Standards 1–6 (a tenfold dilution series of a linear, 452 bp template), 10× Library Quantification Primer Premix with Primer 1 (5′-AAT GAT ACG GCG ACC ACC GA-3′), Primer 2 (5′-CAA GCA GAA GAC GGC ATA CGA-3′) and 2× KAPA SYBR® FAST qPCR Master Mix (available with various passive reference dyes). When using the kit for the first time, add 1 mL of 10× Primer Premix to 5 mL of 2× KAPA SYBR® FAST qPCR Master Mix. Then, make 500 μL aliquots (to minimize freeze-thaw cycles) of the combined KAPA SYBR FAST/Primer Premix solution, protect from light and store at −20 °C (*see* **Note 8**).
28. MiSeq or NextSeq 1000 or 2000 sequencing system (Illumina) (*see* **Note 9**).
29. MiSeq® or NextSeq® Reagent Kit for 300 cycles (Illumina), including 200 mM NaOH (to denature libraries), HT1 Hybridization Buffer (to dilute denatured libraries prior to sequencing) and 200 mM Tris–HCl, pH 7.0.

30. MiniSeq System Denature and Dilute Libraries Guide (Illumina).
31. Bioinformatic analysis and interpretation are performed using ARCHERDx/INVITAE software, distributed by Integrated DNA Technologies (IDT). It is available either as a Software as a Service (SaaS) product hosted by IDT ("Archer Analysis Unlimited") or as a locally installed virtual machine (VM). To run Archer Analysis on a VM, the server must have at least 10 GB of memory (RAM) and one available processor core (CPU). IDT recommends a N+1 model for CPU provisioning, where N is the number of concurrent samples to process, plus an additional core for the operating system to ensure optimal performance. Disk space must be sufficient for the initial setup of the VM (about 10 GB), as well as for running multiple analyses. IDT recommends at least 200 GB of free space to accommodate VM growth during data analysis.

3 Methods

3.1 TNA Extraction from FFPE Samples

TNA extraction should be preferred to RNA extraction, as recommended by the supplier (*see* **Note 10**). Before starting manipulation, pre-heat hybridization oven at 80 °C and dry-heat bath at 56 °C (*see* **Note 11**).

1. For FFPE samples dewaxing, add 300 μL of mineral oil in 2 mL microtubes containing FFPE sample and mix by vortexing for 10 s. Heat microtubes in hybridization oven at 80 °C for 2 min and then place on ice (*see* **Note 12**).
2. For sample lysis, prepare the following "lysis solution" on ice: 224 μL of lysis buffer, 25 μL of proteinase K, 1 μL of blue dye (volumes are for one sample and should be multiplied by the number of samples +1). Mix gently by pipetting (*see* **Note 13**).
3. Add 250 μL of the lysis solution in each sample tube, vortex for 5 s, and centrifuge at 10,000× *g* for 20 s. Incubate overnight in the dry-heat bath at 56 °C with agitation, and then for 1 h in the hybridization oven at 80 °C.
4. After the heating step, dispose microtubes in a new tubes rack (*see* **Note 14**), incubate for 15 min at room temperature, and centrifuge at 10,000× *g* for 2 min. After sample lysis, the solution should present a blue aqueous phase.
5. Change gloves before automatic RNA extraction (*see* **Note 15**), and then put the number of cartridges corresponding to the sample number and press cartridges firmly into their slots.
6. Place a 0.5 mL elution microtube in the corresponding slot opposite each cartridge and add 50 μL of nuclease-free water.



Fig. 1 Presentation of the Maxwell® CSC Cartridge and the deck tray used for TNA extraction. (**a**) Wells of the cartridge contain (1) binding solution, (2) magnetic beads, (3–7) successive wash buffers, (8) empty well. The preprocessed FFPE sample is added to well (1) and a plunger is added to well (8). (**b**) Cartridges containing the previously added FFPE sample and inserted plunger are placed on the deck tray. Nuclease-free water is added to the elution tubes as shown. Elution microtubes are shown closed to avoid contamination, but must be opened before deck tray insertion in Maxwell extractor

7. Remove the plastic film of each cartridge.
8. Pipet the blue aqueous phase (volume near 250 µL) from the previously lysed FFPE sample and transfer in the well N°1 of the cartridge (Fig. 1a). Also place a plunger in the well N°8 of the cartridge (Fig. 1a and b).
9. Place deck tray with cartridge on the extractor platform, open all elution tubes and add 50 µL of nuclease-free water in each tube (Fig. 1b). Leave elution tubes open and run extraction following the screen instruction (*see* **Note 16**).
10. At the end of the run, collect the elution microtubes, centrifuge briefly, and place on ice until quantification.
11. Proceed to system decontamination after carefully removing the cartridges from the tray: clean the tray with 70% ethanol, and then rinse it and proceed with UV decontamination of the extractor according to the supplier's recommendations.

3.2 TNA Quantification and Qualification

This step is carried out using a fluorimetric method. As this method is based on the incorporation of a fluorochrome into the nucleic acids, it not only quantifies but also qualifies the RNA: if the RNA is degraded, the fluorochrome will not be incorporated. The amount of fluorochrome measured is directly proportional to the amount of undegraded RNA available for sequencing (*see* **Note 17**).

1. Prepare extemporaneously blank, RNA standard for instrument calibration and RNA samples, in special Quantus microtubes.
2. Blank: 200 µL of QuantiFluor® RNA Dye working solution.
3. Standard: mix 200 µL of QuantiFluor® RNA Dye working solution with 5 µL of RNA Standard.
4. RNA samples: mix 200 µL of QuantiFluor® RNA Dye working solution with 1 µL of RNA sample.
5. Vortex and centrifuge briefly all the tubes, and then leave for 5 min at room temperature in the dark.
6. On the fluorimeter system, select RNA High Standard, and proceed to calibration with blank and standard and then to RNA quantification, following supplier's recommendations.
7. Usually dilute RNA samples up to 10 ng/µL in a final volume of 20 µL with ultra-pure water (*see* **Note 18**).
8. Then proceed to cDNA libraries construction following Subheadings 3.3, 3.4, 3.5, 3.6, 3.7, and 3.8. An overview of the workflow of cDNA libraries construction, which outlines the main steps and tube color as a guide for each step, is presented in Fig. 2.

3.3 Construction of cDNA Libraries: Random Priming and First Strand cDNA Synthesis

1. Before starting the random priming step, preheat a thermocycler at 65 °C, with the lid heated to 100 °C (*see* **Note 19**).
2. For random priming, dissolve the lyophilized reagents of the Random Priming 2.0 tube (green tube) with 20 µL of diluted RNA sample (*see* **Note 5**).
3. Incubate at 65 °C for 5 min in the preheated thermocycler (with the lid heated to 100 °C).
4. Place tubes for 2 min on ice and centrifuge briefly.
5. Before starting the step of First Strand cDNA Synthesis, preheat the thermocycler at 25 °C, with the lid heated to 100 °C (*see* **Note 19**).
6. Place First Strand cDNA Synthesis tubes (purple tube) on ice and transfer 20 µL of the previously random-primed RNA (*see* **step 4**), which is used to dissolve the lyophilisate (*see* **Note 5**).

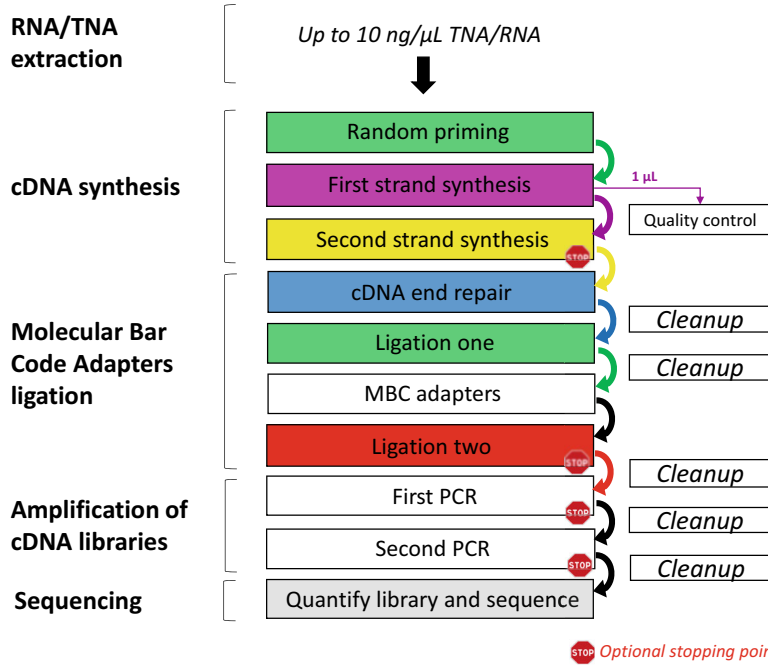


Fig. 2 Overview of the workflow for the preparation of libraries from a TNA solution, prior to sequencing. Note that the times for the first and second PCRs may vary depending on the specific panel and cycling conditions in each laboratory

7. Run the following program in the thermocycler (with the lid heated to 100 °C): 25 °C for 10 min, 42 °C for 30 min, and 80 °C for 20 min.
8. Centrifuge briefly and place tubes on ice.

3.4 Construction of cDNA Libraries: Quality Control of the First Strand cDNA

1. Let reagents (iTaq Universal SYBR Green Supermix and 10× VPC primer mix tubes) to return to room temperature prior to proceeding to quality control assessment of the first strand cDNA (*see* **Note 20**).
2. Prepare blank and sample for the quantification in new 0.2 mL microtubes: for the blank, add 10 μL of ultra-pure water (*see* **Note 21**); for samples, add 1 μL of the previously obtained First Strand cDNAs (*see* **step 8** in Subheading 3.3) to 9 μL of ultra-pure water.
3. Prepare the following PCR mix: 5 μL of iTaq Universal SYBR Green Supermix and 1 μL of 10× VPC primer mix (volumes are for one sample and should be multiplied by the number of samples +2).
4. In a 96-well microplate (or strips of eight tubes) specific to QS5 Real-time PCR system, mix 6 μL of PCR mix with 4 μL of sample or blank (*see* **step 2**). Seal microplate well with plastic film.

5. Proceed to thermocycling: 95 °C for 20 s, followed by 35 cycles (95 °C for 3 s and 60 °C for 30 s) with fluorescence acquisition at the end of each cycle.
6. A First Strand cDNA quantity is optimal for cycle threshold value between 23 and 28 (*see* **Note 22**).

3.5 Construction of cDNA Libraries: Second Strand cDNA Synthesis

1. Before starting the step of Second Strand cDNA Synthesis, set the thermocycler at 16 °C, with the lid heated to 100 °C (*see* **Note 19**).
2. Add 21 μL of ultra-pure water to tubes containing 19 μL of RNA sample after the First Strand cDNA Synthesis (*see* **step 8** in Subheading 3.3). Mix by aspiration/reflux and centrifuge briefly.
3. Drop off the First Strand cDNA mixture (40 μL) in the Second Strand cDNA Synthesis microtube (yellow tube) and dissolve the lyophilisate as described (*see* **Note 5**).
4. Run the following program in the thermocycler (with the lid heated to 100 °C): 16 °C for 60 min and then 75 °C for 20 min.
5. Place tubes for 2 min on ice and centrifuge briefly.
   Optional: stopping point (*see* **Note 23**).

3.6 Construction of cDNA Libraries: cDNA End-Repair Step

1. Before starting the step of cDNA end-repair, preheat the thermocycler at 25 °C, with the heating lid off (*see* **Note 19**).
2. Transfer 40 μL of Second Strand cDNA Synthesis mixture (*see* **step 5** in Subheading 3.5) to the End Repair tube (blue tube) and dissolve the lyophilisate as described (*see* **Note 5**).
3. Incubate at 25 °C for 30 min in the thermocycler, with the heating lid off.
4. Centrifuge briefly and keep samples at room temperature.
5. For the Cleanup step (*see* **Note 24**), add 100 μL (2.5 volumes) of HighPrep™ PCR beads to 40 μL of End-repaired cDNA (*see* **step 4**). Gently aspirate/reflux at least 10 times to thoroughly mix the bead suspension with the End-repaired cDNA. Incubate for 5 min at room temperature and then centrifuge briefly.
6. Place the tubes on a magnetic plate and incubate for 4 min or until the solution runs clear.
7. Gently remove and discard the supernatant; leave a volume of 5–10 μL in the bottom of the tube without touching the pellet.
8. Add 200 μL of 70% ethanol without resuspending the beads (*see* **Note 25**).
9. Gently remove and discard the supernatant; leave a volume of 5–10 μL in the bottom of the tube without touching the pellet.

10. Perform a second wash by adding 200 µL of 70% ethanol without resuspending the beads and remove all the supernatant at this step (*see* **Note 26**).
11. Allow the droplets to air dry at room temperature for 5 min (*see* **Note 27**).
12. Resuspend the beads in 20 µL of Tris solution by gentle aspiration/reflux at least 10 times.
13. Place the tubes on the magnetic support for at least 2 min or until the solution runs clear.

3.7 Construction of cDNA Libraries: Molecular Bare Code (MBC) Adapter Ligation Steps

1. Before starting the first ligation (Ligation STEP1), preheat the thermocycler at 37 °C with the lid heated to 100 °C.
2. Transfer 20 µL of purified End-repaired cDNA (*see* **step 13** in Subheading 3.6) into the Ligation STEP1 tube (green tube) and dissolve the lyophilisate as described (*see* **Note 5**).
3. Incubate in the thermocycler for 15 min at 37 °C.
4. Centrifuge briefly and keep samples at room temperature.
5. Proceed to cleanup as described (*see* **steps 5–13** in Subheading 3.6) except adding 50 µL (2.5 volumes) of HighPrep™ PCR beads to the Ligation STEP1 tube (*see* **step 2**), and eluting with 42 µL of Tris solution.
6. Preheat the thermocycler at 22 °C, with the heating lid off, before proceeding to the ligation of MBC Adapters.
7. Transfer 40 µL of cleaned Ligation STEP1 cDNA (*see* **step 5**) into the tube containing MBC Adapters (transparent tube) and dissolve the lyophilisate as described (*see* ***Note 5***).
8. Transfer the entire volume of this mixture into the Ligation STEP2 tube (pink cap) and dissolve the lyophilisate as described (*see* **Note 5**).
9. Incubate for 5 min at 22 °C in the thermocycler, with the heating lid off.
10. Centrifuge briefly and keep Ligation STEP2 cDNA samples at room temperature.

    Optional: stopping point (*see* **Note 23**).
11. Before starting ligation cleanup and elution steps below, preheat the thermocycler at 75 °C with the lid heated to 100 °C.
12. Resuspend Ligation Cleanup Beads by vortexing and pipette 50 µL into a new 0.2 mL microtube. Place the microtube on a magnetic plate and incubate for 1 min or until solution runs clear. Carefully remove and discard the supernatant.
13. Remove the microtube containing Ligation Cleanup Beads from the magnetic plate and add 50 µL of Ligation Cleanup Buffer. Resuspend by aspiration/reflux.

14. Immediately transfer the whole solution into the tubes containing Ligation STEP2 cDNA samples (*see* **step 10**).
15. Mix by vortexing and incubate for 5 min at room temperature.
16. Repeat the last step.
17. First cleanup step: centrifuge briefly the tubes and place them on a magnetic plate and incubate for 1 min. Carefully remove and discard supernatant. Add 200 µL of Ligation Cleanup Buffer and mix samples by vortexing.
18. Second cleanup step: repeat the last step.
19. Third cleanup: repeat the last step but resuspend with 200 µL of ultra-pure water instead of Ligation Cleanup Buffer.
20. Centrifuge briefly, place the tubes on the magnetic plate and incubate for 1 min.
21. Carefully remove and discard all the supernatant (~200 µL) (*see* **Note 28**).
22. Proceed to cDNA denaturation by resuspending the beads with 18 µL of 5 mM NaOH.
23. Incubate at 75 °C for 10 min in the thermocycler with the lid heated to 100 °C.
24. Centrifuge briefly and place the tubes on the magnetic plate.

3.8 Amplification of cDNA Libraries: First and Second PCR Steps

1. Before starting the First PCR step, preheat the thermocycler at 95 °C with the lid heated to 100 °C.
2. Into the First PCR tubes (transparent tube), add 2 µL of FusionPlex panel GSP1 solution, centrifuge, and place on ice. Then, add 18 µL of the supernatant containing denatured cDNAs (*see* **step 24** in Subheading 3.7) without touching the beads. Dissolve as described (*see* **Note 5**).
3. Proceed to thermocycling (lid heated to 100 °C): 95 °C for 3 min, 15 cycles (95 °C for 30 sec, 65 °C for 5 min), and a final amplification step at 72 °C for 3 min (*see* **Note 29**). Then, centrifuge briefly.
4. Proceed to First PCR cleanup as described (*see* **steps 5–13** in Subheading 3.6), except adding 24 µL (1.2 volumes) of High-Prep™ PCR beads to 20 µL of First PCR products (*see* **step 3**) and eluting with 24 µL of Tris solution.
5. Transfer 22 µL of the purified First PCR products into new 0.2 mL PCR tubes. Make sure no beads are transferred.
   Optional: stopping point (*see* **Note 23**).
6. Before starting the Second PCR step, preheat the thermocycler at 95 °C with the lid heated to 100 °C.
7. Add 2 µL of FusionPlex panel GSP2 solution to the Second PCR tubes (transparent tube) and centrifuge (*see* **Note 7**).

8. Transfer 18 µL of the purified First PCR Products (*see* **step 5**) to the Second PCR tubes and dissolve as described (*see* **Note 5**).
9. Proceed to thermocycling (lid heated to 100 °C): 95 °C for 3 min, 20 cycles (95 °C 30 sec, 65 °C 5 min), and a final amplification step at 72 °C for 3 min (*see* **Note 29**). Then, centrifuge briefly.
10. Proceed to Second PCR cleanup (*see* **steps 4** and **5**), except eluting with 20 µL of Tris solution, and transfer 18 µL of the purified Second PCR products into new 0.2 mL PCR tubes as described.

    Optional: stopping point (*see* **Note 23**).

3.9 Quantification of the Libraries and Next-Generation Sequencing

According to the supplier's recommendations, library quantification is performed by amplifying the set of six pre-diluted DNA Standards and diluted library samples by qPCR, using the KAPA SYBR FAST qPCR Master Mix and primers targeting the Illumina P5 and P7 flow cell oligo sequences. The average Cq (quantification cycle) score for each DNA Standard is plotted against $\log_{10}$(-concentration in pM) to generate a standard curve. The concentrations of diluted library samples are then calculated against the standard curve, using absolute quantification. This step is very important to establish an equimolar pool of libraries before loading on sequencer.

1. Prepare the following serially diluted libraries for each sample: (i) 1:100 dilution by mixing 2 µL of library (i.e., the purified Second PCR products, *see* **step 10** in Subheading 3.8) to 198 µL of Tris solution, (ii) 1:10,000 dilution by mixing 2 µL of 1:100 dilution to 198 µL of Tris solution, (iii) 1:100,000 dilution by mixing 10 µL of the 1:10,000 dilution to 90 µL of Tris solution and, (iv) 1:500,000 dilution by mixing 20 µL of the 1:100,000 dilution to 80 µL of Tris solution. This last dilution is used for quantification.
2. Prepare a quantitative PCR mix: for one reaction, add 12 µL of the combined KAPA SYBR FAST/Primer Premix solution to 4 µL of Nuclease-free water. Prepare this mix in triplicate for all reactions, i.e., six standards, one blank, and n samples (e.g., for 10 libraries to be sequenced, prepare a mix for 17 × 3 reactions).
3. Centrifuge the PCR mix and dispense 16 µL of mix into each well of a 96-well plate.
4. According to the plate plan (Fig. 3), add 4 µL of nuclease-free water to all NTC (Non-Template Control) wells, of each DNA standard into the appropriate wells, note working from the most diluted tubes (Standard 6) to the most concentrated (Standard 1), and of each previously 1:500,000 diluted library samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	NTC	SPL1	SPL1	SPL1	SPL9	SPL9	SPL9			
B	STD6	STD6	STD6	SPL2	SPL2	SPL2	SPL10	SPL10	SPL10			
C	STD5	STD5	STD5	SPL3	SPL3	SPL3	SPL…	SPL…	SPL…			
D	STD4	STD4	STD4	SPL4	SPL4	SPL4						
E	STD3	STD3	STD3	SPL5	SPL5	SPL5						
F	STD2	STD2	STD2	SPL6	SPL6	SPL6						
G	STD1	STD1	STD1	SPL7	SPL7	SPL7						
H	-	-	-	SPL8	SPL8	SPL8						

Fig. 3 Example of a 96-well plate plan for quantification of libraries, with layouts of NTC (No Template Control), STD (Standards) from the most diluted (STD6) to the most concentrated (STD1), and each sample (SPL) library to be quantified

5. Seal plate with a plastic film and place in real-time thermal cycler. Proceed to PCR with the following program: 95 °C for 5 min, 35 cycles (95 °C for 30 s, 60 °C for 45 s).
6. At the end of the PCR, enter Ct values in a pre-filled excel table (provided by the supplier) to calculate Intercept and Slope values of the standard curve. Then, enter Ct values for each library, to calculate library concentrations and the volumes required for dilutions.
7. Dilute each library in ultra-pure water according to the values indicated in the table to obtain a final library concentration of 4 nM (*see* **Note 30**).
8. Pool 5 µL of each diluted library in a 1.5 mL microtube.
9. Mix 10 µL of the pool of libraries and 10 µL of 200 mM NaOH in a tube. Leave for 5 min at room temperature.
10. Add 10 µL of 200 mM Tris–HCl followed by 970 µL of HT1 Hybridization Buffer: resulting in a final library concentration of 40 pM.
11. Perform sequencing according to MiSeq sequencing system's instructions. The libraries are denatured and loaded on the sequencer following the MiniSeq System Denature and Dilute Library Guide, using final library concentrations of 13 pM to obtain an average of 105 sequencing clusters per sample.
12. Proceed to bioinformatic analysis with ARCHER Dx/INVITAE software. This analysis software utilizes MBC (*see* **Note 7**) for duplicate read binning, error correction, and read deduplication to support quantitative multiplex data analysis and confident mutation detection. Results are delivered in the form of a report with both sequencing metrics and number of unique observations supporting called variants. After validation of quality requirements, fusion transcripts are interpreted according to supplier's instructions (*see* **Note 31**).

4 Notes

1. We work in separate rooms for pre-PCR (before amplification) and post-PCR (after amplification) handling, with laboratory coats specific to each sector, to avoid any inter-sample contamination. The direction of circulation in the premises must always be unidirectional from pre-PCR to post-PCR areas. No backtracking is possible during the same day. Ideally, although this is not always possible, pre-PCR rooms can be over-pressurized and post-PCR rooms under-pressurized. Before and after manipulations, clean the workstation and pipettes with the RNase AWAY nuclease/nucleic acid cleaning product.
2. FFPE tissue to be placed in microtubes for TNA extraction can be obtained from a cytology sample, a biopsy, or a surgical specimen. To be within the sensitivity limits of the RNA-Seq method, tissue sections should contain at least 20% tumor cells, as estimated by a pathologist. There are three different ways to prepare tissue sections: (i) 5 μm tissue sections prepared using a microtome and placed directly in microtubes, (ii) scalpel blade scraping of the tumor area of interest on a slide, and (iii) scalpel blade-macro-dissection of the tumor area of interest in the paraffin block. In the last two cases, the scalpel blade must be changed between each sample. Caution: FFPE samples can be a particular source of inter-sample contamination as paraffin is a light component which can be deposited on surrounding elements, particularly when scratched. Wear gloves throughout sample preparation and be sure to change gloves or clean them with 70% ethanol between samples. When sectioning FFPE blocks with a microtome, use a new blade for each sample. Decontaminate the microtome between samples with clean absorbent paper soaked in 70% ethanol.
3. TNA is extracted rather than just RNA in order to preserve the DNA in the sample for analysis, but the qualification steps relate to the RNA contained in the TNA (*see* **Note 10**).

   Automated TNA extraction from FFPE blocks is a relatively simple and efficient routine technique. However, certain precautions must be taken, such as the use of powder-free nitrile gloves, and the use of a benchtop centrifuge for short centrifugation to prevent the dispersion of microdroplets and aerosols when opening microtubes are opened, thus avoiding inter-sample contamination. RNA extraction is performed using the Maxwell® RSC RNA FFPE Kit, in the Maxwell 16 Automated Extraction System (for up to 16 samples). Carefully prepare the automated system by following the supplier's instructions for the RNA extraction protocol, omitting the

DNA removal step with DNase I. All equipment must be carefully installed and checked to eliminate any risk of malfunction in the system rack.

4. High-throughput sequencing using RNA templates is a rapid and simple method to identify the translocation partners but other alternatives exist to detect gene fusions, hotspot mutations, or short Indels. The choice of methodology depends on the biological matrix or context (pathology), the tissue preservation, the number of genes required in the panel, and also on the equipment and skills available in the laboratory.
5. Lyophilized reagents should always be kept at room temperature in the aluminum bag containing the strips and a desiccant sachet to prevent condensation in the tubes. At the start of each step, the appropriate tubes should be removed from the bag, placed on ice until use, and briefly centrifuged before opening. Never touch the lyophilisate with pipette tips! To dissolve it, add the appropriate volume of sample or reagent (specific to each step) to the tube on ice, wait for 5 s for complete dissolution, and then aspirate/reflux eight times. Centrifuge briefly and place the tubes on ice.
6. Please note that compliance with incubation temperatures is essential for successful sequencing. Use a thermal cycler with a heated lid and follow the lid heating recommendations when indicated. When using a thermal cycler with a 100% ramp rate >6 °C/s, set ramp rate to go no higher than 6 °C/s for PCR 1 and PCR 2.
7. Index 1 P7 consists of eight different indexes, numbered from 1 to 8, each with a precise sequence to be entered on the sample sheet in parallel with the sequence of MBC Adapters.
8. Always ensure that components have been fully thawed and thoroughly mixed before use. The KAPA SYBR FAST qPCR Master Mix may not solidify after freezing from −15 °C to −25 °C. All components of KAPA Library Quantification Kits, as well as the combined KAPA SYBR FAST/Primer Premix solution, are stable through 30 freeze-thaw cycles. The SYBR Green I dye (contained in the KAPA SYBR FAST qPCR Master Mix) and ROX dyes are light sensitive. Exposure to direct light for an extended period of time will result in loss of fluorescent signal intensity. Ensure that all reagents are stored properly (see also Technical Data Sheet KAPA Library Quantification Kit Illumina® Platforms).
9. Only sequencing systems allowing a minimum read length of 300 bp (i.e., 2 × 150 bp) should be used for analysis with Invitae FusionPlex™ Lung v2 panel (sequencing system specifications are available on https://emea.illumina.com/systems/sequencing-platforms.html).

10. For Invitae FusionPlex™ RNA-Seq analysis, TNA instead of only RNA is recommended by the supplier because the DNA component can be used as an internal control for the assay. Indeed, when RNA expression of a target is very low, the DNA in a TNA input will generate reads that can be used to verify that the panel is functioning correctly. Without the DNA, it's difficult to assess whether low read counts are due to low RNA expression or lack of panel functionality.
11. These devices must be switched on long enough before use to be at the correct temperature for the dewaxing and lysis steps. These are critical steps to ensure TNA/RNA extraction efficiency, particularly from FFPE samples.
12. To prevent RNA degradation, it is important to keep RNA samples and reagents on ice.
13. Do not vortex, as the presence of surfactant can cause the solution to foam if stirred too vigorously.
14. Replace with a clean rack, previously washed and decontaminated, to avoid any risk of inter-sample contamination by splashes or micro-droplets.
15. Although this recommendation may seem superfluous, it is essential to reiterate it whenever necessary, particularly as certain steps in TNA extraction and FFPE sample preparation can be a source of inter-sample contamination.
16. Always check the plungers. If incorrectly positioned or not securely attached, the instrument may malfunction or the TNA extraction from the sample may fail.
17. It is crucial to quantify RNA by a fluorimetric method, regardless of the system used (which can be left to the user's choice). Indeed, this method allows the simultaneous assessment of both the quantity and quality of the RNA, indicating whether the RNA has sufficient integrity for RNA-Seq analysis. In contrast, conventional spectrophotometric quantification is not suitable as it only quantifies the nucleotides present in the solution, regardless of whether the nucleic acid is degraded or intact.
18. To obtain reliable sequencing quality, the supplier recommends using a 20 µL volume input containing 20–250 ng RNA, corresponding to initial concentrations of 1–12.5 ng/µL, respectively. Note that higher amounts of RNA can be used if available.
19. The thermocycler should be switched on before handling so that it is at the correct temperature when tubes are inserted and the reaction is started. This is an important precaution for the success of the entire RNA-Seq protocol.

20. This step is critical to evaluate the quality of the first-stranded complementary DNA (cDNA) generated after the reverse transcription step. If quality is inadequate, handling cannot be continued.
21. These controls (Blanks), corresponding to PCR without cDNA matrix, are mandatory to ensure absence of contamination.
22. Optimal Ct values of 23–28 are recommended. However, the supplier indicates that Ct values up to 28.3 are acceptable. In our personal experience, since NSCLC samples are valuable because they are often biopsies with little material, experiments are pursued for Ct values up to 30.
23. Stopping points can be carried out where indicated, overnight at 4 °C or long term at −15 °C to −25 °C.
24. All cleanup/washing steps are identical throughout the protocol, except for the elution volume, which varies according to each specific step. All the cleanup steps must be performed at room temperature. Caution: The washing steps are particularly critical to the success of the manipulation. The recommended volumes, times, pipetting, and mixing procedures must be scrupulously respected. An error or inaccuracy in washing the beads can result in detachment of the fixed cDNAs, and thus in loss of material for library preparation. If "centrifuge briefly" is indicated, it is important not to centrifuge too fast and/or for too long as this may cause sedimentation of the beads.
25. Caution: do not resuspend beads! If the beads have been resuspended, even partially, return the tubes to the magnetic support for 5 min or until the supernatant is clear, then remove and discard the supernatant without touching the bead pellet (leave 5–10 μL).
26. Caution: ensure that the wells are free of ethanol droplets by leaving the plate on the magnet. Any remaining ethanol drops may inhibit amplification. If necessary, centrifuge the tubes and remove the residual volume with a micropipette (ideally a 10 μL pipette volume -P10-).
27. The appearance of the bead pellet should remain shiny and not be dull, indicating that it has dried too much. Beads with cDNAs on the surface must not be allowed to dry excessively; otherwise cDNAs will not be eluted properly.
28. Caution: ensure that the wells are free of water droplets. To remove all droplets, the tubes can be briefly centrifuged and then returned on the magnetic plate. Any droplets remaining at the bottom of the tube can be aspirated using a P10 micropipette. If necessary, pull remaining droplets to the top of the tube for faster drying.

29. The end of the program can coincide with the end of the first day of experiments. Consequently, the thermocycler should be set to 4 °C to keep the samples cold until the next step at the beginning of the second day of experiments.
30. The protocol can also be adapted for sequencing using a NextSEQ Illumina instrument.
31. A detailed procedure for analyzing high-throughput sequencing data is included in the supplier's software.

Acknowledgments

This work was supported by grants from INCa (N°2020-157, grant for implementation of the RNA-SEQ analysis in routine Heathcare). The authors would like to thank the Dupuytren University Hospital Center for financial and logistical support during the analysis implementation, and the Information systems management for providing the computing infrastructure, the pole of Biology-Pharmacy, the pre-analytical sector of the Pathology department, and the Cancer Molecular Genetics, Sequencing and Bioinformatics Platforms.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Stransky N, Cerami E, Schalm S et al (2014) The landscape of kinase fusions in cancer. Nat Commun 5:4846. https://doi.org/10.1038/ncomms5846
2. Hanahan D (2022) Hallmarks of cancer: new dimensions. Cancer Discov 12:31–46. https://doi.org/10.1158/2159-8290.CD-21-1059
3. Tan AC, Tan DSW (2022) Targeted therapies for lung cancer patients with oncogenic driver molecular alterations. J Clin Oncol 40:611–625. https://doi.org/10.1200/JCO.21.01626
4. Gálffy G, Morócz É, Korompay R et al (2024) Targeted therapeutic options in early and metastatic NSCLC-overview. Pathol Oncol Res 30:1611715. https://doi.org/10.3389/pore.2024.1611715
5. Friedlaender A, Perol M, Banna GL et al (2024) Oncogenic alterations in advanced NSCLC: a molecular super-highway. Biomark Res 12:24. https://doi.org/10.1186/s40364-024-00566-0
6. Yoshihara K, Wang Q, Torres-Garcia W et al (2015) The landscape and therapeutic relevance of cancer-associated transcript fusions. Oncogene 34:4845–4854. https://doi.org/10.1038/onc.2014.406
7. Pisapia P, Pepe F, Sgariglia R et al (2021) Methods for actionable gene fusion detection in lung cancer: now and in the future. Pharmacogenomics 22:833–847. https://doi.org/10.2217/pgs-2021-0048
8. Chen M, Xue J, Sang Y et al (2023) Highly sensitive droplet digital PCR for detection of RET fusion in papillary thyroid cancer. BMC Cancer 23:363. https://doi.org/10.1186/s12885-023-10852-z
9. Melchior L, Hirschmann A, Hofman P et al (2024) Multicenter evaluation of an automated, multiplex, RNA-based molecular assay for detection of ALK, ROS1, RET fusions and MET exon 14 skipping in NSCLC. Virchows Arch 484:677–686. https://doi.org/10.1007/s00428-024-03778-9

10. Reguart N, Teixidó C, Giménez-Capitán A et al (2017) Identification of ALK, ROS1, and RET fusions by a multiplexed mRNA-based assay in formalin-fixed, paraffin-embedded samples from advanced non-small-cell lung cancer patients. Clin Chem 63:751–760. https://doi.org/10.1373/clinchem.2016.265314

11. Bruno R, Fontanini G (2020) Next generation sequencing for gene fusion analysis in lung cancer: a literature review. Diagnostics (Basel) 10:521. https://doi.org/10.3390/diagnostics10080521

Chapter 10

Simultaneous Profiling of Transcripts and Genomic Regions Associated with Nuclear Bodies Using the RNA–DNA High-Salt Recovered Sequence (RD-HRS) Method

Nathan Lecouvreur, Cosette Rebouissou, Annick Lesne, and Thierry Forné

Abstract

Regulation of many nuclear functions in eukaryotic cells, such as transcription and splicing, relies on the association of specific genomic loci and/or transcripts with nuclear microenvironments having high molecular crowding, such as nuclear bodies. These membrane-less organelles are typically self-assembled through mechanisms of phase separation, and because of their liquid-like nature, analyzing their composition remains challenging. Here we present a detailed protocol of the RNA–DNA High-salt Recovered Sequence (RD-HRS) method, an extended version of the High-salt Recovered Sequence-sequencing (HRS-seq). Using high-salt treatments to make nuclear bodies insoluble, this extended version allows the profiling of both their RNA and genomic DNA contents, simultaneously, from the same biological sample.

Key words Nuclear bodies RNAs, High-order chromatin organization, Phase separation

1 Introduction

Nuclear bodies are membrane-less cellular organelles typically self-assembled and disassembled through mechanisms of phase separation. They play a crucial role in 3D genome organization, promoting interactions between distant chromatin regions located in distinct topologically associating domains (TADs) and sometimes even on different chromosomes [1]. They appear to be crucial for the regulation of gene expression [2], in particular transcription and splicing. However, because of their liquid-like nature, it remains challenging to purify them in order to examine their composition.

Based on experimental approaches developed in our laboratory [3, 4], we have developed a genome-wide experimental method, High-salt Recovered Sequences sequencing (HRS-seq), which

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_10,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

allows the identification of chromatin regions associated with nuclear microenvironment with high molecular crowding, like nuclear bodies [5]. Here we describe the detailed protocol of an extended version of this method, named the RNA–DNA High-salt Recovered Sequence (RD-HRS) method, that allows the simultaneous profiling of both the RNA and genomic DNA associated with nuclear-bodies from the same biological sample (Fig. 1).

Using Triton X-100 permeabilization of purified cell nuclei on a filtration unit, we first extract nucleoplasmic RNAs (soluble RNA fraction) (Fig. 1). We then use high-salt treatment in order to make nuclear bodies insoluble, resulting in the formation of the so-called nuclear halos. As previously demonstrated [5], a key point of the method is that the trapping of DNA and RNA inside the insoluble nuclear halos (inset in Fig. 1) accurately reflects their in vivo location in nuclear micro-environments with high molecular crowding, like nuclear bodies or other large ribonucleoprotein complexes. To analyze their DNA and RNA contents, these nuclear halos are incubated with a restriction enzyme (StyI) to obtain a soluble DNA fraction that passes through the filter while the RNA and DNA trapped in insoluble nuclear bodies remain on its surface (insoluble RNA and DNA fractions) (Fig. 1). Unbiased high-throughput sequencing or locus-specific quantitative PCR/RT-PCR can then be used to identify the RNAs and DNA regions associated with nuclear bodies as being those enriched in the insoluble fraction compared with their respective soluble RNA/DNA fraction.

2 Materials

1. Cell culture medium (appropriate for cells of interest) and trypsin–EDTA (for adherent cells).
2. Phosphate-buffered saline (PBS).
3. Buffer 1: 20 mM HEPES pH 7.9, 10 mM KCl, 0.1% Triton X-100, 20% glycerol, 1 mM $MnCl_2$, 0.5 mM spermidine, Protease Inhibitor 1× (*see* **Note 1**).
4. Triton buffer: 10 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.3 M sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100.
5. 2 mL centrifugal filter units with 0.22 μm pores and 12 mL collection tubes (e.g., Millipore Ultrafree®-CL GV 0.22 μm).
6. Home-made pistons: pistons of 2 mL syringe modified by adding a seal of interior diameter = 6 mm/exterior = 9 mm or home-made apparatus (HMA) for pressurizing filtration units (*see* **Note 2**).
7. Extraction buffer: 20 mM Tris–HCl pH 7.5, 2 M NaCl, 10 mM EDTA, 0.125 mM spermidine.

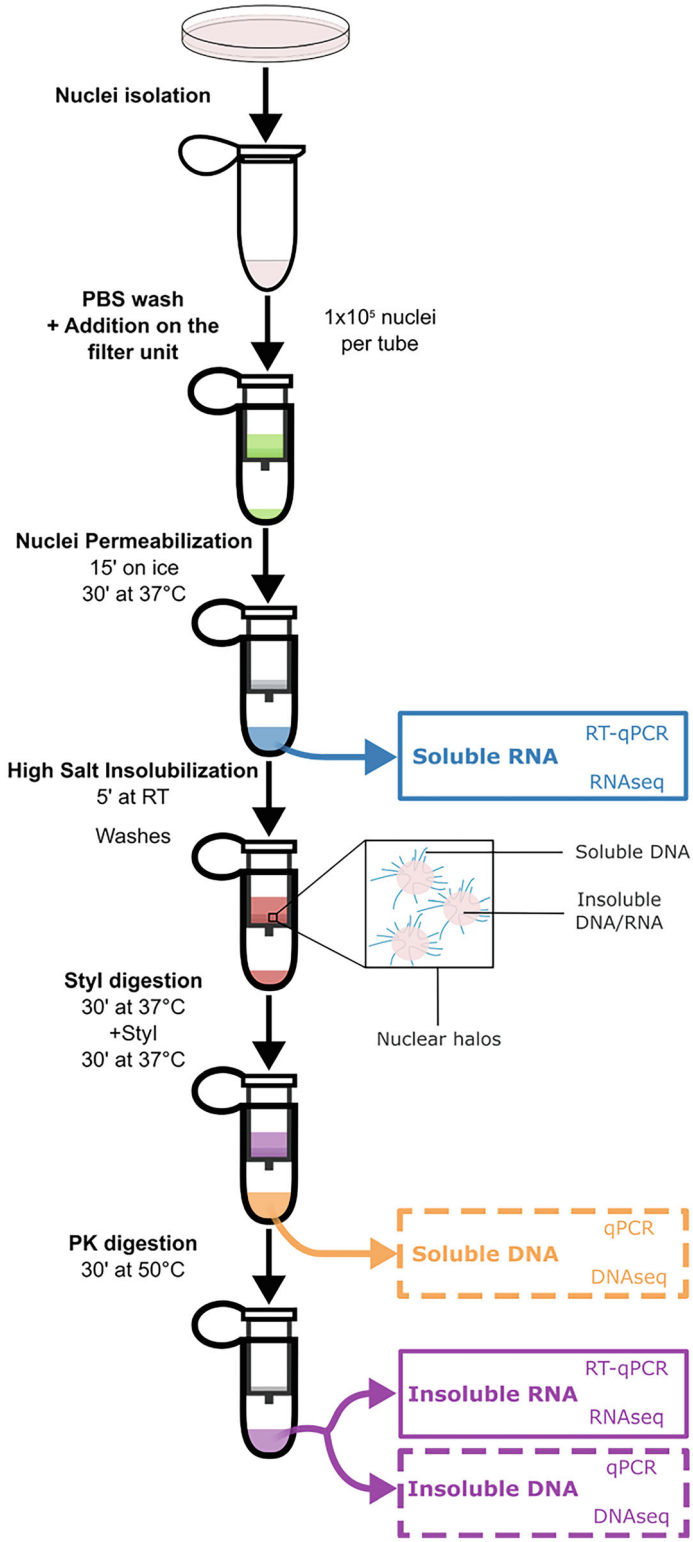


Fig. 1 Principle of the RNA-DNA high-salt recovered sequence (RD-HRS) assay. Starting from purified cell nuclei preparations, nucleoplasmic RNAs are first extracted by gently permeabilizing the nuclei with Triton X-100 on a filtration unit. The principle of the HRS technique then relies on purification of nuclear bodies that

8. 1× StyI enzyme buffer: 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 0.2 mM spermidine (*see* **Note 3**).
9. 10 U/μL StyI enzyme.
10. 10× Proteinase K (PK) buffer: 100 mM Tris–HCl pH 7.5, 50 mM EDTA, 5% SDS. Also diluted to a 2× stock.
11. 20 mg/mL proteinase K (PK).
12. NucleoSpin™ Gel and PCR Clean-up kit compatible with SDS-containing samples (e.g., Macherey-Nagel™, with NTB, NT3, and NE buffers).
13. Vacuum manifold.
14. Qubit fluorimeter and dsDNA/RNA HS assays, or equivalent.
15. Milli-Q and RNase-free water.
16. PCA: Acid phenol pH4.3/chloroform/isoamyl alcohol (25:24:1).
17. SEVAG: chloroform/isoamyl alcohol (24:1).
18. 5 M NaCl.
19. 20 mg/mL glycogen.
20. DNase: DNase I.
21. 10× DNase buffer.
22. 100% and 70% ethanol.

3 Methods

3.1 Cell Nuclei Preparation (See Note 4)

1. If working with non-adherent cultured cells, proceed with **step 2** below. If working with adherent cell cultures, trypsinize cells (*see* **Note 5**), before proceeding with **step 2** below.
2. Wash cells in PBS.
3. Resuspend cells in 100 μL of ice-cold buffer 1 per 500,000 cells.
4. Incubate for 10 min on ice.
5. Centrifuge for 3 min at 600× *g*, 4 °C.
6. Remove supernatant.

Fig. 1 (continued) are made insoluble by high-salt treatments. The soluble DNA can be released after digestion with a restriction enzyme (StyI), while the insoluble RNA and DNA fractions, trapped into the nuclear bodies, can be purified from the biological material that remains on the filter. Accurate quantifications of RNAs and target sequences in the insoluble vs soluble fractions by quantitative PCR/RT-PCR or by high-throughput sequencing are then performed. Significant overrepresentation of an RNA or a restriction fragment in the insoluble fraction reflects its retention into nuclear bodies

7. Resuspend pellets in the same volume of ice-cold buffer 1 as in **step 3** and take a 10 µL aliquot to count nuclei in a Thoma cell, or any other cell counting device, after trypan blue staining.
8. Adjust nuclei preparation to a final concentration of about 20 million nuclei in 100 µL and, from this stock, make 100 µL aliquots containing 100,000 nuclei diluted in buffer 1. Slowly freeze at −80 °C in a room temperature isopropanol-filled chiller.

3.2 Generation of Nuclear Halos by High-Salt Treatment

1. Thaw on ice an aliquot containing 100,000 nuclei in a 1.5 mL Eppendorf tube (important: do not increase the number of nuclei treated during these steps, i.e., 100,000 nuclei, *see* **Note 6**).
2. For 100 µL of aliquot, add 900 µL of PBS on the nuclei and homogenize by pipetting.
3. Transfer the Eppendorf content onto the filter of a centrifugal filtration unit equipped with a 12-mL collector tube below.
4. Apply a mild pressure with the help of the piston or home-made apparatus (HMA) (*see* **Note 2**) to push the liquid through the filter at a drop-by-drop rhythm (no more than one drop per second) until as little liquid remains as possible above the filter (do not dry the filter).
5. Add a band of parafilm under the filter part to obstruct the filtration unit and place it back on the collector tube.
6. Add 1 mL of Triton buffer and homogenize by gently pipetting.
7. Incubate for 15 min on ice (mix gently by pipetting three times during incubation).
8. Incubate for 20 min at 37 °C (*see* **Note 7**).
9. Remove the parafilm and put the filtration unit on a 12-mL collector tube.
10. Use the piston (or HMA) to pass the entire volume as described in **step 4**. This fraction is the *permeabilization fraction* containing soluble RNAs (Fig. 1).
11. Add 100 µL of 10× Proteinase K (PK) buffer as well as 3 µL of 20 mg/mL PK to the permeabilization fraction, and incubate for 30 min at 50 °C. Proceed to Subheading 3.6 to extract total RNA from this fraction.
12. Add 1 mL of extraction buffer onto the filter of the filtration unit and homogenize gently by pipetting. Push six drops out (dead volume of the column).
13. Incubate for 5 min at room temperature.
14. Use the piston (or HMA) to pass the entire volume of extraction buffer through the filter at a drop-by-drop rhythm.

15. Add 1.9 mL of 1× StyI enzyme buffer. Pass all the liquid through the filter at a drop-by-drop rhythm. Repeat one more time, passing all the liquid through the filter and emptying the collection tube to the waste (these washings allow removal of high salt concentration present in the extraction buffer).

3.3 Digestion of Nuclear Halos

1. For each filtration unit, add a band of parafilm under the filter part to obstruct the filtration unit and place it back on the collector tube.
2. Add a mix containing 960 μL of 1× StyI enzyme buffer and 20 μL of 10 U/μL StyI, and homogenize gently by pipetting (*see* **Note 8**).
3. Incubate the filtration unit for 30 min at 37 °C (take care that all the liquid in the filtration unit should be immersed into the water bath and check regularly that there is no leakage due to a lack of parafilm obstruction).
4. Collect evaporation drops in the tube cap and around tube walls and place them back in the liquid onto the filter. Add 20 μL of 10 U/μL StyI, mix gently by pipetting, and incubate for 30 min more.

3.4 DNA Purification of StyI-Digested DNA from Soluble and Insoluble Fractions

1. Remove tubes from the water bath. Collect evaporation drops in the tube cap and around the tube walls, and place them back with the remaining liquid on the filter. Homogenize gently by pipetting.
2. Remove the parafilm, collect residual drops on the parafilm, and place them back in the liquid on the filter. Place the filter part on a clean collector tube and pass all the liquid through the filter.
3. Add 1 mL of 1× StyI enzyme buffer to rinse the nuclear halos and pass it all through the filter in the same collector tube as **step 2**. Save this eluate, corresponding to the *soluble fraction* (2 mL) containing the soluble DNA (Fig. 1). Proceed to **step 7** to extract DNA from this fraction.
4. Place a parafilm to obstruct the filtration unit and put the filtration unit on a clean collector tube (12-mL).
5. Add 500 μL of 1× StyI enzyme buffer to the filter,
6. Add 500 μL of 2× PK buffer and 10 μL of 20 mg/mL proteinase K to the filter. Homogenize gently by pipetting.
7. Add 200 μL of 10× PK buffer and 10 μL of 20 mg/mL proteinase K to the *soluble fraction* from **step 3**. Homogenize gently by pipetting.

8. Incubate the filtration unit and the tube containing the soluble fraction for 30 min at 50 °C in a water bath (take care to keep them well immersed).
9. Pass all the liquid through the filter of the filtration unit. Save this eluate in a clean collection tube. It corresponds to the *insoluble fraction* (1 mL) containing the biological material made insoluble by the high-salt treatment (Fig. 1).
10. Split the 1 mL of insoluble fraction in two 500 µL aliquots. Proceed to Subheading 3.6 to extract RNA from the first aliquot and to **step 11** to extract DNA from the second aliquot.

    Steps 11–16 refer to the NucleoSpin™ Gel and PCR Clean-up kit protocol.
11. Add 2.5 mL of NTB buffer to the insoluble fraction and 11 mL to the soluble fraction (five volumes). The soluble and insoluble fractions are incubated for 5 min at 50 °C in a water bath (*see* **Note 9**) and then processed in parallel.
12. Add 700 µL of one fraction to a NucleoSpin™ column and pass all the liquid by aspiration on a vacuum manifold (or by centrifugation 30 s at 11,000× *g*, room temperature). Repeat these 700 µL loadings until all of one fraction has passed through the same column.
13. Pass 700 µL of NT3 buffer through the column using the vacuum manifold (or centrifuge for 1 min at 11,000× *g*, room temperature) and empty collector tube in the waste.
14. Pass again 700 µL of NT3 buffer through the column as in **step 13**.
15. Place the column on a clean Eppendorf tube properly labeled.
16. Add 100 µL of NE buffer to the column corresponding to the soluble fraction and 50 µL to the column corresponding to the insoluble fraction. Incubate for 5 min minimum at room temperature. Centrifuge for 1 min at 11,000× *g*, room temperature.
17. Quantify the DNA within each fraction with a Qubit fluorometric DNA assay or equivalent (*see* **Note 10**).
18. Repeat Subheadings 3.2, 3.3, and 3.4 on further aliquots until at least 150 ng DNA and 150 ng total RNA are obtained for each fraction. Add milli-Q water to each fraction to make a final volume of 500 µL.

3.5 Re-Digestion of Genomic DNA (See Note 11)

1. Add 55 µL of 10× digestion buffer (*see* **Note 3**) and 1 µL of 10 U/µL StyI to each fraction from **step 18** in Subheading 3.4. Incubate for 30 min at 37 °C.

2. Purify the DNA with NucleoSpin™ columns as in **steps 11–16** in Subheading 3.4, except that 2.5 mL NTB buffer (five volumes) is first added.
3. Quantify the DNA within the fractions with a Qubit fluorometric assay or equivalent. If required, concentrate each fraction by ethanol precipitation and resuspend the DNA to reach a final concentration of at least 10 ng/μL (*see* **Note 12**).
4. HRS-seq DNA libraries are then made and analyzed as previously described [6]. Each library requires 150 ng of StyI-digested genomic DNA (soluble or insoluble fraction) as starting materials (*see* **Note 13**).

3.6 RNA Purification from Permeabilization and Insoluble Fractions

1. Add two volumes of PCA to each fraction (i.e., 2 mL for the permeabilization fraction from **step 11** in Subheading 3.2 and 1 mL to the first aliquot of the insoluble fraction from **step 10** in Subheading 3.4). Shake vigorously for 15 s.
2. Centrifuge for 15 min at 13,000× *g*, 4 °C. Save the supernatants into clean tubes.
3. Add two volumes of SEVAG to each fraction (i.e., 2 mL of SEVAG to the permeabilization fraction and 1 mL to the insoluble fraction aliquot).
4. Centrifuge for 15 min at 13,000× *g*, 4 °C. Save the supernatants into clean tubes.
5. Add 0.05 volume of 5 M NaCl to each fraction (i.e., 50 μL to the permeabilization fraction and 25 μL to the insoluble fraction), as well as 1 μL of glycogen to each fraction.
6. Add 2.5 volumes of cold 100% ethanol to each fraction (i.e., 1.25 mL to the permeabilization fraction and 2.5 mL to the insoluble fraction). Incubate for 1 h at −80 °C (or overnight at −20 °C).
7. Centrifuge for 20 min at 17,000× *g*, 4 °C and remove ethanol.
8. Add 1 mL of cold 70% ethanol on the pellets and centrifuge for 10 min at 17,000× *g*, 4 °C.
9. Add 30 μL of RNase-free water to the dried pellets and shake carefully for 5 min.
10. To each resuspended pellet, add 1 μL of DNase, 25 μL of 10× DNase buffer, and 194 μL of RNase-free water (i.e., 250 μL final volume).
11. Incubate for 20 min at 34 °C and repeat **steps 1–9** (adapt the volumes of reactants to a sample volume of 250 μL).
12. Quantify the RNA within the permeabilization and insoluble fractions with a Qubit fluorometric RNA assay or equivalent.

13. Purified total RNA can then be analyzed either by standard RNA-seq procedures (*see* **Note 14**) and/or by reverse transcription and quantitative PCR (RT-qPCR) targeting transcripts of interest.

4 Notes

1. Spermidine and protease inhibitors should be added extemporaneously to this buffer.
2. A home-made apparatus (HMA) for pressurizing filtration units was developed. Such apparatus is making use of a hydrostatic pump. It enables users to exert a fixed and constant pressure on the liquid above the filter which can be easily set-up by controlling the speed of the hydrostatic pump. This apparatus was previously described in a publication on the former HRS-seq protocol [6].
3. We recommend using the home-made restriction buffer since commercial buffers usually contain reducing agents like DTT that may alter the insoluble fraction thus affecting DNA retention.
4. The present protocol, adapted from the EpiCypher Cutana™ CUT&RUN Library Prep kit, is intended for cultured cells as previously described [7]. If working with mammalian tissues, prepare nuclei as previously described in references [8, 9] and proceed with **step 2** in Subheading 3.1. The quality of nuclei preparation is a critical point of the procedure. This protocol is intended for metazoan cultured cells or tissues. If using organoids or plant cells, make sure to use adapted protocols for nuclei preparation.
5. For example, incubate cells for 5 min at 37 °C with (0.25% trypsin, 10 mM EDTA, diluted in PBS), add culture medium containing 5% FCS, centrifuge for 5 min at 290× *g*, or use any other dissociation protocol adapted to the cell type used.
6. *Important note*: do not increase the number of nuclei treated during **step 2** in Subheading 3.2 to **step 11** in Subheading 3.4. It is extremely important to keep a small number of nuclei (100,000) diluted in a big volume during these steps because nuclear halos tend to aggregate together and such aggregation could generate in vitro artifacts. For the same reason, do not pellet nuclear halos by centrifugation, but, as stated in this protocol, always use filtration units and apply gentle pressure on the liquids using a piston or HMA [10].
7. Make sure that the liquid level in the column is fully immersed in the water bath.

8. If using a different enzyme to StyI, the enzyme buffer provided in this protocol will need to be modified accordingly.
9. Samples should be heated at 50 °C to avoid clogging in the purification columns.
10. Quality controls can be made at this stage by qPCR to check for enrichment of specific sequences [3, 5].
11. Partial digestion is a technical concern; Subheading 3.5 ensures full digestion before DNA library preparation.
12. The minimal concentration for DNA samples after Subheading 3.5 is 10 ng/μL. If less than 500 ng have been obtained, the sample will need to be concentrated to at least 10 ng/μL by ethanol precipitation (to a 50 μL sample add 2.5 μL of 5 M NaCl, 1 μL of 20 mg/mL glycogen, and 150 μL of 100% ethanol, mix, keep 45 min at −80 °C, and centrifuge for 15 min at 15,700× *g*, room temperature). If more than 500 ng have been obtained, the sample can be used directly.
13. Critical step: 150 ng corresponds to the amount recommended to ensure a good quality of the final libraries.
14. Use oriented RNA-seq after ribodepletion of total RNA if premature/nascent RNAs are to be investigated.

Acknowledgments

We thank Jean Casanova, leader of the SEM platform, for developing the HMA system, and Hugues Parrinello and all the other staff of the MGX platform (Montpellier) who designed the strategy for building the HRS-seq DNA libraries. This work was supported by grants from the *Mission pour les initiatives transverses et interdisciplinaires* (MITI) of the *Centre National de la Recherche Scientifique* (projet 80|Prime Argecor), the *Ligue contre le cancer (comité de l'Hérault, contract 269152)*, the *ARC Fondation* (*contract* ARCPJA2023080006958), and the *Fondation pour la Recherche Médicale (contract MND202310017917)*.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Ea V, Baudement M-O, Lesne A, Forné T (2015) Contribution of topological domains and loop formation to 3D chromatin organization. Genes 6:734–750. https://doi.org/10.3390/genes6030734
2. Lesne A, Baudement M-O, Rebouissou C, Forné T (2019) Exploring mammalian genome within phase-separated nuclear bodies: experimental methods and implications for gene expression. Genes 10:1049. https://doi.org/10.3390/genes10121049

3. Braem C, Recolin B, Rancourt RC et al (2008) Genomic matrix attachment region and chromosome conformation capture quantitative real time PCR assays identify novel putative regulatory elements at the imprinted Dlk1/Gtl2 locus. J Biol Chem 283:18612–18620
4. Weber M, Hagège H, Lutfalla G et al (2003) A real-time polymerase chain reaction assay for quantification of allele ratios and correction of amplification bias. Anal Biochem 320:252–258. https://doi.org/10.1016/S0003-2697(03)00396-8
5. Baudement MO, Cournac A, Court F et al (2018) High-salt-recovered sequences are associated with the active chromosomal compartment and with large ribonucleoprotein complexes including nuclear bodies. Genome Res 28:1733–1746
6. Rebouissou C, Baudement M-O, Forné T (2022) The high-salt recovered sequence-sequencing (HRS-seq) method: exploring genome association with nuclear bodies. In: Sexton T (ed) Spatial genome organization. Springer, New York, pp 187–197
7. Rebouissou C, Sallis S, Forné T (2022) Quantitative chromosome conformation capture (3C-qPCR). In: Sexton T (ed) Spatial genome organization. Springer, New York, pp 3–13
8. Milligan L, Forné T, Antoine E et al (2002) Turnover of primary transcripts is a major step in the regulation of mouse *H19* gene expression. EMBO Rep 3:774–779. https://doi.org/10.1093/embo-reports/kvf142
9. Milligan L, Antoine E, Bisbal C et al (2000) H19 gene expression is up-regulated exclusively by stabilization of the RNA during muscle cell differentiation. Oncogene 19:5810–5816. https://doi.org/10.1038/sj.onc.1203965
10. Weber M, Hagège H, Murrell A et al (2003) Genomic imprinting controls matrix attachment Regionsin the *Igf2* gene. Mol Cell Biol 23:8953–8959. https://doi.org/10.1128/MCB.23.24.8953-8959.2003

Part III

RNA Maturation: Splicing, Polyadenylation, and Epitranscriptomic Modifications



Chapter 11

Functional Evaluation of Splice Variants Using a Minigene Strategy

Angélique Nizou, Lana Mahfoud, Alexandre Janin, and Anne-Sophie Lia

Abstract

Suspected splicing variants can be very challenging to evaluate more particularly when RNA samples are not available to perform RNA sequencing or when the gene of interest is expressed in specific tissues not easily reachable. One alternative strategy to study the potential splicing effect of a variant is to use the patient's DNA. Indeed, it is easily possible to amplify the region of interest and include it in the reporting system such as hybrid minigene, which could then be transfected in eukaryotic cells in order to estimate the impact of the mutated region in comparison to a wild-type version of the region. This strategy could help to evaluate the impact of genetic alterations on RNA splicing by using only patient's DNA.

Key words Hybrid minigene, RNA splicing, Homologous recombination-mediated cloning, Bacterial transformation, Cell transfection

1 Introduction

After transcription, the vast majority of eukaryotic transcripts are spliced. RNA splicing is based on the presence of specific sequences allowing the cell to identify the precise location to be spliced. This includes the exon–intron borders consensus sequences [1], but also the regulation sequences located in exons and introns that enhance or repress exon usage named exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs) [2]. When a variant is identified in putative region involved in splicing, it could be sometimes very challenging to evaluate its impact, more particularly when RNA samples are not available to perform RNA sequencing. While bioinformatic prediction tools have been largely developed this last decade (SpliceAI for instance [3]), functional tests are often preferred by the molecular biologists in order to conclude. Minigene constructs are important tools to assess the impact of putative

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_11,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

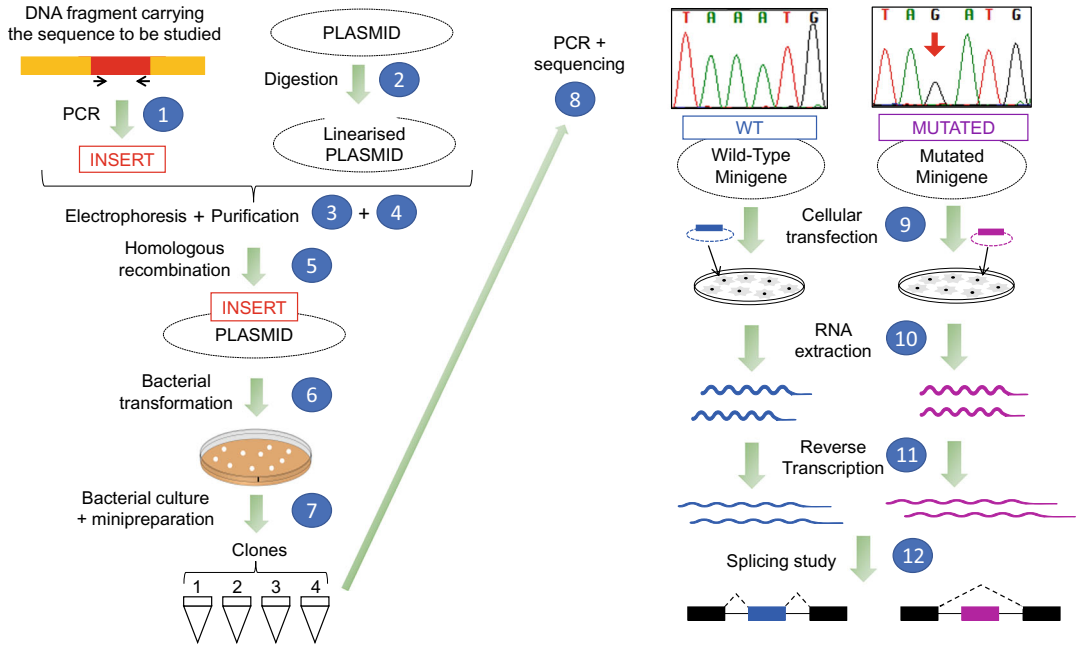


Fig. 1 General overview of the Minigene strategy used to evaluate splicing variants. The 12 steps presented here are in correlation with the 12 steps described in the text. Step 1: The region of interest (usually an exon and the surrounding intronic region) of a patient is amplified by PCR. Step 2: At the same time, the plasmid containing a minigene (generally a hybrid construct containing exons from different genes under the control of a strong promoter) is digested using restriction enzyme in order to linearize it. Steps 3 and 4: The qualities of the PCR product and the linearized plasmid are evaluated with an electrophoresis and the fragments are then purified. Step 5: Homologous recombination is performed in order to introduce the insert within an intronic region of the minigene, leading to a hybrid minigene. Step 6: The preparation is then transformed into bacteria. Step 7: Clones are cultured and plasmid DNA are extracted (minipreparation). Step 8: The plasmid DNA of the clones is screened, by PCR and sequencing, in order to select both kinds of plasmids: a minigene including the mutated amplified region and a minigene including the wild-type region. Step 9: Transfections into eukaryotic cells are performed allowing the minigenes to be transcribed and spliced. Step 10: Total RNA extractions are performed. Step 11: RNAs are then reverse transcribed into cDNAs. Step 12: cDNAs derived from the hybrid minigene are analyzed, by PCR and Sequencing, in order to assess the impact of a genetic alteration on RNA splicing

splicing mutations, by testing splicing efficiency of a specific region. Numerous minigenes have been created these last years, but the principle to use them is the same and is summarized in Fig. 1. A minigene is generally a hybrid construct containing exons from different genes under the control of a strong promoter. The region of interest of a patient is then amplified and introduced within an intronic region of the minigene. The preparation is then transformed into bacteria and clones are screened in order to select, by PCR and sequencing, both kind of plasmids: a minigene including the mutated amplified region and a minigene including the wild-type region. Transfection into eukaryotic cells is then performed allowing the minigenes to be transcribed and spliced. Upon RNA

extraction, mRNA derived from the hybrid minigene are analyzed by RT-PCR using primers located on adjacent (upstream and downstream) exons. PCR products corresponding to the spliced transcripts are first evaluated on agarose gel and then sequenced. The strategy presented here to assess the impact of a genetic alteration on RNA splicing is an adapted method derived from Baralle and Baralle [4].

2 Materials

2.1 Amplification of the Region of Interest on Patient's DNA

1. 50 ng/μL genomic DNA (gDNA) from patients to be studied (*see* **Note 1**).
2. PCR primers designed specifically to (a) amplify the region of interest with a minimum of 200 bp surrounding the exon to be studied and (b) include specific sequences allowing homologous recombination-mediated cloning: Forward primer 5′ [CTAAACAGCCACATA + specific 5′ sequence of the region of interest] 3′ and Reverse primer 5′ [CCCCCCTCGACCATA + specific 3′ sequence of the region of interest] 3′, at 10 μM each (Fig. 2a).
3. Taq polymerase: TransTaq® HiFi DNA polymerase (TransGen Biotech) (*see* **Note 2**).
4. 10× TransTaq® HiFi Buffer I.
5. 10 mM Deoxynucleotide Triphosphate (dNTP).
6. Nuclease-free sterile water.
7. Thermocycler.

2.2 Plasmid Digestion

1. 250 ng/μL plasmid minigene, called here pTB2 (Fig. 2b) [4].
2. 20 U/μL NdeI restriction enzyme.
3. 10× Buffer for NdeI enzymatic restriction (included with the restriction enzyme).
4. Thermocycler.

2.3 Electrophoresis on Agarose Gel

1. 0.5× TAE Buffer: 40 mM Tris-Acetate, 1 mM EDTA.
2. Agarose gel: 1% w/vol prepared with 0.5× TAE Buffer.
3. 6× DNA loading buffer.
4. DNA Ladder 1 kb.
5. Electrophoresis system: RunOne™ electrophoresis system with gel casting (Embi Tec).
6. UV lamp.

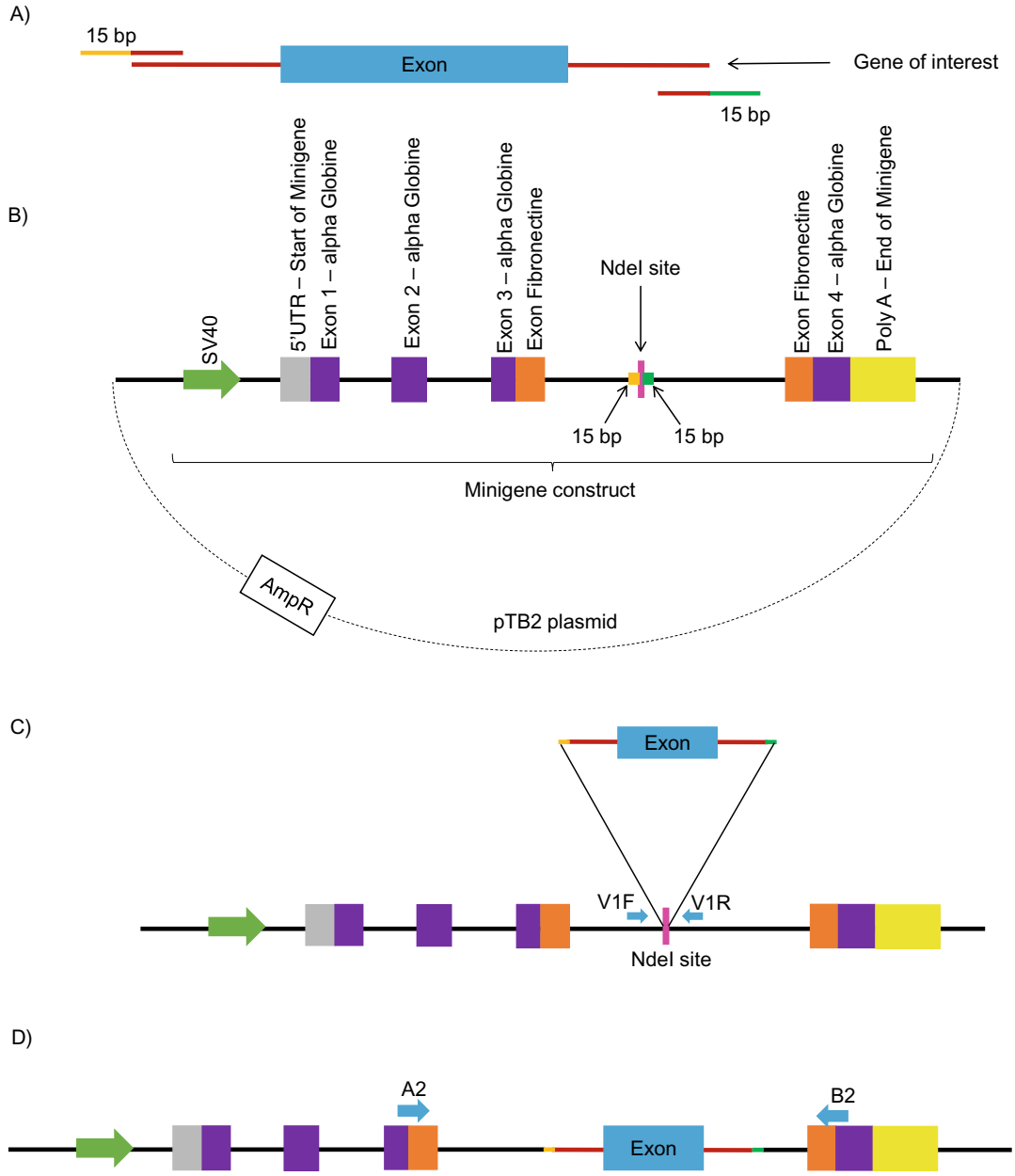


Fig. 2 Overview of the plasmid minigene and primer positions. (**a**) Amplification of the region of interest on patient's DNA. (**b**) Overview of the pTB2 plasmid minigene. (**c**) Visualization of primer positions for screening bacterial clones. (**d**) Visualization of primer positions to study splicing of the insert

2.4 DNA Purification (PCR Products and Linear Plasmid) and Bacterial Cloning

1. PCR product.
2. Linearized pTB2 Plasmid after NdeI digestion.
3. EasyPure® PCR Purification Kit (TransGen Biotech) with Binding Buffer (BB), Wash Buffer (WB), Elution Buffer (EB) and Columns (*see* **Note 3**).

4. EasyPure Quick Gel Extraction Kit (TransGen, Biotech) with Gel Resuspension Buffer (GSB), Wash Buffer (WB), Elution Buffer (EB) and Columns (*see* **Note 3**).
5. Microcentrifuge.
6. In-Fusion® HD Cloning Kit (Takara Bio) with 5× In-Fusion HD Enzyme Premix.
7. Water bath.
8. Ice.
9. Incubation chamber with agitation.
10. Competent bacteria: DH5α competent *E. coli*.
11. Solid and liquid Lysogeny Broth (LB) medium.
12. 25 mg/mL ampicillin.
13. EasyPure Plasmid MiniPrep Kit (TransGen Biotech) with resuspension buffer (RB), lysis buffer, neutralization buffer (NB), wash buffer (WB), and columns.
14. Ethanol 96%.

2.5 Screening of Bacterial Clones by PCR and Sequencing

1. Minipreparation: plasmid DNA from bacterial clones.
2. Primers surrounding the pTB2 plasmid NdeI restriction site (Fig. 2c): Forward primer V1F 5'[AGCATTGCTGATACTTACTTCAGA]3′ at 10 μM, and Reverse primer V1R 5'[CACAGCAAAGAGAAACATAGCAG]3′ at 10 μM.
3. Materials and reagents for PCR analysis (*see* **items 3–7** in Subheading 2.1 and **items 1–6** in Subheading 2.3).
4. Big Dye Terminator V1.1 (Applied Biosystems).
5. Purification kit: DyeEx 2.0 Spin Kit (Qiagen).
6. DNA Sequencer: 3500xL Dx Genetic Analyzer (Applied Biosystems), with suitable sequencing plate.
7. Sequence analysis software.

2.6 Cell Culture and Transfection

1. COS-7 Monkey Kidney Fibroblasts (ATCC) (*see* **Note 4**).
2. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% of fetal bovine serum (FBS), 2 mM L-glutamine, and 1% of 100× non-essential amino acids (Gibco).
3. Phosphate buffered saline (PBS).
4. Trypsin solution: Trypsin-EDTA (0.05%), phenol red.
5. Malassez Counting Chamber.
6. Cell culture flasks (75 cm^2) with filter caps.
7. 50 mL centrifuge tubes.
8. 6-well plates.

9. Plasmid pTB2, either empty or with insertion of wild-type or mutated minigene.
10. Transfection Reagents: JetPrime Transfection Reagent and JetPrime Buffer (OZYME).
11. 1.5 mL microtubes.

2.7 Cell Lysis, RNA Extraction, and RT-PCR

1. Transfected cells.
2. RNA extraction kit (Rapid Animal Total RNA Extraction kit, Bio Basic) with Solution A.
3. Chloroform.
4. Ethanol 75%.
5. RNase-free water.
6. Isopropanol.
7. Reverse transcription (RT) kit: FIREScript® RT cDNA Synthesis MIX (Solis BioDyne) with FIREScript Enzyme Mix and 10× RT Reaction Premix with Oligo (dT) and Random primers.
8. Primers designed specifically to study the splicing of the insert into the plasmid (Fig. 2d): Forward primer A2: 5′[CAACTTCAAGCTCCTAAGCCACTG]3′ at 10 μM, and Reverse primer B2: 5′[GGTCACCAGGAAGTTGGTTAAATCA]3′ at 10 μM.
9. Materials and reagents for PCR analysis and DNA sequencing (*see* **items 3–7** in Subheading 2.5).

3 Methods

Chemical waste should be thrown in specific containers. Do not throw down the sink. The workstation needs to be cleaned with a disinfectant solution or 70% ethanol before and after every experiment. The micropipettes need to be cleaned exclusively with 70% ethanol. After closing the workstation, run a UV cycle.

3.1 Amplification of the Region of Interest on Patient's gDNA

The following instructions concern one tube. A negative control needs to be prepared simultaneously. It is preferable to prepare a mix with all the common reagents.

1. Under the workstation, prepare a mix of the following items (multiply the volumes by the number of tubes): 16 μL of nuclease-free sterile water, 2 μL of 10× TransTaq® HiFi Buffer I, 0.4 μL of dNTP 10 mM, 0.2 μL of TransTaq® HiFi DNA polymerase 5 U/μL, and 0.4 μL of the primer mix (forward and reverse primers, *see* **item 2** in Subheading 2.1).
2. Transfer 19 μL of the mix in a tube.

3. Outside the workstation, add 1 µL of patient's gDNA at 50 ng/µL in the sample tube.
4. For the negative control, transfer 19 µL of the mix and add 1 µL of water.
5. The tubes are ready to be put in the Thermocycler. Prepare the Thermocycler and run the following PCR program: 3 min at 94 °C, 25 cycles (30 s at 94 °C, 30 s at 60 °C, 2 min at 72 °C), 10 min at 72 °C and let cool to 10 °C for as long as needed before the next step.

3.2 Enzymatic Digestion of the Plasmid

1. In parallel, proceed with the linearization of the pTB2 plasmid in a 0.2 mL tube.
2. Add 37.5 µL of nuclease-free sterile water, 5 µL of 10× restriction enzyme buffer, 6 µL of plasmid pTB2 at 250 ng/µL, and 1.5 µL of NdeI at 20 U/µL.
3. Incubate for 1 h 30 min at 37 °C.
4. Then, inactivate the restriction enzyme for 20 min at 65 °C.

3.3 Analysis of PCR Products and Linear Plasmid by Agarose Gel Electrophoresis

1. Use a 1% agarose gel prepared the same day. It should contain at least five wells.
2. Place the gel in the electrophoresis system.
3. Prepare five tubes with 2 µL of 6× DNA loading buffer and 8 µL of nuclease-free water and add 2 µL of each sample (Tubes 1–5).
4. Fill five wells on the agarose gel as follows: PCR products (*see* **steps 3–5** in Subheading 3.1) in Lane 1, negative PCR control (*see* **steps 4–5** in Subheading 3.1) in Lane 2, the linearized pTB2 plasmid (*see* **step 4** in Subheading 3.2) in Lane 3, and undigested plasmid (pTB2 diluted at 25 ng/µL) in Lane 4. In Lane 5, add 1 µL of DNA Ladder 1 kb.
5. Turn on the electrophoresis system and set to 100 V. Migrate for 20–30 min and monitor regularly.
6. When migration is complete, take out the gel and observe under UV light.
7. The expected results for each well are as follows: Lane 1, a single band profile where the expected size of the PCR product depends on the primer pair; Lane 2, no band should appear on the negative control; Lane 3, a single band at ~6500 bp corresponding to the linearized pTB2 plasmid; Lane 4, multiple bands of different sizes (varying according to the level of plasmid supercoiling).

3.4 Purification of PCR Products and Linear Plasmid

The same protocol can be used to purify PCR products and the linearized plasmid with the EasyPure® PCR Purification Kit. For the first use of the kit, prepare the solution WB by adding a volume of ethanol 96% as indicated.

1. Take out a column and its 2 mL collection tube.
2. Add five volumes of BB to your sample (PCR products or linearized plasmid) and homogenize carefully by pipetting up and down.
3. Gently transfer your product to the column.
4. Centrifuge for 1 min at 10,000× *g*. Discard flow-through.
5. Add 650 µL of WB on the column.
6. Centrifuge for 1 min at 10,000× *g*. Discard flow-through and repeat to remove residual WB.
7. Throw the collection tube. Place the column in a clean 1.5 mL tube.
8. Add 20 µL of EB on the column and let stand for 1 min.
9. Centrifuge for 1 min at 10,000× *g*. Throw the column and keep the 1.5 mL tube which contains the purified product.
10. If not used immediately, store at 4 °C.
11. If non-specific bands appear after migration of the PCR products (see **step 7** in Subheading 3.3), use EasyPure Quick Gel Extraction Kit with the following method (*see* **Note 3**).
12. After migration on agarose gel (*see* **steps 6** and **7** in Subheading 3.3), excise the band containing the PCR product of interest using a scalpel.
13. Transfer the excised gel in a 1.5 mL tube and weigh.
14. For every 100 mg of gel, add 300 µL of GSB solution (100 mg of gel corresponds to a volume of 100 µL).
15. Incubate at 55 °C for 6–10 min. Occasionally vortex the tubes briefly to increase solubilization.
16. Transfer the solution with the solubilized gel in the column and centrifuge for 1 min at 10,000× *g*. Discard flow-through. If the solution exceeds 800 µL, it can be transferred on the column in multiple times.
17. Add 650 µL of WB solution on the column.
18. Centrifuge for 1 min at 10,000× *g*. Discard flow-through and repeat.
19. Throw the collection tube. Place the column in a clean 1.5 mL tube.
20. Add 20 µL of EB on the column and let stand 1 min.

21. Centrifuge for 1 min at 10,000× *g*. Throw the column and keep the 1.5 mL tube which contains the purified product.
22. If not used immediately, store at 4 °C.

3.5 Homologous Recombination-Mediated Cloning

In this step, the conditions are calculated with the indications of the In-Fusion® HD Cloning Kit User Manual. A molar ratio of 2:1 (insert/vector) needs to be respected. The volumes of water, insert, and vector need to be determined specifically for each experiment.

The following equation can help to calculate the mass of insert needed:

$$\text{mass}_{\text{insert}} = \text{ratio} \times \frac{\text{size of insert}}{\text{size of vector}} \times \text{mass}_{\text{vector}}$$

1. Add the insert corresponding to the purified PCR product with homology regions (*see* **step 10** or **step 22** in Subheading 3.4) and the linearized pTB2 plasmid vector (*see* **step 10** in Subheading 3.4) in a 2:1 molecular ratio and adjust the volume to 8 µL with nuclease-free water.
2. To each preparation, add 2 µL of 5× In-Fusion® HD Enzyme Premix.
3. Incubate at 50 °C for 15 min.
4. Place on ice for 2 min.
5. If not used immediately, store at −20 °C.

3.6 Bacterial Transformation

1. Heat a bain-marie at 42 °C beforehand.
2. Put at least three tubes containing 100 µL of competent bacteria on ice.
3. After homologous recombination-mediated cloning, add 2.5 µL of cloning product diluted 1:10 (*see* **step 5** in Subheading 3.5) in Tube 1. The second tube serves as a control for plasmid digestion and contains the purified pTB2 linear plasmid at 10 ng/µL (*see* **step 10** in Subheading 3.4). The third tube is used as a control for the efficacy of bacterial transformation and contains undigested pTB2 plasmid diluted at 10 ng/µL.
4. Incubate for 30 min on ice.
5. Incubate for 45 s in the bain-marie at 42 °C to create a thermal shock.
6. Incubate for 2 min on ice.
7. Add 500 µL of liquid LB medium in the tubes.
8. Incubate for 1 h at 37 °C under agitation (225 rpm).
9. Prepare solid LB plates and spread 80 µL of ampicillin at 25 mg/mL on every plate (two plates per sample tube).

10. Once incubation is finished, spread 250 µL of each product in the first plate and 350 µL in the second plate.
11. Incubate at 37 °C overnight.
12. Many colonies should be obtained in the plates containing the product of homologous recombination (Tube 1). No colonies should be observed in the plates containing the sample for plasmid digestion (Tube 2). Whereas, the plates from Tube 3 (control for bacterial transformation efficacy) should contain a lot of colonies.
13. If not used immediately, keep the colonies of the plates containing the product of homologous recombination (Tube 1) at 4 °C.

3.7 Bacterial Culture and Purification of Plasmid DNA

1. In a tube containing 5 mL of liquid LB medium, add 20 µL of ampicillin solution at 25 mg/mL.
2. With a sterile stick, pick multiple colonies (*see* **Note 5**) and transfer inside the cell culture tube.
3. Incubate at 37 °C overnight, under agitation (225 rpm).
4. The next day, transfer 2 mL of the bacterial culture in a 2 mL tube.
5. Centrifuge for 1 min at 10,000× *g*. Discard flow-through.
6. Transfer another 2 mL of the bacterial culture in the same tube.
7. Centrifuge for 1 min at 10,000× *g*. Discard flow-through.
8. Add 250 µL of RB solution and pipet up and down to suspend the pellet.
9. Add 250 µL of lysis buffer and shake four to six times until the lysate turns blue.
10. Incubate for 5 min at room temperature.
11. Add 350 µL of NB solution and shake four to six times until the lysate turns yellow and a precipitate appears.
12. Incubate the lysate 2 min at room temperature.
13. Centrifuge for 5 min at 12,000× *g*.
14. Collect the supernatant and transfer in a column from the kit.
15. Centrifuge for 1 min at 12,000× *g*. Discard flow-through.
16. Add 650 µL of WB solution.
17. Centrifuge for 1 min at 12,000× *g*. Discard flow-through. Repeat to remove residual WB.
18. Transfer the column on a new 1.5 mL tube.
19. Add 50 µL of EB in the middle of the column.
20. Incubate for 1 min at room temperature.

21. Centrifuge for 1 min at 10,000× *g* to elute plasmid DNA. Discard the column.
22. Store the plasmid DNA solution at −20 °C until use.

3.8 Screening of Bacterial Clones by PCR and Sequencing

1. Under the workstation, prepare a mix of the following items (multiply the volumes by the number of tubes): 15.6 µL of sterile water, 2 µL of 10× TransTaq® HiFi Buffer I, 0.4 µL of dNTP 10 mM, 0.2 µL of DNA polymerase TransTaq® HiFi 5 U/µL, 0.4 µL of the forward primer V1F and 0.4 µL of the reverse primer V1R.
2. Transfer 19 µL of the mix in a tube.
3. Add 1 µL of water in a tube for the negative control.
4. Outside the workstation, add 1 µL of the plasmid DNA solution (see **step 22** in Subheading 3.7) at 50 ng/µL.
5. In the Thermocycler, run the following PCR program: 3 min at 94 °C, 25 cycles (30 s at 94 °C, 30 s at 60 °C, 2 min at 72 °C), 10 min at 72 °C, and let cool at 10 °C for as long as needed before the next step.
6. Use a 1% agarose gel to verify the size of every clone by electrophoresis. There are two possible outcomes (Fig. 3a): one band appears at 191 bp corresponding to the empty

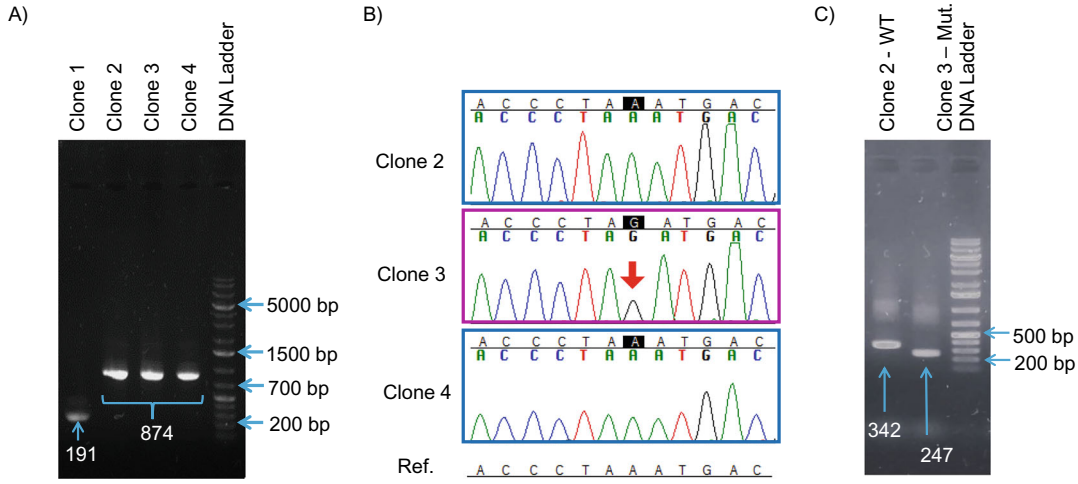


Fig. 3 Examples of expected results. (**a**) Example of PCR products observed at Step 8: one band at 191 bp appears if the insert is not integrated into the plasmid (Clone 1), one band of a higher size appears if homologous recombination has been efficient (presence of the insert in the plasmid). In this case, the band appears at 874 bp (Clones 2, 3, and 4). (**b**) Example of sequencing products observed at Step 8 in comparison to the reference sequence (Ref.), Clones 2 and 4 contain the wild-type (WT) sequence and Clone 3 contains the mutated one, highlighted with a red arrow. (**c**) Example of PCR products obtained in Step 12 from cDNAs: the band corresponding to the WT clone (Clone 2) is not the same size as that of the mutant clone (Clone 3), showing the impact of the mutation on the splicing

minigene plasmid without the insert (Clone 1); one band appears at 874 bp corresponding to the plasmid minigene with the insert added (Clones 2–4).

7. To determine if these positive clones (Fig. 3b, Clones 2–4) integrated a wild-type or a mutated variant from the patient's gDNA, purify the PCR products before sequencing (*see* **steps 1–22** in Subheading 3.4).
8. Under the workstation, prepare two different mixes (multiply the volumes by the number of tubes): Mix "F": 12 µL of sterile water, 4 µL of Big Dye Terminator V1.1 and 1 µL of Forward primer V1F 10 µM; and Mix "R": 12 µL of sterile water, 4 µL of Big Dye Terminator V1.1 and 1 µL of Reverse primer V1R 10 µM.
9. Transfer 17 µL of each mix in a tube.
10. Outside the workstation, add 3 µL of the purify PCR product in each tube.
11. In the Thermocycler, run the following PCR program for DNA sequencing: 3 min at 94 °C, 25 cycles (10 s at 94 °C, 5 s at 50 °C, 4 min at 60 °C), 1 min at 60 °C and let cool to 4 °C for as long as needed before the next step.
12. Before sequencing, a step of purification with the DyeEx 2.0 Spin Kit is needed.
13. Gently vortex the spin column to resuspend the resin.
14. Loosen the cap of the column a quarter turn to avoid a vacuum inside the spin column.
15. Snap off the bottom closure of the spin column, and place the spin column in a 2 mL collection tube (provided).
16. Centrifuge for 3 min at 750× *g*.
17. Carefully transfer the spin column to a clean centrifuge tube. Slowly apply the sequencing reaction directly onto the center of slanted gel bed surface.
18. Centrifuge for 3 min at 750× *g*. Remove the spin column from the microcentrifuge tube. The eluate contains the purified DNA.
19. Load the purified DNA onto the DNA sequencer using suitable sequencing plate (3500xl Dx Genetic Analyzer) and run the sequencing program.
20. Read the sequences using the sequence analysis software and compare to a reference sequence to determine if the plasmid DNA contains a wild-type or a mutated variant from the sequence of interest (Fig. 3b).

3.9 Cell Culture and Transfection

1. Using a flask containing adherent COS-7 cells (*see* **Note 4**), remove the culture medium completely and wash twice with sterile PBS.
2. Add 1 mL of Trypsin solution in the flask.
3. Incubate for 2–3 min at 37 °C.
4. Gently tap the flask to loosen adherent cells.
5. Add 10 mL of DMEM culture medium to the flask and pipet up and down to collect a maximum of cells. Transfer in a tube.
6. Insert a few microliters in a Malassez counting chamber and count cells.
7. Add 150,000 cells/well (6-well plate) in 2 mL of DMEM culture medium. Prepare at least three wells.
8. Incubate cells at 37 °C in 5% CO_2 for 24 h.
9. For transfection, prepare three separate 1.5 mL tubes for the control pTB2 plasmid (empty pTB2), the pTB2 plasmid containing the wild-type sequence (pTB2-WT insert), and the pTB2 plasmid with the mutated variant (pTB2-mutated insert).
10. Add 200 µL of JetPrime Buffer in each tube.
11. Add 4 µL of plasmid DNA (*see* **step 22** in Subheading 3.7) at 500 ng/µL in the corresponding tube. Homogenize for 10 s.
12. Add 3 µL of JetPrime Transfection Reagent in each tube and homogenize.
13. Incubate for 10 min at room temperature.
14. Add 200 µL of the mix to cells cultured in 6-well plate in 2 mL of DMEM culture medium (*see* **step 7** in Subheading 3.9).
15. Incubate at 37 °C in 5% CO_2 for 24 h.

3.10 Cell Lysis and RNA Extraction

This procedure is proposed for adherent cells using Rapid Animal Total RNA Extraction kit (*see* **Note 6**).

1. Discard the culture medium, add 1 mL of Solution A (use a maximum amount of 1×10^7 cells for 1 mL of Solution A) and mix gently by pipetting.
2. Add 0.2 mL of chloroform to the lysate (equivalent to 1 mL lysate) and vortex for 30 s.
3. Centrifuge at 12,000× *g* for 3 min at room temperature (deposit may form at the bottom of the tube).
4. Transfer the supernatant into a new 1.5 mL centrifuge tube.
5. Add equal volume of isopropanol to the supernatant and vortex for 30 s.
6. Centrifuge at 12,000× *g* for 3 min at room temperature.

7. Discard the supernatant carefully.
8. Wash the RNA pellets by adding 1 mL of 75% ethanol. Vortex for 30 s.
9. Centrifuge at 7500× *g* for 3 min at room temperature.
10. Discard the supernatant carefully.
11. Perform a secondary wash by adding 1 mL of 75% ethanol. Vortex for 30 s.
12. Short spin at 7500× *g* and remove the residual ethanol carefully.
13. Incubate the tube with lid open at room temperature for 1–2 min to evaporate the residual ethanol.
14. Add 50–100 μL of RNase-free water to dissolve the pellet, store RNA solution at −80 °C.
15. RNA integrity, yield, and purity can be analyzed via electrophoresis and spectral analysis.

3.11 Reverse Transcription (RT)

For each sample, a negative control without FIREScript Enzyme (RT-) should be prepared simultaneously.

1. Prepare two tubes per sample with 500 ng of RNA in 10 μL RNase-free water.
2. Heat 10 min at 70 °C.
3. Prepare a mix with all the common reagents (multiply the volumes by the number of tubes): 6.5 μL of RNase-free water and 2 μL of 10× RT Reaction Premix with Oligo (dT) and Random primers.
4. Transfer 8.5 μL of the mix in a tube.
5. Add 1.5 μL of RNase-free water in a tube for the negative control (RT−).
6. Add 1.5 μL of FIREScript Enzyme in the sample tube (RT+).
7. Put the tubes in the Thermocycler and the following RT program: 7 min at 25 °C for primer annealing, 30 min at 45 °C (RT), 5 min at 85 °C (inactivation of FIREScript Enzyme), and let cool to 4 °C for as long as needed before the next step.

3.12 Splicing Assessment by PCR and Sequencing

1. Under the workstation, prepare a mix of the following items (multiply the volumes by the number of tubes): 37.5 μL of nuclease-free sterile water, 5 μL of 10× TransTaq® HiFi Buffer I, 1 μL of dNTP 10 mM, 0.5 μL of DNA polymerase TransTaq® HiFi 5 U/μL, 1 μL of Forward Primer A2 (10 μM), and 1 μL of Reverse Primer B2 (10 μM).
2. Transfer 46 μL of the mix in a tube.
3. Add 4 μL of nuclease-free water in a tube for the negative control.

4. Outside the workstation, add 4 μL of cDNA (*see* **steps 6** and **7** in Subheading 3.11) in the sample tube.
5. Put the tubes in the Thermocycler and run the following PCR program: 3 min at 94 °C, 30 cycles (30 s at 94 °C, 30 s at 66 °C, 2 min at 72 °C), 2 min at 72 °C and let cool at 10 °C for as long as needed before the next step.
6. Analyze the PCR products on 1% agarose gel (Fig. 3c).
7. Purify the samples with the purification kit before sequencing (*see* **steps 2–22** in Subheading 3.4).
8. To sequence the purified PCR products, prepare two different mixes: Mix "F", 9 μL of nuclease-free water, 4 μL of Big Dye Terminator V1.1 and 1 μL of Forward primer A2 (10μM); Mix "R", 9 μL of nuclease-free water, 4 μL of Big Dye Terminator V1.1 and 1 μL of Reverse primer B2 (10 μM).
9. Transfer 14 μL of each mix in a tube.
10. Outside the workstation, add 6 μL of the purified PCR product in each tube.
11. Put the tubes in the Thermocycler and run the PCR program for DNA sequencing: 3 min at 94 °C, 25 cycles (10 s at 94 °C, 5 s at 50 °C, 4 min at 60 °C), 1 min at 60 °C and let cool to 4 °C for as long as needed before the next step.
12. Before DNA sequencing, a step of purification with the DyeEx 2.0 Spin Kit is needed (*see* **steps 13–18** in Subheading 3.8).
13. Transfer the purified DNA in the DNA sequencer (3500xl Dx Genetic Analyzer) and run the sequencing program.
14. Analyze DNA sequences to compare the splicing profile from mutated and wild-type alleles.

4 Notes

1. The study of patients' genomic DNA remains the only option when the cells of interest are inaccessible.
2. To avoid sequencing errors, the use of high-fidelity Taq DNA polymerase such as TransTaq® HiFi DNA polymerase is recommended. Any other Taq polymerase with good replication fidelity can also be used.
3. If non-specific bands appear after migration of the PCR products (*see* **step 7** in Subheading 3.3), additional steps are required to purify the band of interest using the EasyPure® Quick Gel Extraction Kit instead of the EasyPure® PCR Purification Kit. This protocol is detailed in the methods section (*see* **steps 13–22** in Subheading 3.4).

4. Other cell types can be used, for example Hela or SH-SY5Y cells, and volumes need to be adapted.
5. We recommend to pick at least four different colonies to maximize the chances of obtaining plasmid containing inserts from both wild-type and mutated sequences.
6. For total RNA extraction using suspension cells: collect cells by centrifugation (300× *g*, 5 min), discard the supernatant, and add 1 mL of Solution A to 1–5 × 10^6 cells, and mix gently by pipetting.

Acknowledgments

We would like to thank Baralle D. and Baralle M. for creating and sharing this plasmid with the scientific community.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Matera AG, Wang Z (2014) A day in the life of the spliceosome. Nat Rev Mol Cell Biol 15:108–121. https://doi.org/10.1038/nrm3742
2. Cooper TA (2005) Use of minigene systems to dissect alternative splicing elements. Methods 37:331–340. https://doi.org/10.1016/j.ymeth.2005.07.015
3. de Sainte Agathe J-M, Filser M, Isidor B et al (2023) SpliceAI-visual: a free online tool to improve SpliceAI splicing variant interpretation. Hum Genomics 17:7. https://doi.org/10.1186/s40246-023-00451-1
4. Baralle D (2005) Splicing in action: assessing disease causing sequence changes. J Med Genet 42:737–748. https://doi.org/10.1136/jmg.2004.029538

Chapter 12

Design and Evaluation of a Truncated Protein Induction Strategy by Antisense Oligonucleotide-Mediated Exon Skipping

Pierre Hirt, Sandrine Le Noir, Laurent Delpy, and Eric Pinaud

Abstract

Antisense oligonucleotide (ASO)-mediated exon skipping is an effective method for generating truncated proteins by altering mRNA splicing. This technique, enabling in situ truncation of proteins, provides a rapid way to investigate the functions of specific domains encoded by the targeted exon(s). In this methodological chapter, we focus on the mouse transcription factor ARID3A (AT-rich interactive domain 3A), which contains a DNA-binding domain (ARID domain) and a nuclear matrix-binding domain (Rekles Beta domain). We describe the design of an ASO targeting the donor splice site of *Arid3a* exon 8, which primarily encodes the Rekles beta domain, to generate a truncated protein lacking nuclear matrix-binding capacity while preserving an open reading frame. The ASO is specifically designed to hybridize only with *Arid3a* pre-mRNA, thus minimizing off-target effects. The effect of the ASO is evaluated first by RT-PCR to measure the proportion of alternative transcripts with skipped exon. Alternative transcripts can be subsequently characterized by Sanger sequencing. RT-qPCR is then performed outside the targeted exon to ensure that the overall transcription of the gene is unaffected and to exclude RNA degradation. Finally, protein analysis techniques, such as cellular imaging using immunofluorescence labeling, are used to assess the production of the truncated protein. This ASO-mediated exon skipping approach provides a reliable method for exploring the functional importance of specific protein domains.

Key words Antisense oligonucleotide (ASO), Exon skipping, Truncated protein, ARID3A

1 Introduction

Antisense oligonucleotides (ASOs) are single-stranded synthetic molecules designed primarily to bind specifically to a target RNA, thereby influencing its fate. Unlike DNA modification-based approaches, ASOs act transiently on RNA, without introducing irreversible genomic alterations, providing a flexible and reversible strategy [1]. These molecules are often optimized for nuclease resistance, which extends their half-life in order to provide measurable activity in biological systems [2].

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_12,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

Steric blocker ASOs are used to create steric hindrance by hybridizing to a target RNA and disrupting critical processes such as splicing. By targeting donor or acceptor splice sites (DSS or ASS) on a pre-mRNA, splice-switching ASOs can induce exon skipping and produce shorter alternative transcripts. When exon skipping induces a frameshift and introduces premature termination codons (PTCs), alternative transcripts can be degraded by the nonsense-mediated mRNA decay (NMD) pathway, resulting in gene knockdown [3, 4]. However, exon-skipping does not necessarily lead to transcript degradation. When an exon whose length is a multiple of 3 nucleotides (nt) is targeted, the resulting mRNA retains an open reading frame. In this case, ASO-mediated exon skipping can lead to the production of a truncated protein [5]. This approach thus allows the study of specific protein domains by assessing the functional impact of their removal.

The transcription factor ARID3A (AT-rich interaction domain protein 3A), also known as BRIGHT (B cell regulator of immunoglobulin heavy chain transcription), was discovered for its ability to bind the flanking regions of the cEμ intronic enhancer within the immunoglobulin heavy chain locus [6, 7]. These specific regions, initially described as nuclear matrix attachment regions (MARs), contain AT-rich DNA motifs described to interact with the ARID domain of ARID3A. Since another domain, the Rekles beta domain of ARID3A is expected to bind components of the nuclear lamina; the function of this factor could be considered as a gene anchor to the nuclear skeleton. The best characterized function of ARID3A is its ability to increase immunoglobulin production and to contribute to the development, differentiation, and function of B cells [8–10]. More recently, ARID3A was considered as broader regulator in hematopoietic precursors; altered expression of this protein has been associated with various pathologies, including certain cancers and autoimmune diseases, such as systemic lupus erythematosus [11–13]. In mice, the *Arid3*a gene is configured such that its Rekles Beta domain, responsible for nuclear matrix binding, is primarily encoded by exon 8 (with a nt length that is a multiple of 3), making the mouse ARID3A protein suitable for ASO-mediated domain truncation (Fig. 1).

In this article, we describe the design of an ASO-based strategy to induce skipping of a specific exon to generate a truncated protein while maintaining the reading frame necessary for protein production. By using *Arid3a* as a candidate, we specifically target the exon 8 DSS to induce exon skipping and remove the nuclear matrix-binding domain of ARID3A. ASO is designed to hybridize specifically to the DSS or ASS of a transcript of interest, thereby limiting off-target effects. To evaluate the efficiency of this approach at both RNA and protein level, we use mouse spleen cells and perform

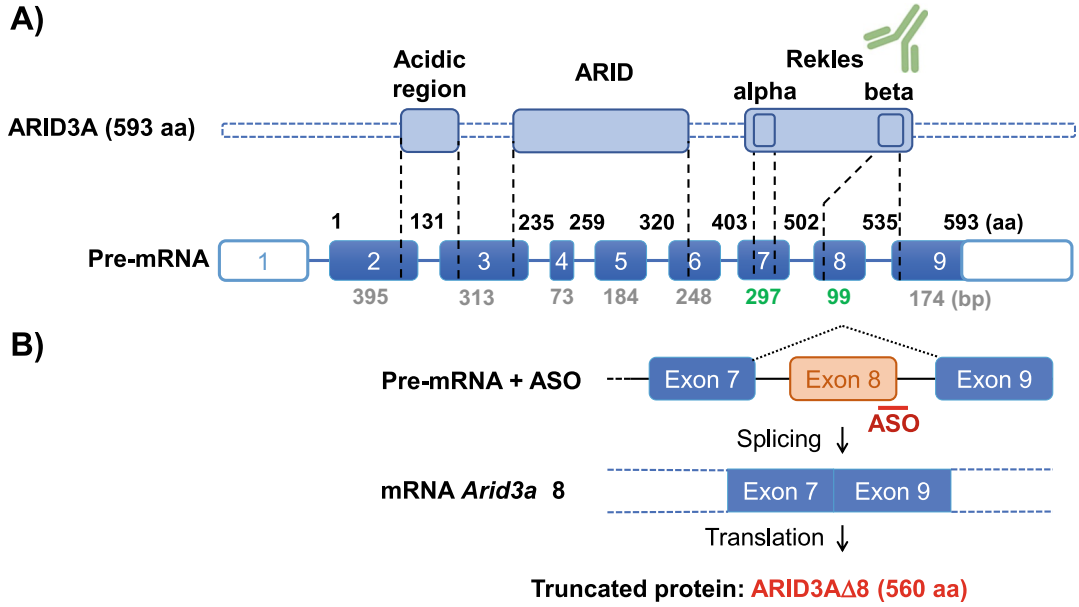


Fig. 1 ASO-mediated exon skipping strategy to produce truncated ARID3A protein. (**a**) Diagram of murine ARID3A protein (top) and pre-mRNA (bottom), highlighting key functional domains and exons. The protein consists of three main domains: the acidic region, ARID DNA-binding domain, and Rekles domain alpha and beta that are recognized by a specific rabbit anti-ARID3A antibody. Rekles domain alpha and beta are mainly encoded by exon 7 and exon 8, respectively. (**b**) Exon-skipping strategy for truncation of ARID3A. The donor splice site of exon 8 is targeted by an antisense oligonucleotide (ASO) to induce alternative splicing. The ASO-mediated skipping of exon 8 results in the Arid3aΔ8 mRNA isoform encoding a truncated ARID3AΔ8 protein lacking the Rekles beta domain

stimulation with lipopolysaccharide (LPS) followed by ASO treatment. To determine the impact of ASO treatment on exon skipping, we first measure the proportion of alternative transcripts by RT-PCR using primers surrounding the targeted exon. RT-qPCR is then performed to verify that the total transcript levels remain unchanged, ruling out RNA degradation. Alternative *Arid3a* transcripts lacking exon 8 (Arid3aΔ8) and encoding a truncated protein devoid of Rekles beta domain are further characterized by Sanger sequencing. Next, we use an antibody recognizing the Rekles beta domain of mouse ARID3A (information provided by the supplier) to perform immunofluorescence labeling and assess the disappearance of this specific domain at the protein level (Fig. 2).

This ASO-mediated exon skipping approach is a versatile tool for exploring the functions of particular protein domains and assessing the impact of their deletion on overall protein function.

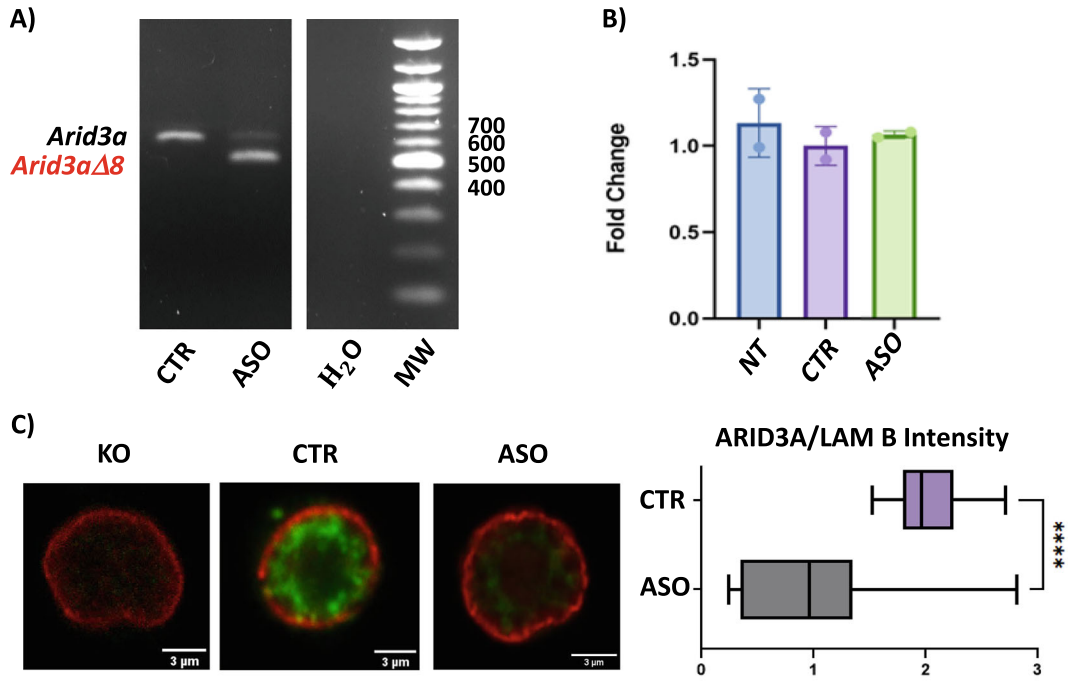


Fig. 2 Qualitative and quantitative validation of ASO-mediated skipping of *Arid3a* exon 8 in mouse B cells. (**a**) RT-PCR analysis using primers located in exon 7 (forward) and 9 (reverse) showing the presence of two bands corresponding to full-length *Arid3a* mRNA (CTR), and *Arid3aΔ8* mRNA lacking exon 8 (ASO). (**b**) RT-qPCR analysis indicates that total *Arid3a* mRNA levels (either full-length *Arid3a* or *Arid3aΔ8*) remain unchanged between the different culture conditions (NT: non-treated, CTR: treatment with control ASO, and ASO: treatment with *Arid3a*-exon8-ASO). (**c**) Immunofluorescence microscopy using an anti-ARID3A antibody (in green) that recognizes the REKLES beta domain mainly encoded by *Arid3a* exon 8. After normalization to Lamin B fluorescence (red), the decreased ARID3A fluorescence intensity observed in ASO-treated cells is consistent with the loss of the REKLES beta domain encoded by exon 8. Quantifications were performed using ImageJ software to measure the mean fluorescence intensity of ARID3A and Lamin B (LAM B) for each nucleus. Statistical analysis using a Mann-Whitney test confirms a significant decrease in ARID3A intensity in ASO-treated cells compared to CTR ($P < 0.0001$; median CTR = 1.962, median ASO = 0.9632)

2 Materials

2.1 ASO Treatments and RNA Extraction

1. Cells expressing significant levels of targeted transcripts: for instance, LPS-stimulated mouse B cell expressing high levels of *Arid3a*.
2. Culture Medium: RPMI 1640, 10% heat inactivated fetal bovine serum (FBS), 50 µM β-mercaptoethanol, 1 µg/mL LPS-EB Ultrapure (InvivoGen) (*see* **Note 1**).
3. 24-well cell culture plates.
4. ASO: the steric blocker ASO targeting Arid3a exon 8 DSS (5′-GGCTATAGGAGCAAGAGATCCACCA) is purchased as

phosphorodiamidate morpholino oligomer coupled with an octaguanidine dendrimer (vivo-morpholino oligos; Gene Tools, LLC). Additionally, a vivo-morpholino irrelevant ASO (5′-CCTCTTACCTCAGTTACAATTTATA) is purchased as control ASO (Gene Tools, LLC) (*see* **Note 2**). Resuspend the lyophilized ASO at a concentration of 500 μM with nuclease-free water. Store the 500 μM ASO stock solution at room temperature, protected from light.

5. 1× phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1 L distilled water.
6. TRIzol™ reagent.
7. Chloroform.
8. Isopropanol.
9. Ethanol 75%.
10. Nuclease-free water.
11. Centrifuge and cooled microcentrifuge.
12. NanoDrop spectrophotometer.

2.2 Reverse Transcription (RT)

1. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with 10× RT Buffer, 10× Random Primers, 100 mM dNTP solution, and 50 U/μL MultiScribe Reverse Transcriptase.
2. 1 U/μL DNase I and 10× DNAse I Buffer.
3. 25 mM EDTA solution.
4. Thermocycler.
5. 1.5 mL tubes and 0.2 mL PCR tubes.

2.3 PCR, Gel Electrophoresis, and Isolation of DNA Bands

1. 10 μM PCR primers: forward et reverse (*see* **Note 3**).
2. Taq PCR Kit (New England Biolabs) with 10× MgCl2 buffer, 10 mM dNTP, and 5 U/μL Taq DNA polymerase.
3. 6× DNA Loading buffer.
4. 1× TBE solution: Tris 0.089 M Tris, 0.089 M Borate, 0.002 M EDTA, pH 8.3 adjusted with HCl.
5. Molecular Biology Grade Agarose powder.
6. Midori Green.
7. 100 bp DNA ladder.
8. Migration chamber.
9. UV lamp.

10. PCR clean-up Gel extraction kit (Macherey-Nagel): Buffer NTI, Buffer NT3, Buffer NE, NucleoSpin columns and collection tubes.
11. Scalpel.

2.4 Sample Purification and Sanger Sequencing

1. Sephadex g-50 superfine.
2. 96-well filtered plate.
3. 96-well sequencing plate
4. BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), with BigDye Terminator v3.1 Ready Reaction Mix and 5× BigDye Buffer included.
5. DNA sequencer.

2.5 Quantitative PCR

1. SensiFAST™ SYBR® Hi-ROX Kit (Meridian Bioscience).
2. 2 μM forward and reverse primers stock solution (*see* **Note 4**).
3. Microplates.
4. Adhesive film seals for microplates.
5. Quantitative PCR instrument.

2.6 Nuclear Immunofluorescence Staining

1. Poly-L-lysine-coated glass slides.
2. Fixation solution: 2% paraformaldehyde (PFA) in 1× PBS.
3. Permeabilization solution: 0.5% Triton in 1× PBS.
4. Blocking solution: 2.5% BSA, 10% FBS, and 0.1% Tween-20 in 1× PBS.
5. Primary antibodies: Rabbit polyclonal anti-ARID3A antibody targeting the 500—540 aa sequence in the C-terminal part (ab227274, Abcam), and Goat polyclonal anti-Mouse Lamin B antibody (sc-6217, Santa Cruz Biotechnology).
6. Secondary antibody: Donkey anti-Rabbit IgG (H + L) Alexa Fluor™ 488 and Donkey anti-Goat IgG (H + L) Texas Red (Thermo Fisher).
7. PBST: 1× PBS and 0.1% Tween-20.
8. 1000× DAPI solution.
9. Mowiol.
10. Coverslips.
11. Coverslip Sealant.
12. Fluorescent or confocal microscope.

3 Methods

3.1 Target Exon Selection

1. Choose an exon coding for a specific protein domain (or at least a large part of the protein domain to be studied). The length of the targeted exon must be a multiple of 3 in order to respect the reading frame after its elimination and not to induce frameshift.
2. Select a 50-nt sequence encompassing the ASS or DSS of the targeted exon. In general, 25 nt are taken from the intron and 25 nt from the exon.
3. Submit this sequence to a supplier for the design of a steric blocker ASO. When the ASO design request is made with Gene Tools company, select "Splice Modifying (pre-mRNA target)" and 37 °C as working temperature.
4. Once the ASO sequence has been obtained, it is important to check that it does not hybridize significantly with a transcribed sequence in the organism (use an alignment search tool such as Blast).
5. (Optional) Use splicing prediction tools to predict that no strong ASS or DSS is located in the vicinity of the targeted splice site, as this could prevent exon skipping and generate an unwanted alternative transcript (*see* **Note 5**).

3.2 ASO Treatments and RNA Extraction

1. For short-term culture of mouse primary B cells, seed 2 mL of spleen cells at a density of 5–10 × 10^5 cells/mL in 24-well plates and add LPS-supplemented culture medium. Incubate at 37 °C in a humidified atmosphere with 5% CO_2 for up to 4 days. Make 3 culture wells for comparative analysis of cells in untreated conditions (NT), treated with the ASO control (CTR) and the ASO of interest (ASO).
2. We usually treat stimulated B cells with ASO concentration of 2 μM from 24 h to 72 h (*see* **Notes 6** and **7**).
3. After 72 h of culture, harvest the cells by centrifuging at 300× *g* for 5 min, and then wash once with 1× PBS.
4. Discard the supernatant and resuspend the pellet in 0.5—1 mL TRIzol. Shake vigorously (*see* **Note 8**) and incubate for 5 min at room temperature. Proceed to the next step or store at 4 °C overnight or at −20 °C for up to one year.
5. Thaw RNA at room temperature if necessary, and then add 0.2 mL of chloroform for every 1 mL of TRIzol used.
6. Mix vigorously for 2–3 min.
7. Centrifuge at 12,000× *g* for 15 min at 4 °C.
8. Carefully transfer the clear upper aqueous phase to a fresh tube (*see* **Notes 9** and **10**).

9. Add 0.5 mL of isopropanol per 1 mL of TRIzol and mix by vortexing.
10. Incubate at 4 °C for 10 min.
11. Centrifuge at 12,000× *g* for 10 min at 4 °C.
12. Gently remove the supernatant using a pipette.
13. Wash the pellet with 1 mL of cold ethanol 75% (stored at −20 °C) per 1 mL of TRIzol (*see* **Note 11**).
14. Vortex the pellet briefly to loosen it.
15. Centrifuge at 7500× *g* for 5 min at 4 °C.
16. Discard the supernatant carefully.
17. Air-dry the RNA pellet for 5–10 min.
18. Dissolve the pellet in 20–30 μL of nuclease-free water.
19. Heat the solution to 55–60 °C for 10–15 min to ensure complete dissolution.
20. Measure RNA concentration and purity using NanoDrop spectrophotometer (*see* **Note 12**).
21. Proceed to reverse transcription (RT) or place RNA samples at −80 °C for long-term storage.

3.3 Reverse Transcription

1. Add 200 ng to 2 μg of RNA to PCR tubes, adjusting the final volume to 10 μL with nuclease-free water. Perform two tubes per sample with identical RNA amounts for reverse transcription reaction (RT+) and control reaction without reverse transcriptase (RT-) (*see* **Note 13**).
2. Prepare a DNase Mix with 1.2 μL of 10× DNAse I Buffer and 1 μL of DNase I per tube.
3. Add 2.2 μL of the DNase Mix to the side of each tube containing RNA, and then briefly spin to mix.
4. Incubate at room temperature for 15 min.
5. Add 2 μL of EDTA to the side of the tube, then briefly spin and incubate for 10 min at 65 °C.
6. Prepare the RT+ mix with 2 μL of 10× RT Buffer, 2 μL of 10× Random Primers, 0.8 μL of 100 mM dNTP solution and 1 μL Reverse Transcriptase per tube.
7. Add 5.8 μL of RT+ Mix into each tube for the RT+ reaction.
8. For RT- reactions, prepare a similar mix, replacing Reverse Transcriptase with 1 μL nuclease-free water and add 5.8 μL of RT- Mix into each tube for the RT- reaction.
9. Incubate all tubes in a thermocycler with the following program: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min.
10. Store cDNA at 4 °C for short-term use or −20 °C for long-term storage.

3.4 PCR and Gel Electrophoresis

1. Prepare a PCR Mix with 2 μL of 10× MgCl2 buffer, 0.4 μL of 10 mM dNTP, 2 μL of 10 μM forward primer, 2 μL of 10 μM reverse primer, 0.2 μL of 5 U/μL Taq DNA polymerase, and 12.4 μL of nuclease-free water per tube.
2. Add 19 μL of PCR mix in each tube and add 1 μL of cDNA in RT+ tubes or 1 μL of nuclease-free water in RT- tubes.
3. Place tubes in a thermocycler with the following program: 94 °C for 2 min, 38 cycles (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s), 72 °C for 10 min and then cool to 15 °C (*see* **Note 14**).
4. Prepare a 2% (w/v) agarose gel in 1× TBE and add Midori Green dye (2 μL/100 mL).
5. Mix PCR products with 4 μL of 6× loading buffer and load 20 μL into each well.
6. Add 5 μL of a 100 bp DNA ladder into a separate well.
7. Run electrophoresis at 120 V for approximately 45 min.
8. Visualize PCR products under UV light (Fig. 2a).

3.5 Characterization of Alternative Transcripts by Sanger Sequencing

For further validation of ASO-mediated exon skipping use Sanger sequencing to analyze the nucleotide sequence of the alternatively-spliced mRNAs.

1. Wear protective UV equipment during this procedure. Excise the band corresponding to the exon skipping amplification products from the agarose gel using a scalpel, cutting the gel slice finely (*see* **Note 15**). Weigh the gel slice and place it into a 1.5 mL tube.
2. Add 200 μL of Buffer NTI for every 100 mg of gel.
3. Incubate the mixture at 50 °C for 30–60 min, vortex periodically until the gel is fully dissolved.
4. Transfer up to 700 μL of the DNA-containing solution into a NucleoSpin column placed inside a collection tube. If the volume exceeds 700 μL, repeat this step for the remaining liquid.
5. Centrifuge the column for 30 s at 11,000× *g*, and then discard the flow-through. Reinsert the NucleoSpin column into the collection tube.
6. Add 700 μL of Buffer NT3 to the column and centrifuge for 30 s at 11,000× *g*. Discard the flow-through and repeat the washing step with 700 μL of Buffer NT3.
7. Centrifuge the column for 1 min at 11,000× *g* to dry the silica membrane.
8. Transfer the NucleoSpin column to a new 1.5 mL microcentrifuge tube. Elute the DNA by adding 20 μL of Buffer NE or

Table 1
DNA template quantities used for Sanger sequencing based on the size of PCR products

DNA Template (bp)	Quantity (ng)
100–200	1–3
200–500	3–10
500–1000	5–20
1000-2000	10–40
>2000	20–50

nuclease-free water directly to the column. Wait for 1 min, and then centrifuge for 1 min at 11,000× *g*.

Stop point: purified PCR products can be store at −20 °C until Sanger sequencing is performed.

9. Prepare a 1 μM solution by diluting forward and reverse PCR primers (*see* **item 1** in Subheading 2.3) with nuclease-free water and keep on ice.
10. For Sanger sequencing of purified PCR products, add 2.5 μL of 1 μM primer solution (forward or reverse), the purified PCR product (*see* Table 1), 1.5 μL of BigDye Terminator v3.1 Ready Reaction Mix, 3 μL of 5× BigDye Buffer and nuclease-free water up to 15 μL, per reaction (*see* **Note 16**).
11. Place the tubes in a thermocycler and run the following sequencing program: 96 °C for 1 min, 25 cycles (96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min), 15 °C for 10 min.
12. To purify the sequencing reactions, distribute Sephadex powder evenly into the wells of a 96-well filter plate.
13. Add 350 μL of nuclease-free water into each well and incubate for 3 h at room temperature or overnight at 4 °C.
14. Position a recovery 96-well plate beneath the filter plate and centrifuge at 1500× *g* for 7 min.
15. Discard the contents of the recovery plate.
16. Add 15 μL of sample (*see* **step 11** in Subheading 3.5) to each well of the filter plate.
17. Place another 96-well plate (sequencing plate) beneath the filter plate and centrifuge at 1500× *g* for 7 min.
18. Sequence purified samples on a DNA sequencer.

3.6 Analysis of Gene Expression by qPCR

A qPCR is carried out with primers that do not include the targeted exon in order to comparatively analyze the levels of full-length and alternative (with exon skipping) mRNAs (Fig. 2b, *see* **Note 4**).

1. Prepare a qPCR mix with 10 µL of SensiFAST SYBR® Hi-ROX Mix, 2 µL of Forward Primer (2 µM), 2 µL of Reverse Primer (2 µM), and 1 µL of nuclease-free water.
2. Dispense 15 µL of the qPCR mix into the wells of a 96-well microplate.
3. After diluting the cDNA (*see* **step 10** in Subheading 3.3) to a ratio of 1:20 with nuclease-free water, add 5 µL of diluted cDNA to each well.
4. For the NTC (No Template Control), add 5 µL of nuclease-free water instead of diluted cDNA.
5. Seal the plate tightly with an adhesive film to prevent evaporation.
6. Briefly centrifuge to ensure that all the volume is at the bottom of the wells.
7. Run the following qPCR program: 95 °C for 2 min, 40 cycles (95 °C for 10 s, 60 °C for 30 s), 95 °C for 15 s, 60 °C for 1 min (Melt Curve), 95 °C for 15 s (with a ramp rate of 0.3 °C per sec). Then, cool to 15 °C until the samples are recovered.

3.7 Nuclear Immunofluorescence Staining

1. Seed 300,000 cells in 200 µL of 1× PBS per spot on a poly-L-lysine-coated glass slide. Place the slide on a polystyrene support within a moist chamber to maintain a humid environment.
2. Incubate at room temperature for 30 min to allow cells to adhere.
3. Carefully remove 1× PBS by blotting on paper towels.
4. Add 250 µL of fixation solution per spot (*see* **Note 17).**
5. Incubate for 10 min at room temperature.
6. Perform two washes of 5 min each in a 1× PBS bath.
7. Add 250 µL/spot of the permeabilization solution.
8. Incubate for 10 min at room temperature.
9. Perform two washes of 5 min each in a 1× PBS bath.
10. Add 250 µL/spot of the blocking solution.
11. Incubate for 30 min at room temperature.
12. Add 100 µL/spot of the primary antibodies mix diluted at 1/500 in blocking buffer.
13. Incubate overnight at 4 °C.
14. Perform two washes of 5 min each in a PBST bath.
15. Add 100 µL/spot of the secondary antibodies mix diluted at 1/1000 in blocking buffer (*see* **Note 18).**
16. Incubate for 1 h at room temperature. From this step onwards, work in the dark to preserve fluorescence.

17. Perform two washes of 5 min each in a PBST bath.
18. Add 100 µL/spot of DAPI solution diluted to 1:1000 with 1× PBS.
19. Incubate for 5 min at room temperature.
20. Perform two quick rinses with 1× PBS.
21. Add 50 µL of Mowiol on the spots.
22. Place a coverslip on top and seal the coverslip (*see* **Note 19).**
23. Store the slides at 4 °C until observation under a microscope (Fig. 2c).

4 Notes

1. Avoid using a culture medium with excessive serum enrichment, as this may interfere with the ASO and reduce its efficacy. For cell cultures requiring high percentages of FBS, higher concentrations of ASO may be required.
2. Controls other than the irrelevant ASO shown here can be used: a "sense control ASO" will have the same nt sequence as the ASO to be studied but in the opposite orientation, and a "scramble control ASO" will have a random sequence composed of the same nt content as the ASO used for exon skipping. As with the ASO to be studied, care must always be taken to ensure that the control ASO cannot hybridize significantly with a transcribed genomic sequence.
3. Primers are designed to span the skipped exon, allowing for the detection of both the full-length transcript and the exon-skipped variant. Additional bands may indicate alternative splicing, suggesting the activation of a new splice site.
4. The primer pairs used to assess global expression of the gene of interest by RT-qPCR should not include the targeted exon in order to amplify full-length and alternatively-spliced mRNA isoforms in the same way.
5. This splice site prediction step is optional because the use of cryptic splice site is often hard to predict and must be tested empirically.
6. For each cell type, a preliminary dose-effect study using a control ASO is recommended to establish the optimal working concentration (with limited cytotoxic effects).
7. Primary B cells are very sensitive to treatment with vivo-morpholino control ASO during the first few hours of culture. We recommend treating cells at least 24 h after LPS stimulation.

8. Immediately vortex or pipette the pellet vigorously to lyse it before letting it rest; delays can lead to clumping that becomes difficult to resuspend.
9. To recover the clear upper aqueous phase containing RNA, tilt the tube to a 45-degree angle and carefully transfer it to a fresh tube. Avoid withdrawing too quickly or excessively to prevent contamination from the red phenol/chloroform layer or DNA at the interface.
10. Add 5–10 μg of nuclease-free glycogen as a visual aid for the RNA pellet. For small samples ($<10^6$ cells), this also serves as a carrier, co-precipitating with RNA without interfering with downstream applications.
11. RNA can be stored in 75% ethanol for at least a year at −20 °C or up to a week at 4 °C.
12. The 260/280 and 260/230 ratios indicate nucleic acid purity. For RNA, a 260/280 ratio above 1.8 (ideally ~2.0) suggests minimal protein or phenol contamination, while a 260/230 ratio above 1.8 reflects low levels of organic solvents or salts.
13. A negative control (RT-) proves very useful for ensuring the absence of genomic DNA contamination. Amplification of contaminating genomic DNA during PCR can generate additional bands of greater size on the gel, particularly if the primers surround small introns that are necessarily absent on the mRNA.
14. Use a high number of PCR cycles to visualize any unwanted splice variants. These splice variants generally contain PTCs and are actively degraded by the NMD pathway.
15. Perform this step quickly to minimize UV-induced DNA damage.
16. Upon thawing, store BigDye reagents at 4 °C for up to a month. Do not refreeze.
17. Handle PFA under a chemical hood with gloves and eye protection.
18. From the secondary antibody incubation onwards, all steps should be carried out in the dark to preserve fluorescence.
19. Ensure there are no air bubbles when placing the coverslips.

Acknowledgments

This work was supported by the Fondation Pour la Recherche Médicale, the "Institut français en Grèce" (to EP), La Ligue Contre le Cancer (comités 87, 23 to EP and SLN), Agence Nationale de la Recherche (ANR-21-CE15-0001-0) and Fondation ARC (ARCPJA2023070006749).

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Gagliardi M, Ashizawa AT (2021) The challenges and strategies of antisense oligonucleotide drug delivery. Biomedicines 9:433. https://doi.org/10.3390/biomedicines9040433
2. McClorey G, Wood MJ (2015) An overview of the clinical application of antisense oligonucleotides for RNA-targeting therapies. Curr Opin Pharmacol 24:52–58. https://doi.org/10.1016/j.coph.2015.07.005
3. Marchalot A, Lambert J-M, Boyer F, et al (2020) Splice switching oligonucleotide mediated gene knockdown in B cells and plasma cells 2020.09.18.302984
4. Zammarchi F, de Stanchina E, Bournazou E et al (2011) Antitumorigenic potential of STAT3 alternative splicing modulation. Proc Natl Acad Sci USA 108:17779–17784. https://doi.org/10.1073/pnas.1108482108
5. Matsuo M (2021) Antisense oligonucleotide-mediated exon-skipping therapies: precision medicine spreading from Duchenne muscular dystrophy. JMA J 4:232–240. https://doi.org/10.31662/jmaj.2021-0019
6. Kim D, Schmidt C, Brown MA, Tucker H (2017) Competitive promoter-associated matrix attachment region binding of the Arid3a and Cux1 transcription factors. Diseases 5:34. https://doi.org/10.3390/diseases5040034
7. Webb CF, Smith EA, Medina KL et al (1998) Expression of bright at two distinct stages of B lymphocyte development. J Immunol 160:4747–4754
8. Webb CF, Bryant J, Popowski M et al (2011) The ARID family transcription factor bright is required for both hematopoietic stem cell and B lineage development. Mol Cell Biol 31:1041–1053. https://doi.org/10.1128/MCB.01448-10
9. Kim D, Probst L, Das C, Tucker PW (2007) REKLES is an ARID3-restricted multifunctional domain. J Biol Chem 282:15768–15777. https://doi.org/10.1074/jbc.M700397200
10. Guo C, Xu HE, Ma X (2023) ARID3a from the ARID family: structure, role in autoimmune diseases and drug discovery. Acta Pharmacol Sin 44:2139–2150. https://doi.org/10.1038/s41401-023-01134-2
11. Ratliff ML, Ward JM, Merrill JT et al (2015) Differential expression of the transcription factor ARID3a in lupus patient hematopoietic progenitor cells. J Immunol 194:940–949. https://doi.org/10.4049/jimmunol.1401941
12. Zheng W, Lai G, Lin Q et al (2021) A miR-129-5P/ARID3A negative feedback loop modulates diffuse large B cell lymphoma progression and immune evasion through regulating the PD-1/PD-L1 checkpoint. Front Cell Dev Biol 9. https://doi.org/10.3389/fcell.2021.735855
13. Ho T (2018) FOXP1 and ARID3A collaborate to activate transcription of Core embryonic stem cell factors in activated B – cell diffuse large B cell lymphoma. JES 2:1–13. https://doi.org/10.23880/JES-16000110

Chapter 13

Modifying 3′-End RNA Isoforms Using Antisense Oligonucleotides

Antoine Praité, Catherine Horiot, Anne Marchalot, Sandy Al Hayek, and Laurent Delpy

Abstract

Alternative polyadenylation (APA) represents a pivotal post-transcriptional mechanism, exerting a significant influence on gene expression through the generation of diverse 3′-end RNA isoforms. The present protocol delineates a streamlined approach for modulating APA using antisense oligonucleotides (ASOs), with a focus on their design, application, and analytical evaluation. By targeting specific polyadenylation signals, ASOs enable the reprogramming of isoform proportions, as demonstrated by the regulation of immunoglobulin heavy chain transcript isoforms. The protocol delineates the sequence of steps involved in the method, commencing with the design and validation of ASOs, followed by treatment of cells, RNA purification, and quantitative PCR-based isoform analysis. Key factors, such as ASO design and treatment optimization, are considered to ensure reproducibility and robustness. This workflow offers a robust platform to assess the therapeutic potential of ASOs in APA modulation across various cell types.

Key words RNA, Polyadenylation, Antisense oligonucleotides, Alternative polyadenylation, RNA-based therapeutics, 3′-end isoforms

1 Introduction

The majority of genes express different isoforms, whose proportions vary both spatially and temporally. Beyond alternative splicing, at least 70% of mammalian genes harbor multiple cleavage and polyadenylation sites, generating distinct 3′-end isoforms [1, 2]. The impact of alternative polyadenylation (APA) can be divided into two main categories [3]. The first category is termed "terminal exonic APA", whereby transcript isoforms produce the same protein but with different lengths of the 3′ untranslated region (UTR), which influences mRNA metabolism [4] (Fig. 1). The second category is APA in the coding regions of pre-mRNA, which results in the production of different transcripts and protein isoforms (Fig. 1). A well-documented example is the APA of the

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_13,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

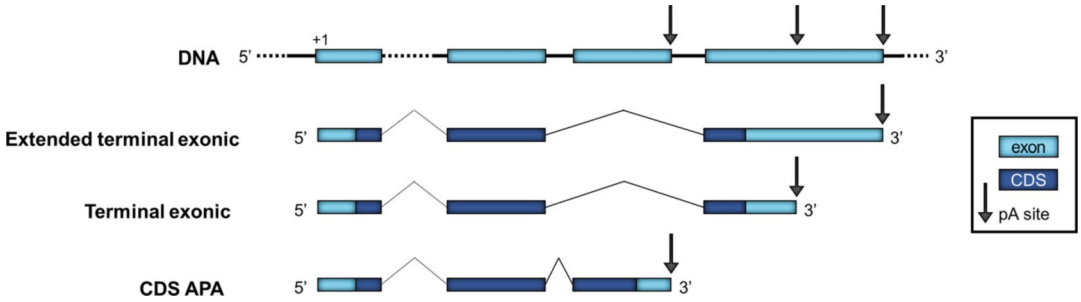


Fig. 1 Schematic representation of alternative polyadenylation (APA) generating multiple 3′-end mRNA isoforms. Exons (light blue), coding sequences (CDS, dark blue), and cleavage and polyadenylation (pA) sites (arrows) are shown, highlighting splicing and polyadenylation processes leading to various mRNA isoforms

immunoglobulin heavy chain (*IGH*) transcript. Depending on the polyadenylation site, B cells express either the secreted or membrane-bound isoform [5, 6].

One strategy to modulate APA is the application of antisense oligonucleotides (ASOs) [7]. ASOs are defined as short, single-stranded, synthetic nucleic acid sequences, which have the capacity to modify gene expression by hybridizing specifically to a portion of a transcript [8]. ASO treatment can disrupt the recognition of the polyadenylation signal by steric hindrance and induce APA. As shown by Marchalot et al., targeting the polyadenylation signal of secreted *IGHE* transcripts with an ASO reduces IgE secretion and increases membrane *IGHE* RNA expression at the same time [9]. Similar results are obtained using a consensus ASO hybridizing to the polyadenylation signal of all secreted *IGHG* isotypes. Interestingly, this ASO treatment disrupts the stochiometric assembly of immunoglobulin heavy and light chains, thereby affecting protein homeostasis and myeloma cell survival [10].

ASO treatments are therefore of therapeutic interest for selectively reducing immunoglobulin secretion, with broad applications in IgE-dependent allergic manifestations and plasma cell disorders. In addition, the targeting of polyadenylation signals by ASOs offers promising avenues for therapeutic intervention, particularly in cases where genetic disorders are caused by the gain of a polyadenylation signal, such as Wiskott-Aldrich syndrome [11].

The present study proposes a streamlined analytical approach to demonstrate the feasibility of using ASOs to modulate APA. We use an ASO targeting the polyadenylation site of secreted *IGHE* transcripts and an IgE-expressing myeloma cell line (U266) as a model. We assess APA by quantifying the amounts of secreted and membrane *IGHE* mRNA isoforms in ASO-treated cells compared to an irrelevant control ASO (Fig. 2). The subsequent sections provide a comprehensive overview of the materials and methods employed, encompassing the treatment of cells and the analysis of transcripts.

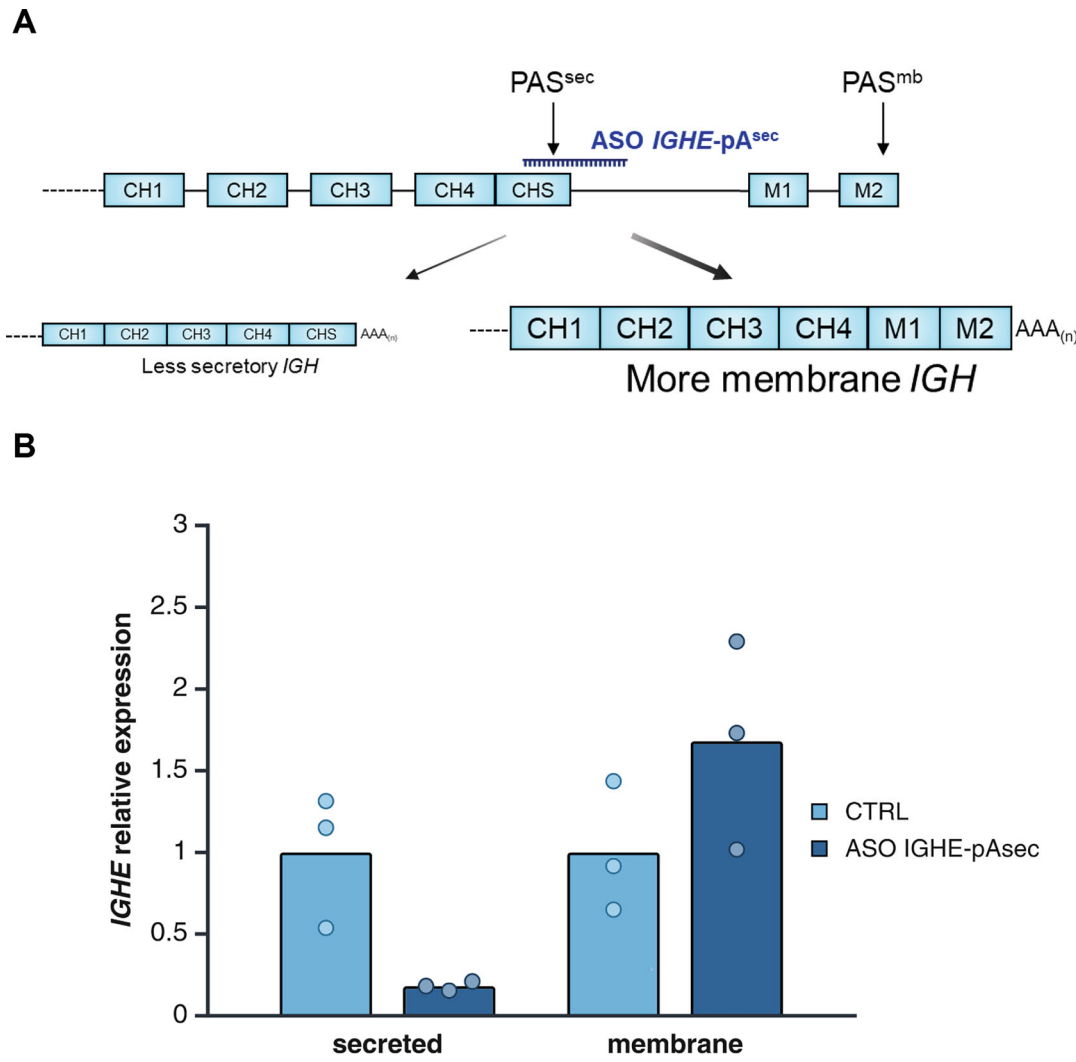


Fig. 2 Impact of *IGHE*-pA^{sec} ASO treatment on alternative polyadenylation and expression of secreted and membrane *IGHE* mRNA isoforms. (**a**) Schematic representation of human *IGHE* transcripts with secreted and membrane polyadenylation signals (PAS^{sec} and PAS^{mb}, respectively). The position of ASO *IGHE*-pA^{sec} targeting the PAS^{sec} on secreted *IGHE* pre-mRNAs is depicted. The effect of ASO *IGHE*-pA^{sec} treatment on the expression of secreted or membrane *IGHE* mRNA isoforms is illustrated by the size of the letters. (**b**) Effects of ASO treatment on IgE-expressing cells. U266 myeloma cells were treated with 6 μM of ASO *IGHE*-pA^{sec} or Control ASO (CTRL) for 48 h, and RT-qPCR were performed on RNA samples. The bar graph represents the relative levels of secreted and membrane *IGHE* mRNAs from three different experiments (light blue circle)

2 Materials

To guarantee the validity of the results, it is recommended that materials and reagents dedicated exclusively to ASO treatments be used. Transcriptomic analyses must be performed in an RNase-free working environment. All these materials and reagents should be stored in designated compartments for exclusive use.

2.1 Antisense Oligonucleotides

1. Gene-specific (GS) ASO: the ASO targeting the polyadenylation signal of human secreted *IGHE* transcripts (ASO IGHE-pAsec: 5′-GCTCTGAGCAGGCACAGTTTATTG-3′) is purchased as phosphorodiamidate morpholino oligomer coupled with an octaguanidine dendrimer (Vivo-Morpholino oligos; Gene Tools) (*see* **Note 1**). Resuspend the lyophilized ASO at a concentration of 500 μM with sterile nuclease-free water (*see* **step 2** in Subheading 3.2). Store at room temperature protected from light.
2. Irrelevant control (CTRL) ASO: Vivo-Morpholino standard control oligo (Ctrl ASO: 5'-CCTCTTACCTCAGTTACAATTTATA-3′; Gene Tools) (*see* **Notes 2** and **3**). Resuspend the lyophilized ASO at a concentration of 500 μM with nuclease-free water (*see* **step 2** in Subheading 3.2). Store at room temperature protected from light.
3. Sterile nuclease-free water (not DEPC-treated) (*see* **Note 4**). Store at room temperature.
4. Parafilm.
5. Cell culture hood.

2.2 Cell Culture and Viability Test

This chapter describes ASO treatment on suspension cell cultures, but this aspect depends on the choice of cell model and must be adapted to each study. All reagents and consumables used for cell culture (e.g., tips, tubes, and micropipettes) must be sterile or sterilized.

1. Primary cells or cell lines adapted to the study model. Here, U266B1 (U266) cells, as IgE-expressing human myeloma cell line, are culture at 37 °C in a humidified atmosphere containing 5% CO_2.
2. Culture medium: RPMI 1640, 10% heat inactivated fetal bovine serum (FBS). Depending on the cell model, a different medium, percentage of FBS or other additives may be used for optimal cell (*see* **Note 5**).
3. 1× phosphate-buffered saline (PBS).
4. CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) (*see* **Note 6**). Store aliquots protected from light at −20 °C.
5. Sterile 96-well and 24-well plates.
6. Multichannel pipette.
7. Autoclavable reagent reservoirs for multichannel pipettes.
8. 96-well plate reader.
9. Centrifuge.

2.3 RNA Extraction and Purification

1. RNase surface decontamination reagent such as RNase AWAY.
2. TRIzol reagent (Ambion). Store at 4 °C (*see* **Note 7**).
3. Absolute ethanol molecular biology grade. Store at room temperature or at 4 °C to prevent evaporation.
4. Column-based RNA purification: Direct-zol™ RNA Microprep (Zymo Research) (*see* **Note 8**).
5. RNase-free 1.5 mL tubes.
6. Nuclease-free water.
7. Cooled microcentrifuge.
8. RNA quantification apparatus: spectrophotometer system such as NanoDrop (Thermo Scientific) or a fluorometric quantification system such as Qubit (Invitrogen).

2.4 Gene Expression Analysis

1. 1 U/μL DNase I amplification grade, 10× DNase I Reaction Buffer, and 25 mM EDTA, pH 8.0 (Invitrogen). Store at −20 °C.
2. 0.2 mL PCR tubes.
3. Thermal cycler.
4. 200 U/μL SuperScript IV Reverse Transcriptase (SSIV RT), 5× SSIV Buffer and 100 mM dithiothreitol (DTT) (Invitrogen), or alternatively High Capacity cDNA Reverse Transcription kit (Applied Biosystems) (*see* **Note 9**). Store at −20 °C.
5. 10 mM dNTP mix: 10 mM each. Store at −20 °C.
6. 50 μM Random hexamers 50 μM. Store at −20 °C.
7. Ribonuclease inhibitor such as RNaseOUT (Invitrogen). Store at −20 °C.
8. SensiFAST™ Probe Hi-ROX Kit if using probe-based quantitative PCR (qPCR) or SensiFAST™ SYBR® Hi-ROX Kit (Meridian Bioscience) if using SYBR green-based qPCR (*see* **Note 10**). Always store protected from light at −20 °C; then once thawed, it can be kept at 4 °C but must be used within a month.
9. Primers or primer/probe pairs specific to isoforms impacted by ASO treatment (isoforms A and B). For the secreted *IGHE* mRNA isoform, we use a forward primer located in the CH4 exon (5′- CGAGCGGTGTCTGTAAATCC -3′) and a reverse primer located in the CHS exon (5′- -CACTGCACAGCTGGATGG-3′). For the membrane *IGHE* mRNA isoform, we use a Taqman qPCR assay with an M1-M2 exon spanning probe (*see* Fig. 2 and [9]).
10. Quantitative PCR system: QuantStudio 3 qPCR thermocycler (Applied Biosystems).

3 Methods

3.1 Gene-Specific Targeting Poly(A) Signal ASO Design

The design of the GS ASO necessitates a meticulous in silico analysis as a preliminary step. A significant concern associated with antisense strategies pertains to the potential for off-target effects. This analysis enables the production of a highly specific ASO for the transcript isoform of interest. The majority of these steps are analogous to the process of designing a reverse primer.

1. Retrieve the sequence from a database such as Ensembl (*see* **Note 11**).
2. Select at least 20 nucleotides upstream and downstream of the poly(A) signal. If the poly(A) signal is not annotated, use a prediction tool.
3. Make a list of all 24-mer, 25-mer, and 26-mer sequences that fully cover the poly(A) signal (*see* **Note 12**). Perform the next steps for all sequences.
4. Blast the nucleotide sequences on blastn (NCBI) or BLAST (Ensembl). Select sequences that are not homologous to others besides the sequence of interest.
5. Use an oligonucleotide analysis tool online to exclude sequences with too many secondary structures.
6. Finally, combine the analysis results to choose the most relevant sequence. It is possible to design and test multiple sequences (*see* Note **13**). Don't forget to order the reverse complement of the chosen sequence, not the forward.

3.2 Toxicity and Viability Test

This entire procedure must be carried out under a cell culture hood under sterile conditions. All surfaces and materials must be thoroughly disinfected.

1. When working with cell lines, use cells that have not undergone too many passages to avoid genetic drift. To prevent biological variability between replicates, keep and use cells in their exponential growth phase: typically, between 0.5 and 1.5 × 10^6 cells/mL (*see* **Notes 5** and **14**).
2. Work in a cell culture hood and wear a lab coat and gloves to resuspend the lyophilized ASOs (GS and CTRL). Remove the metal seal from the glass vessel and tap the tube to prevent any powder remaining on the cap and then open the tube (*see* **Note 15**). Add the volume of nuclease-free water required for a final concentration of 500 μM. Do not pipette up and down or touch the powder with the tip. Close the vial and mix gently. Wait at least 30 min for complete ASO reconstitution. Wrap the cap with parafilm and store at room temperature protected from light.

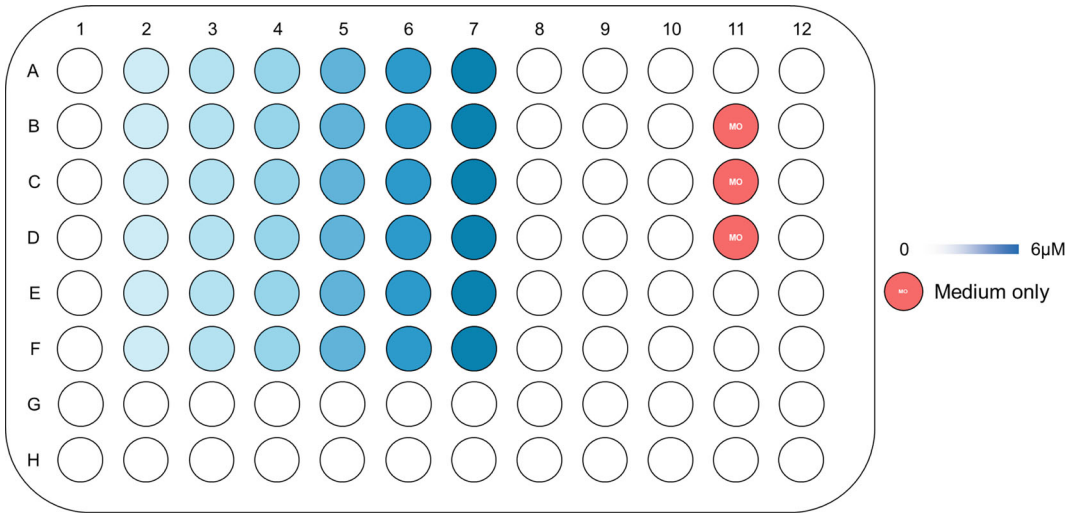


Fig. 3 96-well plate design used to test the cellular toxicity of ASO treatment. Cells are incubated with increasing concentrations of ASO (0–6 μM) and MTS assays are performed to assess cell viability and determine working ASO concentrations

3. In a 96-well plate, conduct a pilot study to assess the cellular toxicity of the CTRL ASO chemistry in order to determine the optimal concentrations for testing the effects of GS ASO treatment (*see* **Note 16**). Incubate 50,000 cells in 100 μL of complete culture medium with increasing final concentrations of Ctrl ASO, usually between 0.25 and 6 μM (*see* **Note 17**). For an accurate assessment of viability, we recommend making six replicates/conditions and comparing to the untreated condition (NT). Also add three blank wells containing only complete culture medium. A typical experimental design is presented in Fig. 3.
4. Incubate for 24–48 h under appropriate culture conditions (*see* **Note 17**). For U266: 37 °C, 5% CO_2 and humidified atmosphere.
5. After the incubation period with CTRL ASO, add 20 μL of MTS to each culture well and the blanks with a multichannel pipette (*see* **Note 18)**.
6. Return the plate to the incubator for 1–4 h. Regularly check the evolution of the purple compound appearance to avoid saturation (*see* **Note 19**).
7. Gently shake the plate to homogenize the formazan in each well. Read the plate's absorbance at 490 nm in a 96-well plate reader.
8. For analysis, subtract the mean of the blank wells from all values, then average the technical replicates and compare to the untreated condition.

9. Among the CTRL ASO concentrations that are not or minimally toxic to the cells, choose a low dose and a high dose for the next comparative assessment of GS- and CTRL-ASO treatments.

3.3 Gene-Specific ASO Treatment

This entire procedure must be carried out under a cell culture hood under sterile conditions.

1. To prevent cross-contamination of ASO vials, thoroughly disinfect surfaces and materials.
2. In a 24-well plate, for each condition (NT, CTRL ASO, and GS ASO), incubate 1×10^6 cells in 1 mL of culture medium with the previously determined low dose and high dose ASO concentrations (*see* **Note 20**). Gently mix the 500 μM ASO vials before deposit.
3. Gently tap the 24-well plate to homogenize ASO/cell mixture and incubate for 24–48 h.
4. After 24–48 h incubation, gently mix the well volume by pipetting five to six times, and then transfer the entire volume to RNase-free 1.5 mL tubes (*see* **Note 21**).
5. Keep the tubes on ice until RNA extraction.

3.4 RNA Extraction and Purification

All subsequent steps must be carried out within a fume hood in an RNase-free environment. Wearing gloves is essential, and avoiding contact with skin or hair prevents nuclease contamination. The use of filtered tips is also imperative.

1. Clean the workstation with an RNase surface decontamination reagent.
2. Set the microcentrifuge to 4 °C.
3. Centrifuge the cells in a 1.5 mL tubes (*see* **step 5** in Subheading 3.3) at 300× *g* for 5 min, at 4 °C.
4. Wash the pellet once in cold 1× PBS. Centrifuge at 300× *g* for 5 min, at 4 °C.
5. Let the centrifuge warm up to room temperature. Discard the supernatant. Resuspend the cell pellet in 350 μL of TRIzol (*see* **Note 22**). Pipette vigorously and gently vortex. Incubate for 5 min at room temperature. At this stage, cells in TRIzol can be stored at −80 °C for long-term storage.
6. Work now at room temperature. Add an equal volume of absolute ethanol to the TRIzol volume: here, 350 μL. Vigorously shake the tubes for 1 min. Do not vortex.
7. Transfer the entire volume to the Direct-zol™ RNA Microprep purification column. Follow the manufacturer's instructions. At the final step, to recover total RNA with nuclease-free water, use a maximum of 30 μL and wait for 1 min before centrifuging

at 12,000× *g* for 1 min. Immediately place the tubes containing the RNA on ice.

8. Measure RNA concentrations with a spectrophotometer system such as NanoDrop (Thermo Scientific) or a fluorometric quantification system such as Qubit (Invitrogen) (*see* **Note 23**). Store RNA samples at −80 °C or process immediately.

3.5 DNA Digestion, Reverse Transcription, and Quantitative PCR

All subsequent steps must be carried out in an RNase-free environment. Wearing gloves is essential, and avoiding contact with skin or hair prevents nuclease contamination. The use of filtered tips is also imperative.

1. Clean the workstation with an RNase surface decontamination reagent.
2. In a 0.2 mL PCR tube, set up the DNase I reaction as described in Table 1 (*see* **Note 24**).
3. Add 1 μL of dNTP and 1 μL of random hexamers to each DNase I-treated RNA samples.
4. Heat for 5 min at 65 °C. Then, put on ice for at least 1 min.
5. Dilute each tube to 1:2 with 13 μL of nuclease-free water and mix well. Transfer 13 μL of this mixture to the corresponding "RT+" and "RT-" tubes with or without SSIV RT, respectively (*see* **Note 25**).
6. Prepare a mix for reverse transcription as described in Table 2, per one tube.
7. Add 7 μL of the corresponding mix to each tube (i.e., RT+ and RT- tubes).
8. In a thermal cycler, heat for 10 min at 23 °C, 10 min at 55 °C, and 10 min at 80 °C.
9. After reverse transcription, we recommend to dilute cDNAs to 1:2 by adding 20 μL of nuclease-free water. This 1:2 dilution

Table 1
Protocol for DNase I digestion of RNA samples

Reagents	Volume per reaction (μL)
1 μg total RNA (*see* note 24) in nuclease-free water	8.0
10× DNase I reaction buffer	1.0
1 U/μL DNase I	1.0
Incubate for 15 min at room temperature	
25 mM EDTA	1.0
In a thermal cycler, heat for 10 min at 65 °C.	

Table 2
Reverse Transcription Mix

Reagents	Volume per reaction (μL)	
	RT+	RT−
5× SSIV buffer	4.0	4.0
100 mM DTT	1.0	1.0
RNaseOUT	1.0	1.0
Nuclease-free water	–	1.0
200 U/μL SSIV RT	1.0	–

reduces the concentration of reagents from the previous enzymatic reactions (DNase I and SSIV RT) which could interfere with qPCR amplification.

10. Determine the impact of ASO treatment by qPCR using the SensiFAST™ Probe Hi-ROX Kit or SensiFAST™ SYBR® Hi-ROX Kit following the manufacturer's recommendations. We recommend that cDNA volume is at least 10% of final qPCR reaction volume (i.e., ≥2 μL of cDNA for a 20 μL qPCR reaction). Measure the expression of the different RNA isoforms in the GS ASO-treated condition compared to control conditions (*see* Fig. 2 for the relative level of secreted and membrane *IGHE* mRNAs) (*see* **Note 26**).

4 Notes

1. A modified ASO with an RNA-like structure is essential to induce steric hindrance at the polyadenylation signal: a DNA-like chemistry leads to the recruitment of RNase H, which cleaves RNA in the DNA heteroduplex. Other chemistries than morpholino exist. The "vivo" compound is a positively charged conjugate that allows the ASO to be attracted to cell surfaces and internalized. This chemistry-conjugate association provides an effective model to study ASO effects on its target.
2. The control ASO must have the same chemistry and conjugation as the gene-specific one. However, it has an irrelevant sequence that cannot hybridize significantly with any human genomic sequence.
3. There are other controls that can be used: a Sense control ASO has the same nucleotide sequence as the gene-specific ASO being studied but in the opposite orientation, and a Scramble control ASO has a random sequence with the same nucleotide

content as the gene-specific ASO. As with the ASO to be studied, it is important to make sure that the control ASO cannot bind strongly with a transcribed sequence.

4. If the nuclease-free water is not sterile, filter it with a 0.2 μm filter in sterile conditions. DEPC could interfere with some cellular processes.
5. Avoid using a culture medium with high percentage of FBS, as proteins in FBS can quench the ASO and reduce its efficacy. For cell cultures requiring excessive FBS percentages, higher concentrations of ASO may be required.
6. This test is based on the reduction of the MTS compound to formazan and is equivalent to the MTT test.
7. The TRIzol reagent can also be stored at room temperature.
8. Adapt the type of column to the number of cells resuspended in TRIzol. In this kit, the columns are compatible with extracting up to 1×10^6 cells. Other RNA purification kits can be used, but we recommend this one as it allows recovery of all types of RNA.
9. SSIV RT allows reverse transcription to conserve proportions between weakly and strongly expressed transcripts and operates at a high temperature to denature RNA secondary structures. If the ASO target gene is highly expressed by the cell, a less sensitive RT such as the High Capacity cDNA Reverse Transcription kit works properly. This kit already provides dNTP and random hexamers.
10. This is an example of an optimized master mix for qPCR. Use the usual laboratory reagents with which you are familiar. We use the QuantStudio 3 qPCR thermocycler which uses ROX fluorescence as a reference, justifying the presence of ROX in the mix. Use reagents compatible with your thermocycler and analysis software.
11. We recommend Ensembl as it gives easy access to 3'UTR sequences. RefSeq is correct, but beware of potential polymorphism in the region.
12. A morpholino ASO usually has a length of around 25 nucleotides. Depending on the ASO chemistry, other rules may apply.
13. Alternatively, the targeted sequence can be submitted to the supplier for the ASO design. When this request is made with Gene Tools company, select a 50-nucleotide sequence encompassing the targeted polyadenylation site (usually AAUAAA) and submit this sequence to the supplier's website for the design (Tab: oligo-design-form). Once the ASO sequence has been obtained, it is important to check that it does not hybridize significantly with a transcribed sequence from the organism.

14. Pay attention to the FBS percentage in the culture medium. This is important to consider this parameter when comparing ASO effects on different cell lines (*see* **Note 5**).
15. GeneTools ASOs are supplied as lyophilized powder in glass vessel with a metal seal on top. For ease of use, we recommend removing this seal and wrapping the lid with parafilm to prevent evaporation.
16. In our experience, some cell lines are highly sensitive to vivomorpholino treatment while others seem far less sensitive to this chemistry. This pilot study clarifies this aspect by consuming only small amounts of the molecule.
17. This concentration range is given as an example of effective doses according to our experience. The duration of ASO treatment can also be adjusted according to experience.
18. Try to work as much as possible protected from light. When dispensing the volume in each well with a multichannel pipette, be careful to deposit the volume without touching cells. Start with the least concentrated conditions.
19. The supplier recommends between 1 and 4 h. For most lymphoid cell lines including U266, an absorbance slightly above 1 is usually obtained after 90 min incubation under untreated conditions.
20. Replicates (e.g., NT, NT', CTRL ASO, CTRL ASO', GS ASO, and GS ASO') or triplicates are recommended. To prevent evaporation of the culture volumes, fill empty wells of the plate with sterile 1× PBS.
21. If relevant for the study, a viability MTS test can also be performed. In this case, use 300 µL from each well to make six replicates of 50 µL, adjust to 100 µL with culture medium and proceed to **steps 5–8** in Subheading 3.2. The remaining 700 µL volume is transferred to 1.5 mL tubes and placed on ice until RNA extraction. We always recommend keeping 10–20 µL of each well for Trypan blue counting.
22. The solution should not be too viscous and the pellet should be well resuspended. If not, increase the TRIzol volume. The value of 350 µL corresponds to the volume needed for 1×10^6 cells. If the initial cell quantity is higher, follow the supplier's recommendations.
23. Fluorometric systems are more sensitive and allow specific quantification of total RNA but require an additional costly reagent kit for quantification.
24. Starting with 1 µg of RNA allows measuring the expression of most transcripts by qPCR. However, the starting amount can be less than 1 µg. If using larger amounts of total RNA, scale up the reaction.

25. Although, using half of the DNase I-treated RNA samples for RT-tubes reduce the amount of RNA used for reverse transcription (i.e., 500 ng of RNA used for RT+ tubes), RT-tubes are important RT-qPCR controls that assess the amplification of residual contaminating DNA.
26. Depending on the cellular and transcriptomic context, one isoform is often predominantly expressed. In our case, U266 myeloma cells express high levels of secreted *IGHE* mRNA isoform compared to the membrane isoform. The ability of a GS ASO targeting the poly(A) signal to decrease isoform A and increase isoform B is therefore rarely proportional.

Acknowledgments

This work was supported by grants from INCa (PLBIO2022-110, INCA_16734), ANR (2017-CE15-0024-01), Ligue Contre le Cancer (comités Corrèze, Haute-Vienne), Fondation Française pour la Recherche contre le Myélome et les Gammapathies monoclonales (FFRMG), and Région Nouvelle-Aquitaine (AAP ESR 2020 and 2024). The authors would like to thank Dr. Laffleur Brice and Pr. Cogné Michel for their contribution during the initial development of this approach in our laboratory.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Derti A, Garrett-Engele P, MacIsaac KD et al (2012) A quantitative atlas of polyadenylation in five mammals. Genome Res 22:1173–1183. https://doi.org/10.1101/gr.132563.111
2. Hoque M, Ji Z, Zheng D et al (2013) Analysis of alternative cleavage and polyadenylation by 3′ region extraction and deep sequencing. Nat Methods 10:133–139. https://doi.org/10.1038/nmeth.2288
3. Stroup EK, Ji Z (2023) Deep learning of human polyadenylation sites at nucleotide resolution reveals molecular determinants of site usage and relevance in disease. Nat Commun 14:7378. https://doi.org/10.1038/s41467-023-43266-3
4. Gruber AJ, Zavolan M (2019) Alternative cleavage and polyadenylation in health and disease. Nat Rev Genet 20:599–614. https://doi.org/10.1038/s41576-019-0145-z
5. Early P, Rogers J, Davis M et al (1980) Two mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. Cell 20:313–319. https://doi.org/10.1016/0092-8674(80)90617-0
6. Alt FW, Bothwell AL, Knapp M et al (1980) Synthesis of secreted and membrane-bound immunoglobulin mu heavy chains is directed by mRNAs that differ at their 3′ ends. Cell 20:293–301. https://doi.org/10.1016/0092-8674(80)90615-7
7. Vickers TA, Wyatt JR, Burckin T et al (2001) Fully modified 2′ MOE oligonucleotides redirect polyadenylation. Nucleic Acids Res 29:1293–1299
8. Roberts TC, Langer R, Wood MJA (2020) Advances in oligonucleotide drug delivery. Nat Rev Drug Discov 19:673–694. https://doi.org/10.1038/s41573-020-0075-7
9. Marchalot A, Horiot C, Lambert J-M et al (2022) Targeting IgE polyadenylation signal with antisense oligonucleotides decreases IgE

secretion and plasma cell viability. J Allergy Clin Immunol 149:1795–1801. https://doi.org/10.1016/j.jaci.2021.09.039

10. Horiot C, Roussel M, Praité A, et al (2023) Disruption of immunoglobulin heavy and light chain assembly by antisense oligonucleotides impairs protein homeostasis and myeloma cell survival. bioRxiv 2023.08.29.555385. https://doi.org/10.1101/2023.08.29.555385
11. Andreu N, García-Rodríguez M, Volpini V et al (2006) A novel Wiskott-Aldrich syndrome protein (WASP) complex mutation identified in a WAS patient results in an aberrant product at the C-terminus from two transcripts with unusual polyA signals. J Hum Genet 51:92–97. https://doi.org/10.1007/s10038-005-0328-7



Chapter 14

A Mass Spectrometry-Based Pipeline for Analyzing RNA Modifications in Cells and Tissues

Aurore Attina, Jérôme Vialaret, Christophe Hirtz, and Alexandre David

Abstract

Mass spectrometry has become indispensable in studying post-transcriptional modifications of RNA, a.k.a. epitranscriptomics, which are crucial for regulating RNA metabolism, gene expression, and major biological processes. Over 150 chemical modifications have been identified across all RNA subtypes. Dysregulation of RNA mark deposition can lead to disease development and progression. Therefore, accurately detecting and characterizing these modifications are vital for understanding their functional impact and uncovering potential therapeutic and diagnostic biomarkers. In this chapter, we describe a mass spectrometry-based method for detecting and quantifying nearly 40 different RNA modifications.

Key words Multiple reaction monitoring (MRM), Targeted epitranscriptomics, RNA modifications, Biomarker discovery

1 Introduction

Once believed to solely transmit genetic information, RNA has recently been discovered to possess remarkable functional complexity. RNA is a complex, high-molecular-weight compound crucial for cellular protein synthesis and serves as the genetic material in some viruses. RNA is composed of nucleotides, which are nitrogenous bases linked to a ribose sugar and connected by phosphodiester bonds, creating strands of various lengths. The nitrogenous bases in RNA encompass adenine, guanine, cytosine, and uracil (in place of thymine found in DNA). This molecular backbone is complexified by a vast number of chemical modifications, which superimpose on the RNA sequence information (Fig. 1a). Originally found in highly structured RNA species, such as rRNA and tRNA, these chemical marks were thought to be merely involved in RNA stability and/or catalytic function. The discovery of some reversible RNA modifications, coupled with the advent of next-

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_14,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

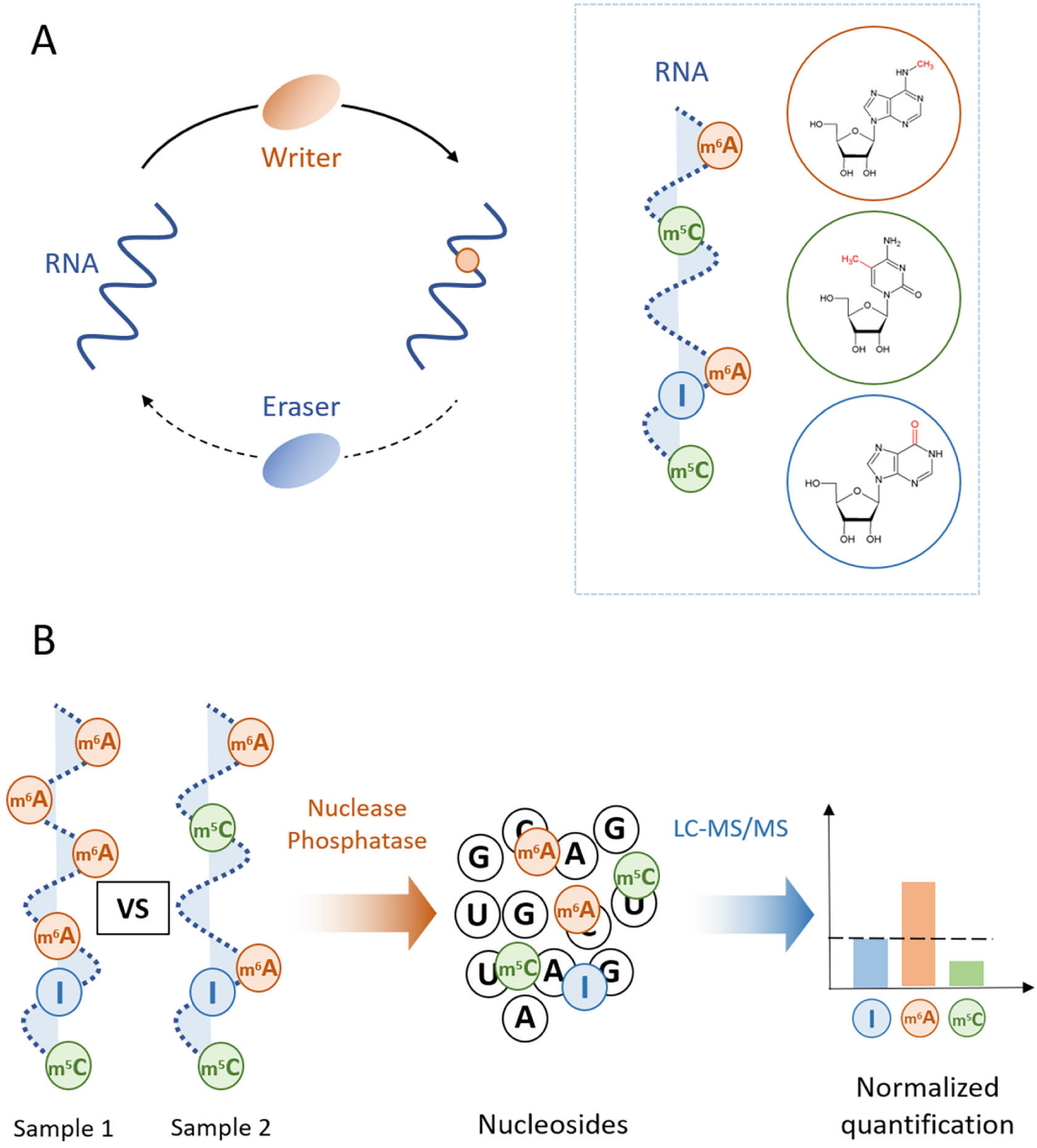


Fig. 1 (**a**) General view of the epitranscriptome regulation. Chemical modifications (e.g., the addition of a methyl group for N6-methyladenosine, m6A) are added to the four bases (or riboses) following RNA synthesis and dynamically regulated by enzymes. Modifications are deposited by "writers" and removed by "erasers" (whenever pertinent). This complex chemical code (>170 modifications to date) is superimposed on the genetic information and controls cellular RNA metabolism in real time. (**b**) Overview of RNA sample processing. RNA species extracted from biological samples (cells or tissues) are isolated and treated with enzymes (Nuclease P1 and alkaline phosphatase) to obtain a mixture of nucleosides. Nucleosides are separated by Nexera LC-40 systems (Shimadzu) using a Synergi™ Fusion-RP C18 column (4 μm particle size, 250 mm x 2 mm, 80 Å) (Phenomenex) and detected by Shimadzu TripleQuad 8060NX in positive ion mode. MS is operated in dynamic MRM mode

generation sequencing (NGS) and mass spectrometry, gave rise to the field of epitranscriptomics. So far, more than 150 types of post-transcriptional modifications have been reported, on various RNA molecules [1]. This complex landscape of chemical marks embodies a novel, invisible code driving the post-transcriptional fate of RNA: stability, splicing, storage, translation. Importantly, RNA epigenetics emerged as a novel layer of gene expression regulation in healthy tissues as well as in other pathologies such as cancer [2] and neurodegenerative diseases [3].

The diversity of RNA modifications is remarkable, both in their nature and distribution across different RNA species. Additionally, RNA modifications can be specific to particular RNA types or nucleotide positions within a sequence. For instance, 5-methoxycarbonylmethyl-2-thiouridine (mcm5s2U) is exclusively found in transfer RNAs, while others are specific to ribosomal RNAs, such as N6,N6-dimethyladenosine ($m^{6,6}A$) [4]. The complexity of RNA modifications reflects their central role in fine-tuning cellular biology.

These modifications, ranging from simple chemical alterations to complex structural changes, regulate a wide variety of biological processes. They can significantly influence RNA functionality, modulating their biological activity and interactions with other cellular molecules. The most prevalent internal modification in eukaryotic messenger RNA (mRNA) is the N^6-methyladenosine (m^6A). This chemical mark has been extensively studied, and its effects can vary widely depending on its location and sequence context. These outcomes range from RNA destabilization to the recruitment of the translation machinery [5]. Similarly, other types of base methylation can regulate RNA stability [6], while pseudouridylation—the conversion of uridine to its isoform pseudouridine (Ψ)—can impact protein translation [7, 8]. In addition to their crucial roles in major physiological functions, specific RNA modifications are linked to disease occurrence and progression, with some actively participating in these processes [9]. These modifications not only serve as potential markers for diagnosing diseases but also pave the way for exploring new treatment avenues [10].

Due to the functional significance of RNA modifications, it is crucial to develop sensitive and specific methods for detecting and characterizing them. NGS-based detection technologies are powerful tools for transcriptome-wide mapping of modified nucleotides, facilitating the elucidation of RNA modification‘s regulatory roles [11]. However, these technologies have significant drawbacks, such as detection biases (especially when using antibodies), loss of stoichiometry information, and the need for large amounts of starting material. Additionally, the simultaneous detection of multiple modifications is quite limited. In contrast, mass spectrometry offers a compelling alternative, with powerful analytical capabilities that allow for targeted, multiplex, and quantitative

analysis of RNA modifications from very small sample amounts (Fig. 1b).

Recently, we have demonstrated that multiplex analysis of RNA modifications is a promising approach for characterizing cell states and stratifying samples [4, 12, 13]. In this context, we have developed an analytical pipeline using LC-MRM (liquid chromatography-multiple reaction monitoring) to study approximately 40 RNA modifications (Table 1). This method can be used for the targeted study of specific marks of interest as well as the untargeted identification of potential disease driver(s).

2 Materials

2.1 Samples, Chemicals, and Standards

1. 200–400 ng of RNA extracted from biological samples as tissues, plants, cells culture, or biological fluid as plasma, serum, or cerebrospinal fluid (*see* **Note 1**).
2. Ammonium acetate ULC-MS (0001244153BS, Biosolve).
3. Water ULC-MS (0023214102BS, Biosolve).
4. Acetonitrile ULC-MS (0001204101BS, Biosolve).
5. Acetic acid glacial ULC-MS (0001074131BS, Biosolve).
6. All standards have been purchased by Biosynth® Ltd. except 7-aminomethyl-7-deazaguanine, 7-aminomethyl-7-deazaguanosine, Queuine, and Queuosine were synthetized by Synthenova SAS (https://www.synthenova.com). The reference of each nucleoside is indicated in Table 1.

2.2 Buffers and Enzymes for Enzymatic Digestion

1. 1 M of ammonium acetate.
2. 100 mM of ammonium acetate pH 5.3.
3. Nuclease P1 from *Penicillium citrinum* (Sigma-Aldrich): resuspended with water at a final concentration of 1 U/μL. Aliquots of 50 μL were done and they storage at −20 °C until use.
4. Alkaline phosphatase from *Escherichia Coli* (Sigma-Aldrich): resuspension with water at a final concentration of 1 U/μL and the solution is storage at 4 °C until use.
5. NP1 solution: dilution of nuclease P1 (1 U/μL) with 100 mM ammonium acetate pH 5.3. To dilute it, add 3 volumes of buffer with 1 volume of enzyme. This solution is prepared just before use and the excess is discarded.
6. AP solution: dilution of alkaline phosphatase (1 U/μL) with 1 M ammonium acetate. To dilute it, add 3 volumes of buffer with 1 volume of enzyme. This solution is prepared just before use and the residue is discarded.

Table 1
Description of the nucleosides present in the analysis method.

Code name	Molecular name	Formula	MW	CAS Number	MODOMICS Database ID	Type of ARN	Organism	Biosynth Reference
ac4C	N4-acetylcytidine	C11H15N3O6	285.0961	3768-18-1	81	tRNA, rRNA	Eubacteria, Eukaryota	NA05753
Am	2′-O-methyladenosine	C11H15N5O4	281.1124	2140-79-6	127	tRNA, rRNA, snRNA, snoRNA	Eubacteria, Eukaryota	NM05694
Cm	2′-O-methylcytidine	C10H15N3O5	257.1012	2140-72-9	84	tRNA, rRNA, snRNA	Eubacteria, Eukaryota	NM06302
D	Dihydrouridine	C9H14N2O6	246.0852	5627-05-4	14	rRNA, tDNA, tRNA	Eubacteria, Eukaryota	FAA62705
Gm	2′-O-methylguanosine	C11H15N5O5	297.1073	2140-71-8	28	tRNA, rRNA, snRNA	Eubacteria, Eukaryota	NM02941
hm5C	5-hydroxymethylcytidine	C10H15N3O6	273.0961	19,235–17-7	119	NA	NA	NH35521
ho5U	5-hydroxyuridine	C9H12N2O7	260.0645	957–77-7	64	NA	Eubacteria	NH32540
ho8A	8-hydroxyadenosine	C10H13N5O5	283.0916	29,851–57-8	NA*	NA	NA	NH08494
I	Inosine	C10H12N4O5	268.0808	58–63-9	113	tRNA	Eubacteria, Eukaryota	NI06297
i6A	N6-isopentenyladenosine	C15H21N5O4	335.1594	7724-76-7	56	tRNA	Eubacteria, Eukaryota	ND08001
io6A	N6-(cis-hydroxyiso-pentenyl)adenosine	C15H21N5O5	351.1543	15,896–46-5	10	tRNA	Eubacteria	XZ181120
m1A	1-methyladenosine	C11H15N5O4	281.1124	15,763–06-1	83	tRNA, rRNA	Archaea, eubacteria, Eukaryota	NM03697

(continued)

Table 1
(continued)

Code name	Molecular name	Formula	MW	CAS Number	MODOMICS Database ID	Type of ARN	Organism	Biosynth Reference
m1G	1-methylcytidine	C11H15N5O5	297.1073	2140-65-0	22	tRNA, rRNA	Archaea, eubacteria, Eukaryota	NM08574
m1I	1-methylinosine	C11H14N4O5	282.0964	2140-73-0	26	tRNA	Eukaryota	FM163160
m2,2,7G	N2,N2,7-trimethylguanosine	C13H19N5O5+	327.1543	40,027–70-1	111	NA	Eukaryota	NT08918
m2,2G	N2,N2-dimethylguanosine	C12H17N5O5	311.123	2140-67-2	13	tRNA, rRNA	Archaea, eubacteria, Eukaryota	ND05647
m2,7G	N2,7-dimethylguanosine	C12H17N5O5+	313.1386	62,122–07-0	4	NA	Eukaryota	ND45563
m2A	2-methyladenosine	C11H15N5O4	281.1124	16,526–56-0	37	rRNA, tRNA	Eubacteria	NM46832
m2G	N2-methylguanosine	C11H15N5O5	297.1073	2140-77-4	36	tRNA, rRNA, snRNA	Archaea, eubacteria, Eukaryota	2140-77-4
m3C	3-methylcytidine	C10H16N3O5+	257.1012	2140-64-9	52	tRNA,	Eukaryota	NM05757
m3U	3-methyluridine	C10H14N2O6	258.0852	2140-69-4	49	rRNA	Eubacteria	NM06185
m3Um	3,2′-O-dimethyluridine	C11H16N2O6	272.1008	7103-27-7	57	NA	NA	NM163076
m5C	5-methylcytidine	C10H15N3O5	257.1012	2140-61-6	18	tRNA, rRNA, mRNA, pre-tRNA, other	Eubacteria, Eukaryota	NM03720
m5U	5-methyluridine	C10H14N2O6	258.0852	1463-10-1	12	tRNA, rRNA	Archaea, eubacteria, Eukaryota	NM04922

m6,6A	N6,N6-dimethyladenosine	C12H17N5O4	295.1281	2620-62-4	41	rRNA	Eubacteria, Eukaryota	ND04708
m6,6Am	N6,N6,2′-O-trimethyladenosine	C13H19N5O4	309.1437	30,891–53-3	124	NA		NT59826
m6A	N6-methyladenosine	C11H15N5O4	281.1124	1867-73-8	96	tRNA, rRNA, mRNA,snRNA	Eubacteria, Eukaryota	NM32281
m6Am	N6,2′-O-dimethyladenosine	C12H17N5O4	295.1281	57,817–83-1	82	snRNA	Eukaryota	NM157460
m7G	7-methylguanosine	C11H15N5O5+	299.123	20,244–86-4	116	tRNA, rRNA	Eubacteria, Eukaryota	NM08037
mcm5s2U	5-methoxycarbonyl-methyl-2-thiouridine	C12H16N2O7S	332.0678	20,299–15-4	68	tRNA	Eukaryota	NM159492
mcm5U	5-methoxycarbonyl-methyluridine	C12H16N2O8	316.0907	29,428–50-0	27	tRNA	Eukaryota	NM45525
mo5U	5-methoxyuridine	C10H14N2O7	274.0801	35,542–01-9	74	tRNA	Eubacteria	NM08028
ncm5U	5-carbamoyl-methyluridine	C11H15N3O7	301.091	29,569–30-0	43	tRNA	Eukaryota	NC159473
oxo8G	8-oxoguanosine	C10H13N5O6	299.0865	3868-31-3	NA*	NA	NA	NO05772
PreQ1base	7-aminomethyl-7-deazaguanine	C7H9N5O	179.0807	69,251–45-2	61	NA	Eubacteria	In house
PreQ1	7-aminomethyl-7-deazaguanosine	C12H17N5O5	311.123	66,048–70-2	123	tRNA	Eubacteria	In house
Y	Pseudouridine	C9H12N2O6	244.0695	1445-07-4	118	tRNA, rRNA, snRNA, snoRNA	Archaea, eubacteria, Eukaryota	NP11297
Qbase	Queuine	C12H15N5O3	277.1175	72,496–59-4	105	NA	NA	In house
Q	Queuosine	C17H23N5O7	409.1597	57,072–36-3	65	tRNA	Eubacteria, Eukaryota	In house

(continued)

Table 1
(continued)

Code name	Molecular name	Formula	MW	CAS Number	MODOMICS Database ID	Type of ARN	Organism	Biosynth Reference
s2C	2-thiocytidine	C9H13N3O4S	259.0627	13,239–97-9	92	tRNA	Eubacteria	NT02911
Um	2′-O-methyluridine	C10H14N2O6	258.0852	2140-76-3	50	tRNA, rRNA, mRNA,snRNA, snoRNA	Archaea, eubacteria, Eukaryota	NM04259
A	Adenosine	C10H13N5O4	267.0968	58–61-7	109			NA01639
C	Cytidine	C9H13N3O5	243.0855	65–46-3	46			NC04070
G	Guanosine	C10H13N5O5	283.0917	118–00-3	38			TVB52519
U	Uridine	C9H12N2O6	244.0695	58–96-8	60			NU06309

All data present in this following table provided by MODOMICS Database. *MW* molecular weight, *NA* data not available

2.3 Mobile Phase Composition

1. Mobile phase A: 5 mM ammonium acetate acidified with acetic acid (glacial) at pH 5.3. Weigh 0.963 g of ammonium acetate powder and mix with 2.5 L of ULC-MS grade water. The pH is fixed at 5.3 by adding 200 µL of acetic acid (glacial). Limit of use is 2 weeks.
2. Mobile phase B: acetonitrile (ACN) pure.

2.4 LC-MS/MS Instrumentation

1. HPLC system: Nexera LC-40 HPLC (Shimadzu).
2. Liquid chromatography column: Synergi™ Fusion-RP C18 column (2.0 × 250 mm, 4 µm, 80 Å, 00G-4424-B0, Phenomenex).
3. Mass spectrometer: Shimadzu 8060NX triple-quadrupole mass spectrometer with new electrospray ionization source (ESI) equipped with IonFocus™ technology is used to collect MRM data.
4. Software: Realtime Analysis (Labsolution version 5.113, Shimadzu, Japan) is used to control all LC-MS system. The Postrun Analysis (Labsolution version 5.113, Shimadzu, Japan) is used to control the signal from quality control (QC) and blank before injection of samples. Skyline, an open-source windows client application, is used for data processing (version 23.1.0.268, University of Washington, USA).

2.5 Other Instrumentations and Materials

1. Centrifuge.
2. pH meter.
3. Vortex.
4. Incubator.
5. Precision weighing balance.
6. 1 mL syringe (BD Plastipak™).
7. Syringe Driven Filter Unit (Millex®-GV, 0.22 µm, Merck).
8. Vials for injection (11 mm PP Vial, Agilent) and Caps (11 mm clear snap, Agilent).

3 Methods

3.1 Enzymatic Digestion

1. Dilute 200–400 ng of RNA in final volume of 20 µL of water (*see* **Note 2**).
2. Add 4 µL of NP1 solution. Vortex quickly and incubate at 37 °C during 2 h with low agitation at 400 rpm (*see* **Note 3**).
3. Add 4 µL of AP solution. Vortex quickly and incubate at 37 °C during 2 h with low agitation at 400 rpm.

Stop Point If the samples are not injected directly, they are stored for a few days at −20 °C, but never for more than a week.

3.2 Sample Preparation for LC-MS Analysis

1. Add 60 µL of Mobile phase A, 5 mM ammonium acetate pH 5.3. Vortex quickly and centrifuge during few seconds to pellet all solution.
2. Take the solution with a 1 mL syringe and filter it using a Syringe Driven Filter Unit directly in the vial of injection.

3.3 LC-MS Settings

1. The MS is set to operate in the ESI-positive mode. The source parameters are described in Table 2.
2. The chromatographic conditions and gradient are described in Tables 3 and 4.
3. Each target is detected in the MRM mode by monitoring the appropriate ionized molecule [M + H] in the first quadrupole and the generated fragments in the second quadrupole. For all target, the main fragmentation is the loss of the sugar. For more complex molecule, additional transition can be detected. A summary of the ions monitored during the MS analysis of RNA modifications is shown in Table 5.
4. A batch queue is designed with the following considerations: (a) include in the batch blank and negative QC samples (*see* **Note 4**), (b) before injection of biological samples, analyze the QC samples to check system performance, and (c) analyze the biological samples in random order. Injection of each of the samples in simplicate.

Table 2
Mass spectrometer parameters

Parameter	Values
Nebulizer gas flow	3 L/min
Heating gas flow	10 L/min
Interface temperature	350 °C
Desolvation temperature	602 °C
DL temperature	225 °C
Heat block temperature	400 °C
Drying gas flow	3 L/min
Interface voltage	0.5 kV
Focus voltage	2 kV

Table 3
Chromatographic Conditions

Parameter	Materials and conditions
Column	Synergi fusion polar-RP, 80 Å, 4 μm, 2 x 250 mm
Mobile phase A	5 mM ammonium acetate pH 5.3
Mobile phase B	ACN pure
Column temperature	35 °C
Sample temperature	4 °C
Run time	30 min
Injection volume	5 μL
Elution	Gradient

Table 4
Binary Pump System Gradient

Time (min)	Flow rate (mL/min)	% Mobile Phase A	% Mobile Phase B
0.0	0.40	100	0
3.0	0.40	100	0
3.1	0.40	99	1
13.0	0.40	92	8
23.0	0.40	60	40
23.1	0.40	20	80
26.0	0.40	20	80
26.1	0.40	100	0
30.0	0.40	100	0

4 Notes

1. The standard RNA extraction protocol is a liquid–liquid extraction protocol using Trizol™. However, for RNA extraction from plasma, we use an extraction kit that uses a silica column to purify the RNA (miRNeasy Serum/Plasma Advanced Kit—microRNA Isolation, Qiagen). Using this kit increases extraction yield and limits the variability due to sample preparation. However, the use of a column to purify RNA induces a bias since only RNA bigger than 18 nt is retained on the column.

Table 5
Description of MS parameters for each MRM targets. For each target, you have the abbreviation of the name (code name), the m/z of precursor, the m/z of selected product(s), the voltage of Q1, collision cell and Q3, the specific retention time (Ret. Time), and the targeted dwell time

Code Name	Precursor m/z	Products m/z	Target Q1 Pre Bias (V)	Target Collision Energy (V)	Target Q3 Pre Bias (V)	Ret. Time (min)	Target Dwell Time (msec)
A	268	136	−12	−16	−15	12	97
ac4C	286.1 286.1	154 112	−10 −13	−10 −27	−16 −12	12	47
Am	282	136	−13	−15	−15	15	184
C	244.1	112	−18	−13	−12	4	147
Cm	258.1	112	−18	−11	−13	9	85
D	247.1 247.1 247.1	133 115 97	−15 −16 −11	−7 −7 −19	−10 −13 −22	3	47
G	284.1	152	−13	−12	−17	9.5	85
Gm	298.1	152	−11	−12	−11	11	85
hm5C	274.1	142	−13	−11	−15	4	147
ho5U	261.2	129	−12	−10	−22	3.5	147
ho8A	284.5 284.5	152 125	−11 −22	−14 −36	−16 −14	12.5 12.5	47 47
I	269.1	137	−13	−11	−15	9	85
i6A	336.15 336.15 336.15	204 148 136	−25 −19 −22	−17 −23 −33	−15 −17 −26	21.5	247
io6A	352.2 352.2 352.2	220 136 118	−15 −12 −15	−17 −35 −45	−25 −27 −28	17.5	68
iodo5U	371	239	−26	−12	−17	12.5	97
m1A	282.1	150.1	−13	−18	−16	4.5	147
m1G	298.1	166	−14	−14	−18	11.5	85
m1I	283.1	151	−18	−10	−17	10	85
m2,2G	312	180	−14	−14	−19	14	163
m227G	326.15	194	−15	−15	−21	12	97
m27G	312.1	180	−14	−16	−13	10	85
m2A	282.1	150	−12	−17	−17	15	184
m2G	298	166	−13	−13	−18	10	85

(continued)

Table 5
(continued)

Code Name	Precursor m/z	Products m/z	Target Q1 Pre Bias (V)	Target Collision Energy (V)	Target Q3 Pre Bias (V)	Ret. Time (min)	Target Dwell Time (msec)
m3C	258.1	126	−12	−14	−14	4	147
m3U	259	127	−12	−11	−14	10	85
m3Um	273.1	127	−20	−10	−14	15	184
m5C	258	126	−12	−12	−14	8	90
m5U	259	127	−30	−11	−14	8	90
m6,6A	296	164.1	−22	−25	−20	18	297
m6,6Am	310.15	164	−20	−25	−20	19.5	372
m6A	282.1	150.1	−13	−20	−16	16	211
m6Am	296	150	−12	−19	−28	17.5	211
m7G	298.3	166	−10	−15	−18	7	133
mcm5s2U	333.1 333.1 333.1	201 169 141	−10 −15 −12	−9 −18 −28	−23 −12 −15	15	59
mcm5U	317.1 317.1 317.1	185 153 125	−15 −14 −15	−9 −18 −28	−10 −16 −21	11.5	26
mo5U	275.2	143	−13	−12	−15	9	85
ncm5U	302.1 302.1 302.1	170 153 125	−15 −14 −13	−10 −17 −29	−18 −11 −28	4.5	47
oxo8G	300.1	168.1	−14	−16	−18	10	85
PreQ1base	180 180 180 180 180	66 91 121 146 163	−15 −16 −17 −15 −16	−39 −36 −26 −26 −13	−27 −19 −20 −25 −17	4.5	27
preQ1	312 312 312	180 295 163	−11 −11 −11	−14 −14 −14	−20 −20 −20	7.5	30
Y	245.05 245.05 245.05	209 179 155	−21 −19 −11	−9 −11 −13	−24 −19 −26	3	47
Qbase	278.1	146	−13	−38	−15	9	85
Q	410.2	295	−11	−13	−15	9	41
Q	410.2	163	−11	−25	−12		

(continued)

Table 5
(continued)

Code Name	Precursor m/z	Products m/z	Target Q1 Pre Bias (V)	Target Collision Energy (V)	Target Q3 Pre Bias (V)	Ret. Time (min)	Target Dwell Time (msec)
s2C	260.3	128	−12	−11	−14	5.5	147
t6A	413.1	281	−15	−35	−15	14.5	184
U	245.1	113	−19	−9	−20	6	163
Um	259.1	113	−12	−9	−13	10.5	85

2. This protocol is valid for all RNA species. However, depending on the modification(s) studied, it is preferable to adjust the quantity of RNA to be digested. Some modifications are poorly represented, such as mcm5s2U, present only at position U34 of tRNAs. If you want to study it, it is preferable to extract the tRNAs upstream or, failing that, to digest 400 ng of total RNA instead.
3. N6,2′-O-dimethyladenosine is a modification found on mRNAs at the second base on the 5′ side of the Cap. However, P1 nuclease is unable to digest the phosphodiester bond between base 1 and base 2 of the mRNA. To study this modification, we used a phosphodiesterase (Phosphodiesterase I from *Crotalus adamanteus venom*, Sigma-Aldrich). We do not use this enzyme routinely because it has a higher molecular weight than P1 nuclease or alkaline phosphatase and accumulates in the chromatographic column and decrease the life time of the column.
4. Negative QC is prepared by digesting a similar volume of ULC-MS water. Negative QC was used to monitor the contamination of nucleosides by the enzymes. Several suppliers for P1 nuclease and alkaline phosphatase were evaluated and Sigma-Aldrich presented the least contaminated enzymes on the evaluation test. The areas of the negative QC analysis correspond to the basal level.

Acknowledgments

Mass spectrometry experiments were carried out using the facilities of the Montpellier Proteomics Platform (PPM, BioCampus Montpellier), a member of the national Proteomics French

Infrastructure (ProFI UAR 2048) supported by the French National Research Agency (ANR-24-INBS-0015, Investments for the future F2030).

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Jonkhout N, Tran J, Smith MA et al (2017) The RNA modification landscape in human disease. RNA 23:1754–1769. https://doi.org/10.1261/rna.063503.117
2. Barbieri I, Kouzarides T (2020) Role of RNA modifications in cancer. Nat Rev Cancer 20:303–322. https://doi.org/10.1038/s41568-020-0253-2
3. Shafik AM, Allen EG, Jin P (2022) Epitranscriptomic dynamics in brain development and disease. Mol Psychiatry 27:3633–3646. https://doi.org/10.1038/s41380-022-01570-2
4. Amalric A, Attina A, Bastide A et al (2024) Mass spectrometry-based pipeline for identifying RNA modifications involved in a functional process: application to cancer cell adaptation. Anal Chem 96:1825–1833. https://doi.org/10.1021/acs.analchem.3c02635
5. Murakami S, Jaffrey SR (2022) Hidden codes in mRNA: control of gene expression by m6A. Mol Cell 82:2236–2251. https://doi.org/10.1016/j.molcel.2022.05.029
6. Macari F, El-houfi Y, Boldina G et al (2016) TRM6/61 connects PKCα with translational control through tRNAiMet stabilization: impact on tumorigenesis. Oncogene 35:1785–1796. https://doi.org/10.1038/onc.2015.244
7. Rodell R, Robalin N, Martinez NM (2024) Why U matters: detection and functions of pseudouridine modifications in mRNAs. Trends Biochem Sci 49:12–27. https://doi.org/10.1016/j.tibs.2023.10.008
8. Jin H, Chen X (2024) Conserved Pseudouridines in helix 69 of the ribosome are important for ribosome dynamics in translation. 2024.02.02.578723
9. Relier S, Ripoll J, Guillorit H et al (2021) FTO-mediated cytoplasmic m6Am demethylation adjusts stem-like properties in colorectal cancer cell. Nat Commun 12:1716. https://doi.org/10.1038/s41467-021-21758-4
10. Amalric A, Bastide A, Attina A et al (2022) Quantifying RNA modifications by mass spectrometry: a novel source of biomarkers in oncology. Crit Rev Clin Lab Sci 59:1–18. https://doi.org/10.1080/10408363.2021.1958743
11. Zhang Y, Lu L, Li X (2022) Detection technologies for RNA modifications. Exp Mol Med 54:1601–1616. https://doi.org/10.1038/s12276-022-00821-0
12. Vignon M, Bastide A, Attina A et al (2023) Multiplexed LC-MS/MS quantification of salivary RNA modifications in periodontitis. J Periodontal Res 58:959–967. https://doi.org/10.1111/jre.13155
13. Relier S, Amalric A, Attina A et al (2022) Multivariate analysis of RNA chemistry marks uncovers Epitranscriptomics-based biomarker signature for adult diffuse glioma diagnostics. Anal Chem 94:11967–11972. https://doi.org/10.1021/acs.analchem.2c01526

Part IV

RNA Surveillance: Nonsense-Mediated mRNA Decay (NMD)



Chapter 15

Inhibition of Nonsense-Mediated Decay in Dorsal Root Ganglion Neurons

Maddy R. Koch, Jenna B. Demeter, Rebecca Chase, Nikesh P. Kunder, Tzu-Fang Lou, Tarjani N. Shukla, and June Bryan I. de la Peña

Abstract

Given the unique morphology of neurons with extensive projections, such as nociceptors, mRNA regulation is crucial for their physiological processes and function. Tools for manipulating post-transcriptional and translational regulatory pathways in nociceptors are essential to study the role of these pathways in pain. Nonsense-mediated decay (NMD) is a key quality-control and regulatory mechanism in which, canonically, mRNA with premature termination codons are targeted and degraded to protect the cell against aberrant truncated proteins. Inhibition of NMD has been shown to lead to nociceptive sensitization in dorsal root ganglion (DRG) sensory neurons, suggesting the relevance of NMD to pain chronification. Here we describe a method to inhibit NMD in cultured murine DRG neurons and analyze NMD-sensitive transcripts.

Key words 11j, SMG1, Actinomycin D (ActD), Post-transcriptional regulation, Chronic pain, Nociception, RNA-seq

1 Introduction

Dorsal root ganglia (DRGs) are clusters of neuronal cell bodies in the peripheral nervous system (PNS) located adjacent to the spine. Neurons in DRGs are responsible for relaying sensory signals to the central nervous system (CNS). During pain sensation, sensory neurons known as nociceptors relay information from the periphery to the spinal cord and onward to the brain. These nociceptors are subject to precise mRNA regulation [1–3]. mRNA regulation in nociception and neuronal plasticity is pervasive [4–6]. In this

Authors Maddy R. Koch and Jenna B. Demeter have equally contributed to this chapter.

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_15,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

chapter, we focus on an important mRNA quality-control and regulatory pathway known as nonsense-mediated decay (NMD).

The canonical role of NMD is mRNA surveillance and degradation. It involves the recognition of transcripts containing premature termination codons (PTCs), which, left to be translated, would result in the accumulation of aberrant truncated proteins in the cell. During splicing, as introns are excised from a transcript and exons are joined, the exon junction complex (EJC) is formed. For a "normal" transcript, the translating ribosome will move along the mRNA and displace these EJCs until it reaches a stop codon followed by a 3′ untranslated region (3′-UTR). However, for a PTC-containing transcript, the ribosome will reach a stop codon while EJCs remain downstream. This atypical phenomenon is thought to be the trigger for NMD [7, 8].

Upon activation of NMD, regulator of nonsense transcripts 1 (UPF1) and serine/threonine-protein kinase SMG1 are recruited to the ribosome. SMG1, UPF1, and eukaryotic peptide chain release factor subunit 1 (eRF1) and 3 (eRF3) collectively comprise the SURF complex [9]. SURF complex formation leads to the recruitment of UPF2 and UPF3B, which form a "bridge" between the SURF complex and the EJC [9, 10]. Together, these complexes form the decay-inducing (DECID) complex, which triggers phosphorylation of UPF1 by SMG1. The phosphorylation and activation of UPF1 is the rate-limiting step of NMD [11, 12]. Once this step is complete, the ribosome dissociates, the helicase UPF1 begins to unwind any mRNA secondary structure, and nucleases are recruited to cleave and degrade the transcript [10, 13].

In addition to specific degradation of PTC-containing transcripts, NMD serves as a universal regulation pathway that acts on about 20% of all mRNAs [14–16]. The method by which NMD regulates transcripts without PTCs is a major area of ongoing research. Multiple hypotheses have been presented regarding the susceptibility of transcripts to NMD, most of which implicate characteristics that increase the distance between the stop codon and the poly(A) tail. For example, it has been proposed that factors such as a high degree of secondary structure in the 3′-UTR, a long 3′-UTR, or the presence of upstream open reading frames (uORFs) make a transcript sensitive to NMD [16].

Due to its wide-reaching role in mRNA regulation, NMD is likely integral to maintaining RNA homeostasis in cells requiring tight translational control. It is known that key NMD targets and machinery are present in DRG neurons. This presents a unique opportunity to probe the role of mRNA regulation via NMD in the context of neuronal plasticity and pain sensation. To study the regulation of mRNA stability by NMD, we can take advantage of the rate-limiting step of NMD, wherein SMG1 phosphorylates UPF1 [8, 12, 17, 18], which then induces mRNA decay mechanisms [10]. Treatment of cells with 11j, a small molecule inhibitor of

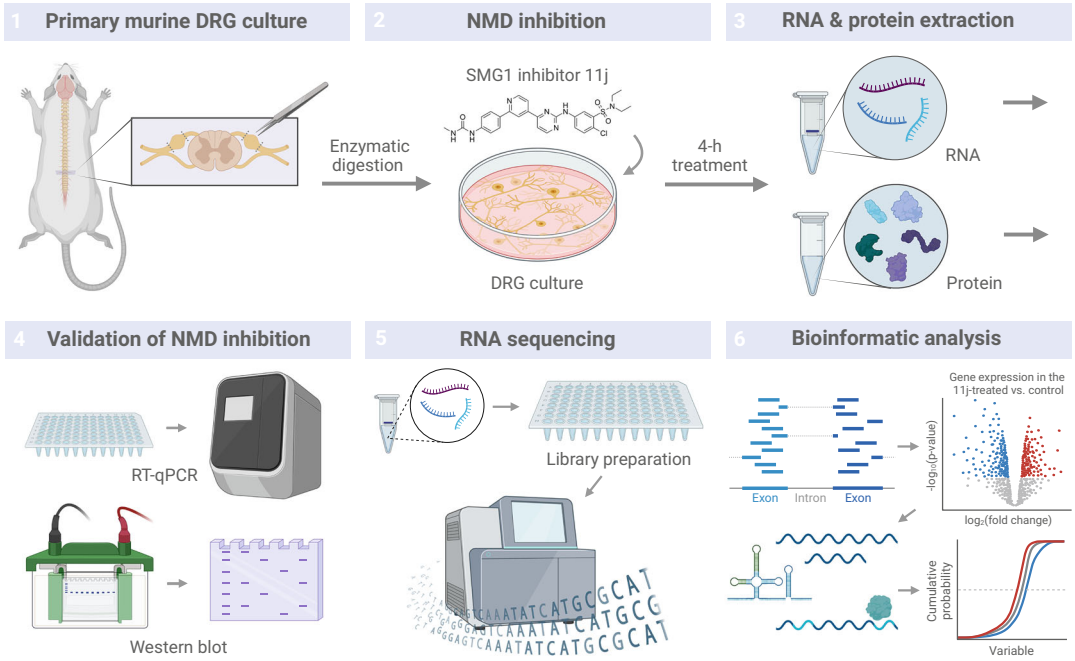


Fig. 1 Summary of the experimental pipeline for inhibiting NMD in primary murine DRG neurons and assessing NMD-sensitive transcripts. Key steps include primary murine DRG culture, NMD inhibition using the SMG1 inhibitor 11j, RNA and protein extraction, validation of NMD inhibition via RT-qPCR and western blot, library preparation and RNA sequencing, and finally bioinformatic analysis of NMD-targeted transcripts. (Created with BioRender.com.)

SMG1 [19], prevents SMG1 from phosphorylating UPF1 and thus inhibits NMD [18]. Among various molecular and phenotypic effects that can be examined in NMD-inhibited, 11j-treated cells, differentially expressed genes are a key outcome that suggests direct or indirect mRNA targets of NMD. These targets can then be funneled into a variety of downstream analyses, including feature characterization of NMD-regulated transcripts.

In this chapter, we present a method to selectively inhibit NMD and thoroughly assess affected mRNA targets. Specifically, we detail methods to (1) dissect and culture primary DRG neurons from mice as described by Smith et al. [20], (2) use the small molecule 11j to selectively inhibit NMD, (3) validate NMD inhibition via western blotting and RT-qPCR, and (4) globally evaluate NMD-sensitive transcripts via RNA sequencing (RNA-seq) and bioinformatic analysis (Fig. 1).

2 Materials

2.1 Primary Murine DRG Culture

1. Wild-type male Swiss Webster mice for in vitro primary DRG cultures (3–4 weeks old) (*see* **Notes 1** and **2**).

2. 6-well tissue culture (TC)-treated plate for protein experiments (western blot) and/or 100-mm TC-treated plate for RNA experiments (RT-qPCR, RNA-seq).
3. 70-μm cell strainer.
4. Paper towels.
5. Absorbent pad.
6. Conical tubes: 15 mL, 50 mL.
7. Ice bucket.
8. Plastic dropper.
9. 5% Isoflurane.
10. Anesthesia induction chamber.
11. Sterile distilled water.
12. 1× Phosphate-buffered saline (PBS).
13. Hanks' Balanced Salt Solution (HBSS).
14. Dissection tools: fine-tip forceps, small dissection scissors, microdissection spring scissors, and decapitation scissors.
15. 70% Ethanol spray.
16. Light source (e.g., gooseneck lamp).
17. Dissection microscope/jeweler's magnifying glasses.
18. Cell culture hood.
19. 0.1 mg/mL Poly-D-lysine (PDL) solution.
20. Filter-sterilized enzymatic solution A: 1 mg/mL collagenase A in HBSS.
21. Filter-sterilized enzymatic solution D: 1 mg/mL collagenase D and 30 U/mL papain in HBSS.
22. Filter-sterilized solution T: 1 mg/mL trypsin inhibitor and 1 mg/mL bovine serum albumin (BSA) in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with GlutaMAX.
23. DRG culture media: DMEM/F-12 with GlutaMAX, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 5 ng/mL nerve growth factor (NGF), 3 μg/mL 5-fluorouridine, and 7 μg/mL uridine.
24. Water bath at 37 °C.
25. Centrifuge.
26. Cell culture incubator (37 °C, 5% CO_2, 90–95% humidity).
27. Inverted microscope.

2.2 NMD Inhibition and Validation

1. Dimethyl sulfoxide (DMSO).
2. Human SMG1 inhibitor, 11j (Chemieliva Pharmaceutical) (*see* **Note 3**). Dissolve 11j in DMSO to make a stock solution of 2 mg/mL (3.533 mM). 11j stock solutions can be stored at −20 °C for short-term storage or −80 °C for long-term storage.
3. Actinomycin D (ActD; Millipore Sigma). Dissolve ActD in DMSO to make a stock solution of 1 mg/mL (796.546 μM). ActD stock solutions can be stored at −20 °C for short-term storage or −80 °C for long-term storage.
4. Radioimmunoprecipitation assay (RIPA) buffer: 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris–HCl (pH 8.0).
5. Pierce Protease Inhibitor Tablets (Fisher Scientific).
6. Pierce Phosphatase Inhibitor Mini Tablets (Fisher Scientific).
7. Sterile cell scrapers.
8. Pierce BCA Protein Assay (Fisher Scientific).
9. 4× Laemmli Sample Buffer (Bio-Rad).
10. 10% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad).
11. Immobilon-P PVDF Membrane (Millipore Sigma).
12. 100% Methanol.
13. Wash buffer: 1× Tris-buffered saline (TBS), 0.05% Tween 20 (TBST).
14. Blocking solution: 5% non-fat dry milk in TBST.
15. Immobilon ECL Ultra Western HRP Substrate (Millipore Sigma).
16. ChemiDoc Imaging System and Image Lab Software (Bio-Rad).
17. Restore PLUS Western Blot Stripping Buffer (Fisher Scientific).
18. GeneJET RNA Purification Kit (Fisher Scientific).
19. ImProm-II Reverse Transcription System (Promega).
20. iQ SYBR Green Supermix (Bio-Rad).

2.3 Antibodies

1. ATF-4 (1:1,000; Cell Signaling Technology).
2. eIF2α (1:1,000; Cell Signaling Technology).
3. p-eIF2α S51 (1:1,000; Cell Signaling Technology).
4. UPF1 (1:2,000; Proteintech).
5. p-UPF1 S1127 (1:1,000; Millipore Sigma).
6. GAPDH (1:10,000; Cell Signaling Technology).

2.4 Primers

1. *Atf4*:
   F: CCTAGGTCTCTTAGATGACTATCTGGAGG
   R: CCAGGTCATCCATTCGAAACAGAGCATCG
2. *Arc*:
   F: AAGTGCCGAGCTGAGATGC
   R: CGACCTGTGCAACCCTTTC
3. *Gadd45b*:
   F: CAACGCGGTTCAGAAGATGC
   R: GGTCCACATTCATCAGTTTGGC
4. *Srp72*:
   F: AGATATTCACACCCTAGCCCAA
   R: AGAGACATGCTATCCGATGAGG
5. *Gapdh*:
   F: TGGAGAAACCTGCCAAGTATGA
   R: GGGATAGGGCCTCTCTTGCT

2.5 RNA-Seq

1. Polysome lysis buffer: 20 mM Tris–HCl (pH 7.5), 250 mM NaCl, 15 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5% Triton X-100, 2.5 U/mL DNase I, 40 U/mL RNasin Ribonuclease Inhibitor (Promega).
2. 22–25-Gauge needle.
3. RNA Clean & Concentrator-5 kit (Zymo Research).
4. Nuclease-free water.
5. NanoDrop Spectrophotometer (Fisher Scientific).
6. RiboCop rRNA Depletion Kit HMR (Lexogen).
7. QuantSeq 3′ mRNA-Seq Library Prep FWD with UDI and REV Kits (Lexogen).
8. qPCR machine and PCR Add-on and Reamplification Kit for Illumina (Lexogen).
9. Qubit Fluorometer (Invitrogen).
10. Fragment Analyzer System and HS NGS Fragment Kit (Agilent).
11. NextSeq 500 System and NextSeq 500/550 Kit (Illumina).
12. Bioinformatic analysis software and tools (*see* Table 1).

3 Methods

3.1 Primary Murine DRG Culture

3.1.1 Cell Culture Plate Preparation

1. Perform all steps in the cell culture hood. Prepare sufficient plates, materials, and cultured DRG for the treatment conditions of each desired assays.
2. Dilute stock PDL with sterile distilled water 1:1000 to a working concentration of 0.1 μg/mL.

Table 1
Bioinformatic analysis

Software/package/tool	Reference website(s) with download options, vignette, manual, etc.	Reference
FastQC	www.bioinformatics.babraham.ac.uk/projects/fastqc/	[28]
BBDuk	github.com/BioInfoTools/BBMap/blob/master/sh/bbduk.sh archive.jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/bbduk-guide/	[29]
TopHat	ccb.jhu.edu/software/tophat/index.shtml	[30]
Bowtie	bowtie-bio.sourceforge.net/index.shtml	[31]
Cufflinks	cole-trapnell-lab.github.io/cufflinks/	[32]
R, RStudio	docs.posit.co/ide/user/; rstudio.github.io/r-manuals/	[33, 34]
biomaRt	bioconductor.org/packages/release/bioc/html/biomaRt.html	[35, 36]
ViennaRNA package (containing RNAfold)	www.tbi.univie.ac.at/RNA/; github.com/ViennaRNA/ViennaRNA	[37]
Party package (containing the random forest algorithm)	cran.r-project.org/web/packages/party/index.html	[25, 26]

3. Cover plates with PDL working solution. For each well in a 6-well plate, 1–2 mL is enough. For a 100-mm plate, 8–10 mL is enough.
4. Incubate at 4 °C overnight or 37 °C for at least 2 h before plating the cells.
5. Wash once with sterile distilled water and allow plate to completely dry before plating the cells.

3.1.2 Dissection and DRG Tissue Isolation

1. Fill a 15-mL conical tube with HBSS as a collection tube for harvested DRG tissues, suitable for tissues from up to 4 mice. Place the tube and 1× PBS on ice.
2. Set up the dissection room with a light source, absorbent pads, paper towels, dissection tools, a dissection microscope, and an anesthesia induction chamber. Follow aseptic technique.
3. Harvest and pool DRGs from 4 mice for each 6-well plate or 100-mm plate as follows.
4. Anesthetize the mouse with 5% isoflurane. Once under deep anesthesia, sacrifice via decapitation. Decapitate the mouse as close to the base of the skull as possible to preserve cervical DRGs.

5. With the help of gravity, allow some time (1–2 min) to exsanguinate the animal after decapitation (*see* **Note 4**).
6. Place the mouse on its ventral side and generously spray the fur with 70% ethanol (*see* **Note 5**).
7. Remove the dorsal skin with fine scissors to visualize the vertebral column.
8. Isolate the vertebral column by cutting between the scapulae and upper rib (rostral), below the lumbosacral joint (caudal), and along both sides of the spine (lateral).
9. Wash the exposed vertebral column with ice-cold 1× PBS to remove hair.
10. Hemisect (cut in half) the vertebral column by locating the opening from the lumbar side and cutting along the midline toward the cervical side (*see* **Note 6**).
11. Once hemisected, you will notice the spinal cord, which is a soft white tissue. Remove the spinal cord as well as the dura, which is a thin membrane underneath the spinal cord (*see* **Note 7**).
12. DRGs are located in regions between 2 vertebrae on either side of the vertebral column. Harvest them with fine tip forceps and place them in the collection tube with ice-cold HBSS. In total, you can get 30–31 pairs of DRGs from a single mouse.
13. Repeat for 4 mice total then immediately proceed to the next section.

3.1.3 Primary Neuron Isolation and Culture

1. Spin down the HBSS collection tube containing DRG tissue at 400 × *g* for 2–5 min (*see* **Note 8**).
2. Carefully aspirate HBSS in a cell culture hood and resuspend tissue in 1 mL enzymatic solution A (*see* **Note 9**).
3. Incubate in a 37 °C water bath for 25 min. Gently flick/tap the tube halfway through the incubation.
4. Spin down the tube containing tissue at 400 × *g* for 2–5 min.
5. Carefully remove enzymatic solution A in a cell culture hood and resuspend tissue in 1 mL enzymatic solution D.
6. Incubate the tube in the water bath for 20 min. Gently flick/tap the tube halfway through the incubation.
7. Spin down the tube containing tissue at 400 × *g* for 2–5 min.
8. Carefully remove enzymatic solution D in a cell culture hood and resuspend the tissue in 1 mL solution T.
9. Gently triturate the tissue in solution T using a P1000 pipette until it is homogenous (*see* **Note 10**).
10. Aspirate the suspension and pass it through a 70-μm cell strainer into a 50-mL conical tube.

11. Add 10–20 mL warm media through the strainer to maximize the passage of DRG cells through the filter.
12. Centrifuge at 400 × *g* for 5 min.
13. Remove media and resuspend in an appropriate volume of DRG culture media. For DRG from 4 mice, resuspend in 12 mL media. Triturate gently until a uniform cell suspension is achieved. Add 2 mL cell suspension to each well of a 6-well plate or 12 mL to a 100-mm plate. This will yield approximately 1,500,000 cells in total (250,000 cells/well), which will yield 50–80 μg total protein or 2–4 μg total RNA.
14. Incubate the cells in a cell culture incubator (37 °C, 5% CO_2, 90–95% humidity).
15. 24 h later, perform a full media change to remove floating debris and dead cells. Add 3 mL DRG culture media to each well of the 6-well plate or 16 mL media to the 100-mm plate.
16. Change media every other day.

3.2 NMD Inhibition

1. Allow DRG cultures to grow and stabilize for 5–7 days in vitro (DIV) before treatment (*see* **Note 11**).
2. On the day of treatment, usually at 6 DIV, dilute 2 mg/mL (3.533 mM) 11j stock in DRG culture media to a final concentration of 1 μM (Fig. 2a).
3. Add an equal volume DMSO to DRG culture media for the vehicle (control) group.
4. For a 6-well plate for protein experiments or a 100-mm plate for RNA-seq, aspirate old media and immediately replace with media containing 11j (1 μM) or vehicle (DMSO).
5. For a 100-mm plate for RT-qPCR, use the transcriptional inhibitor ActD to assay the stability of NMD-targeted transcripts following treatment with 11j as follows: (a) on the day of treatment, dilute 1 mg/mL ActD stock in DRG culture media (1 μM 11j or vehicle) to a final concentration of 5 μg/mL, (b) aspirate old media and immediately replace with media containing 5 μg/mL ActD with either 1 μM 11j or vehicle (DMSO).
6. Incubate cells at 37 °C for 4 h.
7. After 4 h, remove media and wash wells with ice-cold 1× PBS.
8. Proceed with downstream protein or RNA extraction.

3.3 Validation of NMD Inhibition

3.3.1 Protein Extraction and Western Blot

1. After the 4-h 11j treatment and washing of a 6-well plate, add 150–300 μL ice-cold RIPA buffer supplemented with Pierce protease and phosphatase inhibitors into each well of the 6-well plate (*see* **Note 12**).
2. Gently harvest cells using sterile cell scrapers.

3. Transfer the cell lysate to a 1.5-mL tube on ice.
4. Centrifuge samples for 20 min at 18,000 × *g* at 4 °C to pellet cell debris.
5. Transfer the supernatant to a new 1.5-mL tube.
6. Measure protein concentration using the Pierce BCA Protein Assay.
7. Prepare 20 μg protein per well-loading volume in a final concentration of 1× Laemmli Sample Buffer.
8. Incubate for 10 min at 98 °C to denature proteins.
9. Load equal amounts of samples (20 μg/well) to a 10% Mini-PROTEAN TGX Precast Protein Gel.
10. Run for 15 min at 60 V. Increase the voltage to 100 V to finish the run for an additional 1 h.
11. Transfer the gel to a methanol-activated Immobilon-P PVDF Membrane. Transfer at 100 V for 2 h.
12. Block the membrane with blocking solution for 2 h at room temperature.
13. Incubate the membrane overnight at 4 °C with primary antibodies diluted in blocking solution.
14. Wash the membrane 3 times with TBST.
15. Incubate the membrane with secondary antibodies diluted in blocking solution for 1 h at room temperature.
16. Wash the membrane 3 times with TBST.
17. Incubate the membrane with Immobilon ECL Ultra Western HRP Substrate for 1 min before visualizing with a ChemiDoc Imaging System.
18. Strip the membrane with Restore PLUS Western Blot Stripping Buffer for 10 min.
19. Re-incubate the membrane with additional primary and secondary antibodies as outlined above.
20. Perform image analysis using Image Lab Software.
21. Normalize phosphorylated proteins to their respective total proteins (Fig. 2b).

3.3.2 RNA Extraction and RT-qPCR

1. After the 4-h ActD + 11j treatment and PBS wash of a 100-mm plate, extract RNA using the GeneJET RNA Purification Kit according to the manufacturer's protocol. If not immediately proceeding with reverse transcription, purified RNA should be stored at −80 °C.
2. Reverse transcribe 1 μg RNA to cDNA using the ImProm-II Reverse Transcription System according to the manufacturer's protocol (*see* **Notes 13** and **14**).

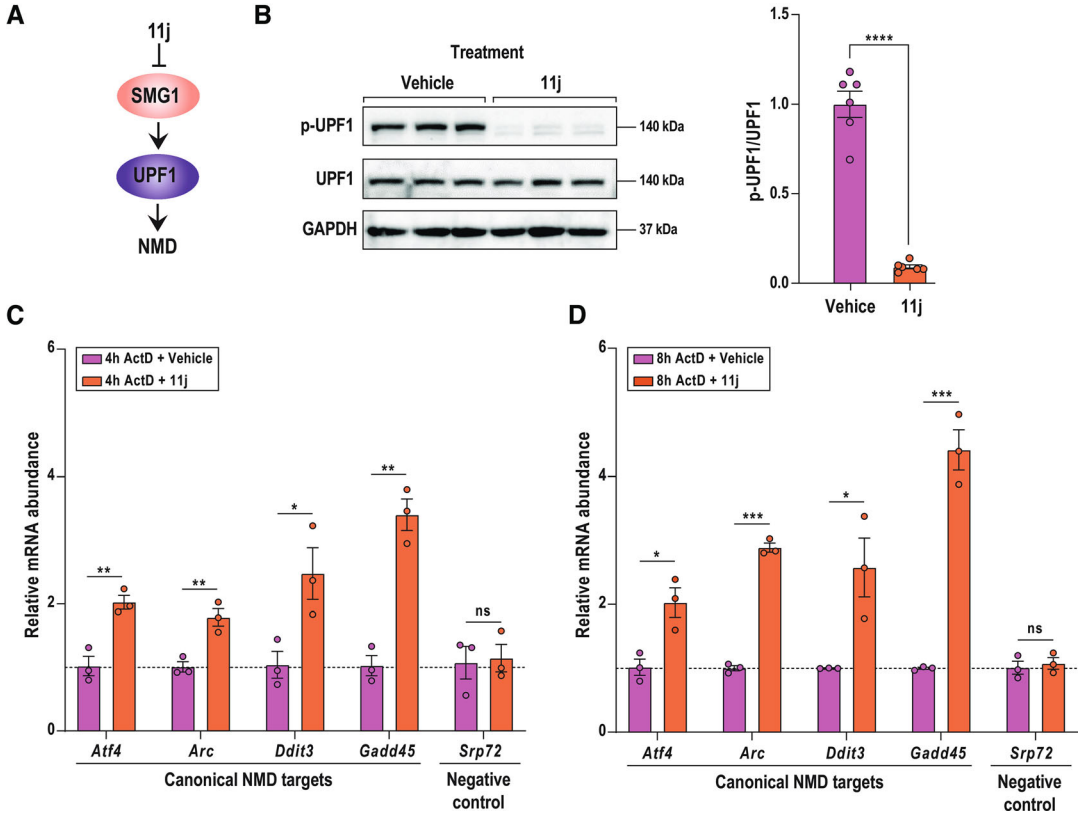


Fig. 2 Example validation of SMG1/NMD inhibition by 11j (from de la Peña et al. [18]). (**A**) A schematic representation of 11j's action on SMG1 kinase activity and its downstream phosphorylation of UPF1. (**B**) (Left) Application of 11j to primary cultured DRG neurons decreased phosphorylated UPF1 (p-UPF1) levels but not total UPF1 or GAPDH. (Right) Quantification showing decreased p-UPF1/UPF1 levels in 11j-treated neurons; $n = 6$ cultures per treatment group. ****$p < 0.0001$ (two-tailed unpaired t-test). (**C** and **D**) qPCR quantification of mRNA levels reveal that 11j treatment stabilized the expression of canonical NMD targets (*Atf4*, *Arc*, *Ddit3*, *Gadd45*) 4 h (**C**) and 8 h (**D**) after transcriptional shutdown by ActD whereas levels of the non-NMD target *Srp72* were unchanged. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; compared with relative vehicle (control) group (multiple unpaired t-test). Data are mean ± SEM; n.s. indicates that the comparison is not significant

3. Perform qPCR using iQ SYBR Green Supermix for several NMD targets. We used *Atf4*, *Arc*, and *Gadd45b* (canonical NMD targets); *Srp72* (negative control); and *Gapdh* (housekeeping gene). Primer sequences for these genes can be found in the materials section.
4. Calculate the fold change in expression for each gene using the ΔΔCt method, where Ct is the threshold cycle. We used *Gapdh* to normalize gene expression (Fig. 2c, d).

3.4 RNA-Seq

3.4.1 RNA Extraction and rRNA Depletion

1. After the 4-h 11j treatment and PBS wash of a 100-mm plate, add 150–200 μL ice-cold polysome lysis buffer to each 100-mm culture dish and scrape the cells extensively with a pipette tip.
2. Use a 22–25-gauge needle to pipette the lysate once to ensure complete lysis.
3. Transfer the lysate to a 1.5-mL tube and keep it on ice for 20 min. Flick/tap the tube periodically for better mixing.
4. Centrifuge the lysate at 20,000 × *g* for 15 min at 4 °C and collect the supernatant.
5. Purify 100 μL RNA extract with the RNA Clean & Concentrator-5 kit. Follow the kit protocol for > 17 nucleotide RNA. Elute in 15 μL nuclease-free water.
6. Use 1 μL of the eluted sample to quantify RNA concentration with a NanoDrop Spectrophotometer.
7. Follow the RiboCop rRNA Depletion Kit protocol to remove rRNA from the total RNA sample. Elute in 10 μL nuclease-free water.

3.4.2 Library Preparation and Sequencing

1. Use 1 μg RNA input to generate libraries using QuantSeq 3′ mRNA-Seq Library Prep FWD with UDI and REV Kits, following the manufacturer's protocol.
2. The number of endpoint cycles needed for the library amplification step is determined by qPCR assay using the PCR Add-on and Reamplification Kit for Illumina. After purification of the double-stranded cDNA library, dilute the 17 μL library with 17 μL elution buffer. Use half of the diluted library for qPCR assay. The exact cycle number is chosen when the fluorescence value for each sample reaches 50% of its maximum.
3. Quantify the concentrations of purified libraries using a Qubit Fluorometer.
4. Pool the respective libraries.
5. Determine the average size of the libraries by fragment analysis with the HS NGS Fragment Kit.
6. Use NextSeq 500/550 Kit reagents for sequencing on a NextSeq 500 System.

3.5 Bioinformatic Analysis

While there are many possible approaches and tools for each step in the bioinformatic analysis of NMD-sensitive transcripts, here we outline the methods we used in our previous work as an example [18] (*see* **Note 15**). However, as bioinformatic analysis tools are constantly evolving, we recommend that you utilize current and recommended approaches, tools, and reference genomes. Additionally, your specific experimental design and available resources

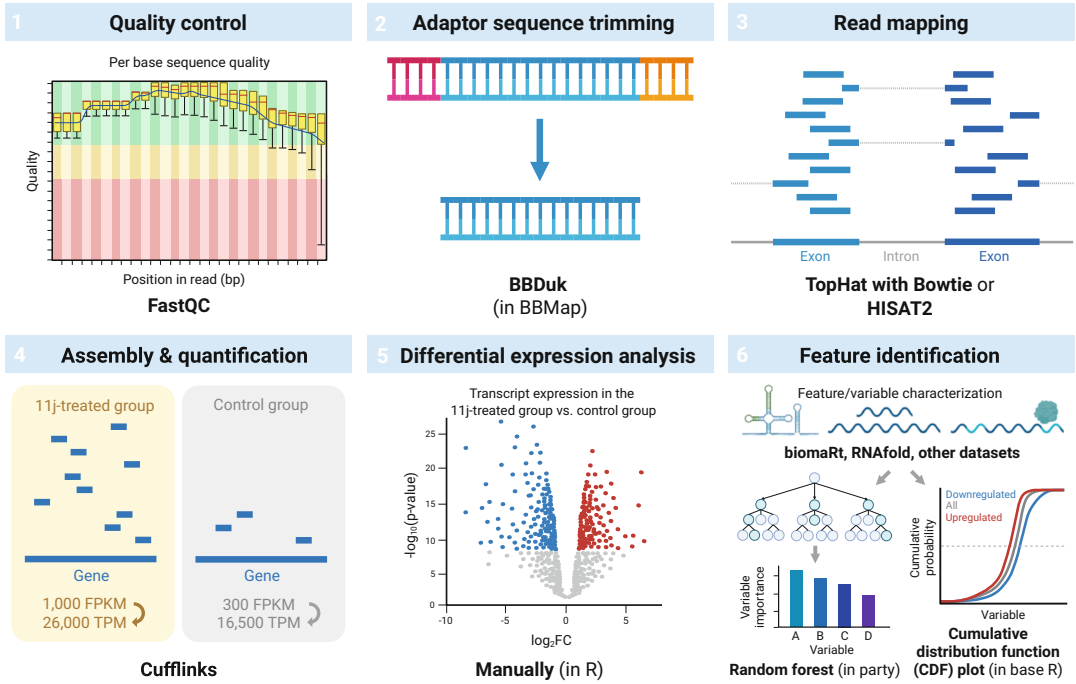


Fig. 3 Summary of the bioinformatic analysis pipeline of NMD-regulated transcript expression and features, as used by the authors (de la Peña et al. [18]). Key steps include quality control, adaptor sequence trimming, read mapping, transcript assembly and quantification, differential expression analysis, and feature identification of NMD-sensitive transcripts. (Created with BioRender.com.)

should inform your analytic approach. As an example, we used the following core pipeline: (a) FastQC for quality control, (b) BBDuk for adaptor sequence trimming, (c) Bowtie and TopHat for read mapping, (d) Cufflinks for assembly and quantification, and (e) manual differential expression analysis (Table 1; Fig. 3). See respective vignettes and manuals for each tool for more detailed instructions on use and interpretation of results.

3.5.1 Quality Control and Adaptor Sequence Trimming

Quality control is a critical early step in the RNA-seq analysis pipeline. If there are errors or abnormalities in the sequencing, then downstream mapping will be unsuccessful. Assessing data quality early prevents erroneous results and wasted resources.

FastQC will identify whether adaptor sequences are present in the reads. If so, it is important to remove them to prevent inaccurate mapping (*see* **Step 3**).

1. Transfer FASTQ files from the Illumina platform to a Linux machine.
2. Use FastQC to perform quality assessment of data. FastQC will generate a report outlining your sequences' basic statistics, per base sequence quality, per tile sequence quality, per sequence quality, per base sequence content, and GC content, among

others. The program will provide summary graphs and highlight problematic areas, if any.

3. If adaptors are present, use BBDuk to trim the adaptor sequences from the reads. If the exact adaptor sequences are known, use BBDuk's built-in tools to find and trim these adaptor sequences. In addition to adaptor trimming, sequences may be trimmed according to base quality or filtered according to length.

3.5.2 Read Mapping

The most computationally intensive step of RNA-seq analysis involves read mapping, in which the short sequencing reads are aligned to a reference genome and/or transcriptome.

1. We used TopHat with Bowtie due to its ability to deal with short reads and coordinate with downstream differential expression analysis (*see* **Note 16**).
2. It is important to use the "transcriptome-index" option (or your software's equivalent) to perform mapping to the transcriptome.
3. After mapping, it is important to evaluate the performance of the mapping. A quick and easy way to do this is to look in the output files for the run statistics, which will show the number of reads mapped compared to the total number of input reads. A low read mapping rate may indicate the presence of upstream issues.

3.5.3 Read Assembly and Quantification

1. To assemble transcripts and quantify the abundance of aligned RNA-seq reads, we used Cufflinks. For our purposes, a key option was the "no-length-correction" flag. As recommended for 3′ end sequencing experiments, this prevents the software from performing length normalization for each count. Cufflinks outputs normalized abundance as fragments per kilobase of transcript per million mapped reads (FPKM) for each mapped transcript.
2. To compare data between samples more confidently, convert FPKM values of each transcript i to transcripts per million (TPM) using the following formula:

$$\mathrm{TPM}_i = \frac{\mathrm{FPKM}_i}{\sum \mathrm{FPKM}} \times 10^6$$

3.5.4 Differential Expression Analysis

1. Differential expression analysis may be carried out manually or using sophisticated tools such as DESeq2, edgeR, or limma. Appropriate approaches will partially depend on your type of input count data.

2. Perform differential expression analysis manually using RStudio with the following steps: (a) remove genes with a 0 TPM value in all samples, (b) average the TPM for each transcript in each condition, and (c) divide the average abundance in the treatment group by the average abundance in the control group. This yields the fold change (FC), which is often reported in $\log_2$ space, (d) using the individual sample data for each condition, perform statistical testing (usually t-tests) to confirm the differences in abundance (TPM) between the samples, and (e) since t-tests are repeated thousands of times, it is recommended to perform multiple testing correction. Use the Benjamini-Hochberg or a comparable method to obtain adjusted p-values.
3. Different thresholds may be used to discern differentially expressed genes (DEGs) in the NMD-inhibited (11j-treated) group relative to the control (vehicle-treated) group. For example, we defined upregulated genes as having an adjusted p-value < 0.05 and a FC > 1.5 ($\log_2$FC > 0.58) and downregulated genes as having an adjusted p-value < 0.05 and a FC < 0.66 ($\log_2$FC < -0.58).

3.5.5 Feature Identification of NMD Targets

To determine characteristics of NMD-sensitive transcripts in murine DRG, perform further downstream analyses on the genes present in the sequencing data, with a particular focus on the DEGs in the NMD-inhibited cultures. Use RStudio for all further data wrangling, statistical analysis, and informatics plot creation unless otherwise stated.

1. Gathering transcript features: (a) make use of many publicly available datasets to identify characteristics of each transcript in your sequencing data, (b) gather information including biotype, transcript length, untranslated regions (UTR) sequences, and CDS sequences using biomaRt, (c) calculate axonal localization scores using, for example, a sub-cellular transcriptomic study of murine sensory neurons [21], (d) identify and count the uORFs present within each transcript by evaluating multiple existing profiling datasets [22–24], and (e) predict minimum free energy (MFE) associated with RNA secondary structure for both coding sequences (CDSs) and UTRs using the RNAfold program, which is found within the ViennaRNA Package. This tool has both a web version and a command line version. Given the large number of sequences to evaluate, use the command line version for efficiency and the "noPS" and "noDP" flags to prevent individual structure drawing and dot-plot files from being generated, greatly reducing the program's runtime.

2. Cumulative distribution function (CDF) plots: (a) to identify differences in features between upregulated or downregulated transcripts vs. all transcripts, generate CDF plots in base R. In short, CDF plots contain upward-trending lines showing the percent of each dataset that is preset at or below each value on the x-axis. It allows for visualization of more discrete differences in the data that cannot be as easily seen with a histogram. (b) To test whether the upregulated or downregulated transcript distributions on a CDF plot are different from the distribution representing all genes, use the Kolmogorov-Smirnov (KS) test.
3. Random forest: To better understand the mechanism of NMD-mediated mRNA regulation, identify features most strongly associated with differential transcript expression in NMD-inhibited DRG culture using a supervised machine learning method known as the random forest via the party package [25, 26]. The random forest model combines the outcomes of many different decision trees, allowing for simple, efficient, and accurate classification of data. While a more detailed outline of the random forest model is out of the scope of this project, many resources exist to explain its core concepts [27] (*see* **Note 17**).

4 Notes

1. In our experience, younger mice (3–4 weeks) yield greater culture efficiency than older mice.
2. Ensure that all protocols regarding laboratory safety and animal use are in place and adhere with local institutional guidelines.
3. 11j is now commercially available and can be bought from a variety of manufacturers (e.g., medchemexpress.com/hsmg-1-inhibitor-11j.html).
4. Exsanguination makes downstream dissection easier as you can visualize anatomic features properly with minimal blood.
5. Generously spray the fur with ethanol. Mouse fur can carry fungal and bacterial spores that can lead to contamination.
6. It is critical to cut along the midline to avoid cutting DRG tissues.
7. There needs to be a balance between sensory neurons and fibroblasts. To minimize the amount of fibroblasts, avoid taking any dura during dissection and, if possible, trim the axons for most DRGs.

8. Optimize centrifugation to ensure that all DRG tissues have pelleted at the bottom of the tube without being too forceful and damaging cells.
9. While aspirating liquid, be careful not to take the tissue. As a tip, tilt the tube so that the liquid comes closer to the Pasteur pipette tip and increases its distance from the tissue.
10. Make sure you are very gentle with trituration to minimize damage to the cells, which will affect viability.
11. Decide on a treatment schedule based on the maturation of the culture. A DRG culture ready for treatment is well-adhered to the plate with extensive neurite outgrowth.
12. RIPA buffer volume is flexible based on the density of cells in the culture.
13. Make a master mix for both the RT and qPCR steps as it helps minimize pipetting errors.
14. Ensure that your pipettes are well-calibrated to minimize errors for precise RNA experiments.
15. An important first step to performing bioinformatic analysis of RNA-seq data is to choose a pipeline suited to the data (Table 1; Fig. 3). Factors that will impact this choice include, for example, experimental methodology, type of sequencing performed, and computational environment. Important considerations for our analyses included the use of murine DRG cultures, 3′ end sequencing, and a Linux environment. To make an informed decision regarding which programs to use, we suggest performing a literature review relevant to your experiment.
16. TopHat has now largely been replaced by HISAT2, which performs the same function as TopHat but does so in a more efficient and efficacious manner.
17. Other machine learning algorithms may be used, depending on the overall structure of the data and the hypothesis being tested. However, for the simple purpose of feature/variable selection, the random forest model is appropriate.

Acknowledgments

We thank Dr. Zachary Campbell and the authors of the source publication titled “Inhibition of Nonsense-Mediated Decay Induces Nociceptive Sensitization through Activation of the Integrated Stress Response” for their support [18].

DoD Disclaimer (TNS) The views expressed are those of the authors and do not reflect the official views or policy of the Department of Defense or its Components.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Alvarez FJ, Fyffe RE (2000) Nociceptors for the 21st century. Curr Rev Pain 4:451–458. https://doi.org/10.1007/s11916-000-0069-4
2. Price TJ, Géranton SM (2009) Translating nociceptor sensitivity: the role of axonal protein synthesis in nociceptor physiology. Eur J Neurosci 29:2253–2263. https://doi.org/10.1111/j.1460-9568.2009.06786.x
3. Khoutorsky A, Bonin RP, Sorge RE et al (2015) Translational control of nociception via 4E-binding protein 1. eLife 4:e12002. https://doi.org/10.7554/eLife.12002
4. Wang H, Tiedge H (2004) Translational control at the synapse. Neurosci Rev J Bringing Neurobiol Neurol Psychiatry 10:456–466. https://doi.org/10.1177/1073858404265866
5. Costa-Mattioli M, Sossin WS, Klann E, Sonenberg N (2009) Translational control of long-lasting synaptic plasticity and memory. Neuron 61:10–26. https://doi.org/10.1016/j.neuron.2008.10.055
6. de la Peña JBI, Song JJ, Campbell ZT (2019) RNA control in pain: blame it on the messenger. Wiley Interdiscip Rev RNA 10:e1546. https://doi.org/10.1002/wrna.1546
7. Le Hir H, Gatfield D, Izaurralde E, Moore MJ (2001) The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. EMBO J 20:4987–4997. https://doi.org/10.1093/emboj/20.17.4987
8. Lejeune F, Maquat LE (2005) Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells. Curr Opin Cell Biol 17:309–315. https://doi.org/10.1016/j.ceb.2005.03.002
9. Kashima I, Yamashita A, Izumi N et al (2006) Binding of a novel SMG-1-Upf1-eRF1-eRF3 complex (SURF) to the exon junction complex triggers Upf1 phosphorylation and nonsense-mediated mRNA decay. Genes Dev 20:355–367. https://doi.org/10.1101/gad.1389006
10. Lejeune F, Li X, Maquat LE (2003) Nonsense-mediated mRNA decay in mammalian cells involves decapping, deadenylating, and exonucleolytic activities. Mol Cell 12:675–687. https://doi.org/10.1016/s1097-2765(03)00349-6
11. Sun X, Perlick HA, Dietz HC, Maquat LE (1998) A mutated human homologue to yeast Upf1 protein has a dominant-negative effect on the decay of nonsense-containing mRNAs in mammalian cells. Proc Natl Acad Sci USA 95:10009–10014. https://doi.org/10.1073/pnas.95.17.10009
12. Yamashita A, Ohnishi T, Kashima I et al (2001) Human SMG-1, a novel phosphatidylinositol 3-kinase-related protein kinase, associates with components of the mRNA surveillance complex and is involved in the regulation of nonsense-mediated mRNA decay. Genes Dev 15:2215–2228. https://doi.org/10.1101/gad.913001
13. Lloyd JPB (2018) The evolution and diversity of the nonsense-mediated mRNA decay pathway. F1000Research 7:1299. https://doi.org/10.12688/f1000research.15872.2
14. Lee LJ, Papadopoli D, Jewer M et al (2021) Cancer plasticity: the role of mRNA translation. Trends Cancer 7:134–145. https://doi.org/10.1016/j.trecan.2020.09.005
15. Nasif S, Contu L, Mühlemann O (2018) Beyond quality control: the role of nonsense-mediated mRNA decay (NMD) in regulating gene expression. Semin Cell Dev Biol 75:78–87. https://doi.org/10.1016/j.semcdb.2017.08.053
16. Nickless A, Bailis JM, You Z (2017) Control of gene expression through the nonsense-mediated RNA decay pathway. Cell Biosci 7:26. https://doi.org/10.1186/s13578-017-0153-7
17. Conti E, Izaurralde E (2005) Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. Curr Opin Cell Biol 17:316–325. https://doi.org/10.1016/j.ceb.2005.04.005

18. de la Peña JB, Chase R, Kunder N et al (2023) Inhibition of nonsense-mediated decay induces nociceptive sensitization through activation of the integrated stress response. J Neurosci 43: 2921–2933. https://doi.org/10.1523/JNEUROSCI.1604-22.2023
19. Gopalsamy A, Bennett EM, Shi M et al (2012) Identification of pyrimidine derivatives as hSMG-1 inhibitors. Bioorg Med Chem Lett 22:6636–6641. https://doi.org/10.1016/j.bmcl.2012.08.107
20. Smith PR, Meyer A, Loerch S, Campbell ZT (2023) Protocol for the isolation and culture of mouse dorsal root ganglion neurons for imaging applications. STAR Protoc 4:102717. https://doi.org/10.1016/j.xpro.2023.102717
21. Minis A, Dahary D, Manor O et al (2014) Subcellular transcriptomics-dissection of the mRNA composition in the axonal compartment of sensory neurons. Dev Neurobiol 74: 365–381. https://doi.org/10.1002/dneu.22140
22. Ingolia NT, Lareau LF, Weissman JS (2011) Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell 147:789–802. https://doi.org/10.1016/j.cell.2011.10.002
23. Johnstone TG, Bazzini AA, Giraldez AJ (2016) Upstream ORFs are prevalent translational repressors in vertebrates. EMBO J 35:706–723. https://doi.org/10.15252/embj.201592759
24. Barragán-Iglesias P, Kuhn J, Vidal-Cantú GC et al (2019) Activation of the integrated stress response in nociceptors drives methylglyoxal-induced pain. Pain 160:160–171. https://doi.org/10.1097/j.pain.0000000000001387
25. Strobl C, Boulesteix A-L, Zeileis A, Hothorn T (2007) Bias in random forest variable importance measures: illustrations, sources and a solution. BMC Bioinformatics 8:25. https://doi.org/10.1186/1471-2105-8-25
26. Strobl C, Boulesteix A-L, Kneib T et al (2008) Conditional variable importance for random forests. BMC Bioinformatics 9:307. https://doi.org/10.1186/1471-2105-9-307
27. Breiman L (2001) Random forests. Mach Learn 45:5–32. https://doi.org/10.1023/A:1010933404324
28. (2015) FastQC
29. Joint Genome Institute (2021) BBTools
30. Kim D, Pertea G, Trapnell C et al (2013) TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 14:R36. https://doi.org/10.1186/gb-2013-14-4-r36
31. Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25. https://doi.org/10.1186/gb-2009-10-3-r25
32. Trapnell C, Williams BA, Pertea G et al (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28:511–515. https://doi.org/10.1038/nbt.1621
33. (2020) The R Project for Statistical Computing
34. R Studio Team (2020) RStudio: Integrated Development for R
35. Durinck S, Moreau Y, Kasprzyk A et al (2005) BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. Bioinforma Oxf Engl 21:3439–3440. https://doi.org/10.1093/bioinformatics/bti525
36. Durinck S, Spellman PT, Birney E, Huber W (2009) Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. Nat Protoc 4:1184–1191. https://doi.org/10.1038/nprot.2009.97
37. Lorenz R, Bernhart SH, Höner Zu Siederdissen C et al (2011) ViennaRNA Package 2.0. Algorithms Mol Biol 6:26. https://doi.org/10.1186/1748-7188-6-26

Chapter 16

Knockdown of Gene Expression Following Activation of the NMD Pathway Using Splice-Switching Oligonucleotides

Pierre Hirt, Anne Marchalot, Catherine Horiot, Jérôme Moreaux, and Laurent Delpy

Abstract

Antisense oligonucleotides (ASOs) are commonly used to induce transient changes in gene expression and do not result in mutagenic effects at the DNA level. In this chapter, we describe a novel gene silencing approach using splice-switching oligonucleotides (SSOs) capable of hybridizing at a splice site to induce exon skipping. This strategy is based on the elimination of an exon whose nucleotide length is not a multiple of 3. In this way, exon skipping induces a frameshift in the reading frame and the appearance of a premature termination codon (PTC) on the alternative transcript. After detection of the PTC, alternative transcripts are degraded by the NMD pathway. Here, we present the steps involved in the SSO design, cell treatment, and assessment of gene silencing. We provide evidence for efficient knockdown of Enhancer of Zeste Homolog 2 (EZH2) expression in lymphoid cells.

Key words Gene silencing, Knockdown, Antisense oligonucleotide (ASO), Splice-switching oligonucleotide (SSO), Exon skipping, Enhancer of Zeste Homolog 2 (EZH2), Nonsense-mediated mRNA decay (NMD)

1 Introduction

Antisense oligonucleotides (ASOs) are synthetic, chemically modified single-stranded oligonucleotides of DNA/RNA type, typically 15–30 nucleotides (nt) in length. By acting at the RNA level, antisense strategies have a transient effect while avoiding irreversible modifications of the DNA. There are two main types of ASOs: "Gapmer ASOs" harboring a DNA-like structure in their center that leads to the recruitment of RNase H, which cleaves RNA in the DNA heteroduplex, and "Steric blocker ASOs" that are unable to recruit RNase H, yet induce steric hindrance upon hybridization with the RNA target. The latter type of ASO is commonly used to modify RNA splicing, polyadenylation, or translation [1, 2].

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_16,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

ASO-mediated modulation of splicing is widely used to repair defective gene expression and numerous examples illustrate exon skipping or inclusion approaches for therapeutic purposes [1, 2]. These strategies are based on targeting splice sites or other cis-regulatory sequences involved in splicing, referred to as exonic/intronic splicing enhancers or silencers (ESE, ISE, ESS, and ISS). However, splicing modulation is not necessarily intended to repair defective gene expression and, conversely, splice-switching oligonucleotides (SSOs) inducing out-of-frame exon skipping can also be used to knockdown gene expression by activating nonsense-mediated mRNA decay (NMD) [3, 4]. This approach known as forced splicing-dependent NMD (FSD-NMD) was first demonstrated with the inhibition of STAT3 expression using cell lines and xenograft models [3]. FSD-NMD is a very attractive alternative to the RNase H-dependent mechanism for inhibiting gene expression.

The NMD pathway is activated upon recognition of a premature termination codon (PTC) during the first round of mRNA translation. To achieve strong mRNA degradation, the position of the PTC must comply with the "50–55 nt" rule, implying that the nonsense codon is located ≥50–55 nt upstream of the last exon–exon junction [5, 6]. Therefore, the choice of out-of-frame exons targeted by SSOs is critical to achieve FSD-NMD. We have described a complete pipeline for FSD-NMD and developed the web application "ExonSkipper 1.0" to help select which exon and splice site (5′ss or 3′ss) can be targeted by SSO on a gene of interest [4]. ExonSkipper 1.0 also predicts the PTC position after out-of-frame exon skipping and assesses the presence of cryptic splice sites along the target gene to minimize the risk of unwanted alternative splicing. We used this pipeline for multiple FSD-NMD approaches and demonstrated efficient SSO-mediated knockdown for NFκB and PIM family members [4, 7, 8].

Enhancer of Zeste Homolog 2 (EZH2) is the catalytic subunit of the Polycomb repressive complex 2 (PRC2). This histone methyltransferase represses gene expression especially through methylation of histone H3 Lysine 27 (H3K27). It is overexpressed and correlates with poor prognosis in numerous cancers [9, 10]. In an attempt to inhibit EZH2 expression, we describe here the complete methodology including the design of a SSO targeting the 5′ss of *EZH2* exon 9 (Fig. 1), the treatment of lymphoid cell lines (e.g., myeloma or diffuse large B-cell lymphoma—DLBCL—cell lines) with EZH2- and control-SSO, and the analysis of EZH2 expression by RT-PCR (Fig. 2), Sanger sequencing (Fig. 3), and Western blot (Fig. 4).

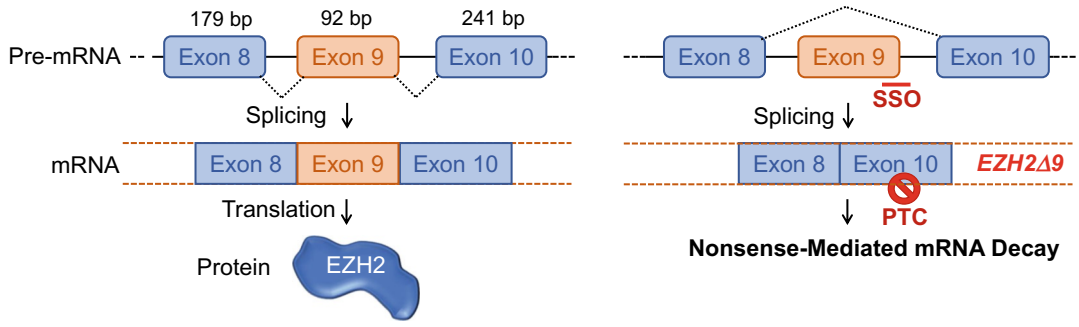


Fig. 1 SSO approach inducing out-of-frame exon skipping to inhibit *EZH2* expression. Schematic representation of exons 8–10 of the *EZH2* (Enhancer of Zeste Homolog 2) transcript, out of a total of 19–20 exons. Normal splicing leading to expression of the EZH2 protein is shown on the left. A splice-switching oligonucleotide (SSO) hybridizing to the donor splice site (5′ss) of *EZH2* exon 9 is shown on the right. Out-of-frame exon skipping results in the appearance of a premature stop codon (PTC) in exon 10 of alternative mRNAs (*EZH2Δ9*). The position of the PTC, located ≥50–55 nt upstream of the last exon–exon junction (EJC), triggers a strong EJC-dependent NMD on *EZH2Δ9* mRNAs

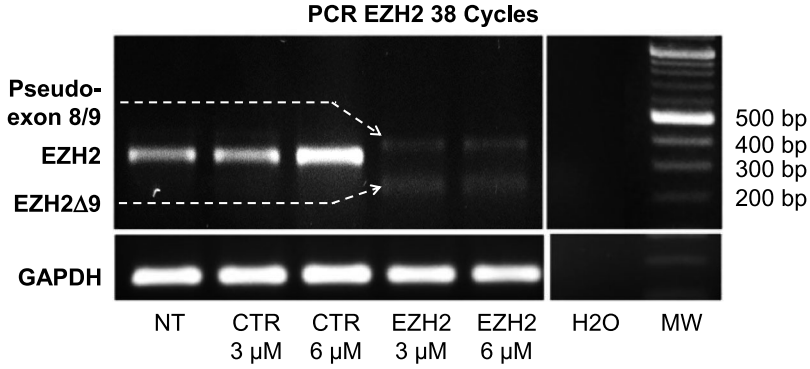


Fig. 2 RT-PCR analysis of *EZH2* expression. U266 cells were either untreated (NT) or treated with a vivo-morpholino SSO targeting EZH2-exon 9-5′ss (EZH2) or an irrelevant vivo-morpholino control SSO (CTR) for 48 h, at the indicated concentrations. RT-PCR analysis of *EZH2* (exon 8 forward and exon 10 reverse primers) and *GAPDH* expression. Normal *EZH2* splicing generates a native band at 353 bp in NT and CTR conditions, whereas two faint bands (dotted lines) appear at 261 bp (*EZH2Δ9*) and 396 bp (pseudo-exon 8/9) in SSO-treated conditions. A high number of PCR cycles is required to detect these weak bands, confirming the strong degradation of the alternative transcripts (*EZH2Δ9* and pseudo-exon 8/9) by NMD. The position of the PTC on these alternative transcripts is depicted in Fig. 3

2 Materials

2.1 Cell Culture, SSO Treatments, and RNA Extraction

1. Cell lines: U266 and OCI-Ly3 as human myeloma and DLBCL cell lines, respectively (*see* **Note 1**).
2. Culture Medium: RPMI 1640 with 10% heat inactivated fetal bovine serum (FBS) (*see* **Note 2**).
3. 24-well cell culture plates.

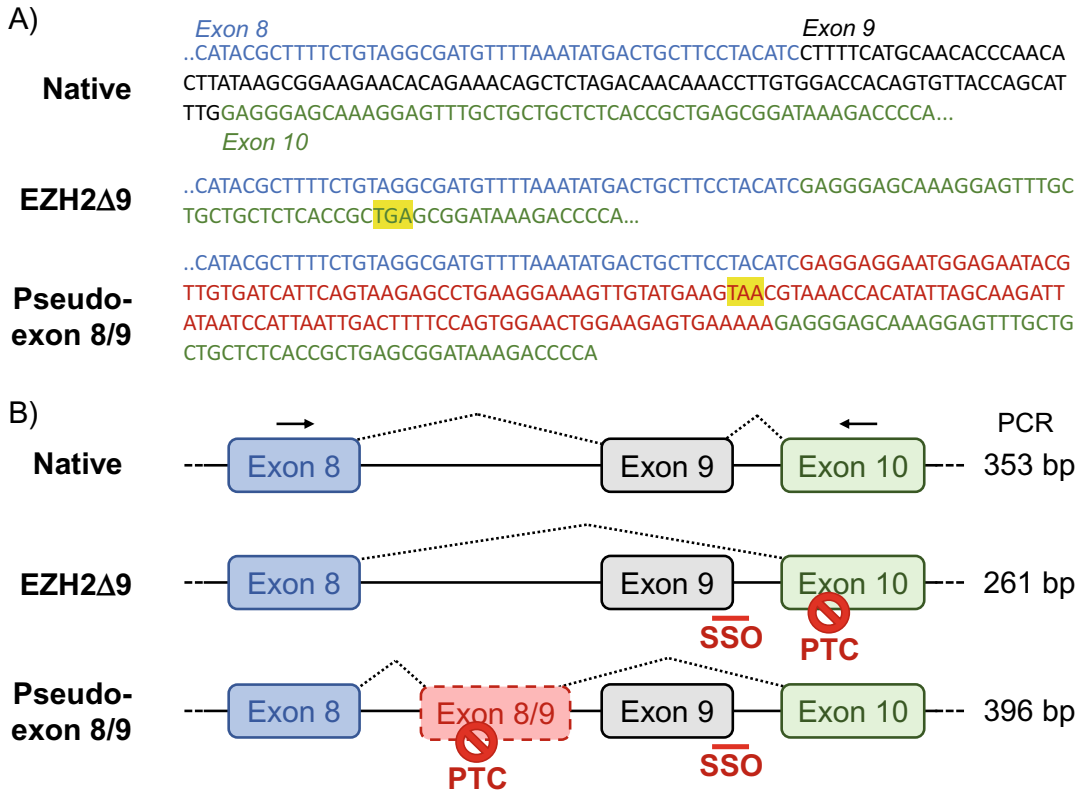


Fig. 3 Sequence analysis of *EZH2* exons 8–10 and identification of the PTC position on alternative transcripts. (**a**) The nucleotide sequence of *EZH2* exons 8 (blue), 9 (black), and 10 (green) and the intron sequence included in the alternative mRNA as pseudo-exon 8/9 (red) are represented. The position of the PTC on alternative mRNAs (EZH2Δ9 and pseudo-exon 8/9) is highlighted (yellow). (**b**) Diagram illustrating the different splicing events occurring on native *EZH2*, *EZH2Δ9* and pseudo-exon 8/9 mRNAs. The position of primers (arrows) used for RT-PCR and the length of the amplification products are also indicated

4. SSO: SSO targeting *EZH2* exon 9 5′ss (5′-GGCACTAAGTCT TACCAAATGCTGG) is purchased as vivo-morpholino (Gene Tools). Resuspend the lyophilized SSO at a concentration of 500 μM with nuclease-free water (*see* **Note 3**). Do not touch the powder with the tip and wait for complete SSO dissolution before pipetting. Store at room temperature protected from light.
5. Control SSO (CTRL): a vivo-morpholino irrelevant SSO (5′-CCTCTTACCTCAGTTACAATTTATA) is purchased (Gene Tools) (*see* **Note 4**). Resuspend the lyophilized SSO at a concentration of 500 μM with nuclease-free water as described in Subheading 2.1, **item 4**. Store at room temperature protected from light.
6. Nuclease-free water.
7. 1× phosphate-buffered saline (PBS).

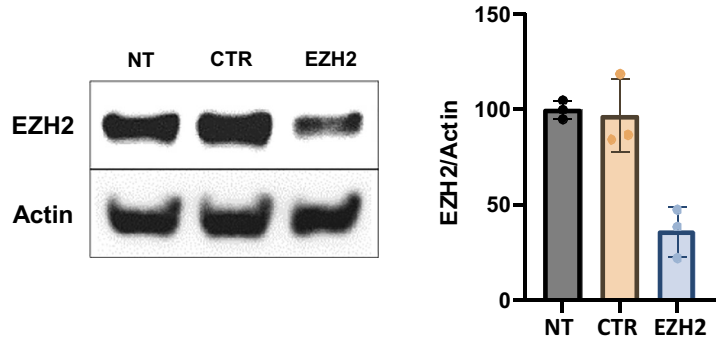


Fig. 4 Western blot analysis of EZH2 expression. OCI-Ly3 cells were either untreated (NT) or treated with 2 μM of a vivo-morpholino SSO targeting EZH2-exon 9-5′ss (EZH2) or an irrelevant vivo-morpholino control SSO (CTR) for 48 h. Representative Western blot image showing the decrease in EZH2 (98 kDa) expression in SSO-treated cells compared to CTR and NT conditions (left). The expression of β-actin (45 kDa) is used for normalization. Bar graph represents a quantitative analysis of EZH2 expression relative to β-actin levels, for three different experiments (right)

8. Parafilm.
9. TRIzol™.
10. Chloroform.
11. Isopropanol.
12. 75% ethanol.
13. 1.5 mL RNase-free tubes.
14. NanoDrop spectrophotometer.
15. Centrifuge and microcentrifuge.

2.2 Reverse Transcription and PCR (RT-PCR)

1. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems): 50 U/μL MultiScribe Reverse Transcriptase, 10× RT Buffer, 10× Random Primers and 25× dNTP Mix (100 mM).
2. 1 U/μL DNase I.
3. 10× DNase I Buffer.
4. EDTA: 25 mM EDTA.
5. RNase-free water.
6. 0.2 mL RNase-free PCR tubes.
7. 10 μM Forward (*EZH2* exon 8 For: 5′-TGCTTCCTACATCG TAAGTGC) and 10 μM Reverse (*EZH2* exon 10 Rev.: 5′-GC TGGGCCTGCTACTGTTAT) primers (*see* **Note 5**).
8. Taq PCR Kit (NEB): 5000 U/mL Taq DNA polymerase, 10× Taq Reaction Buffer and 10 mM dNTP solution mix.
9. Thermocycler.

2.3 Gel Electrophoresis and Isolation of PCR Products

1. Molecular biology grade agarose powder.
2. 6× Orange Dye loading buffer.
3. 1× TBE Buffer: 0.089 M Tris, 0.089 M borate, 0.002 M EDTA, pH 8.3 adjusted with HCl.
4. Midori Green.
5. 100 bp DNA ladder.
6. PCR clean-up Gel extraction kit (Macherey-Nagel): Buffer NTI, Buffer NT3, buffer NE, NucleoSpin columns and collection tubes.
7. Scalpel.
8. Migration chamber and UV lamp.

2.4 Sample Purification and Sanger Sequencing

1. BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), with BigDye Terminator v3.1 Ready Reaction Mix and 5× BigDye Buffer included.
2. Sephadex g-50 superfine.
3. 96-well filtered plate.
4. 96-well sequencing plate.
5. Thermocycler.
6. DNA sequencer.

2.5 Protein Extraction and Quantification

1. Protease Inhibitor Cocktail (PIC).
2. 100 mM sodium orthovanadate.
3. 100 mM PMSF.
4. RIPA lysis buffer: 10 µL of 100 mM sodium orthovanadate, 10 µL of PIC, 10 µL of 100 mM PMSF and 970 µL of nuclease-free water. Freshly made, do not store.
5. Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) with BCA Reagent A, BCA Reagent B and 2 mg/ml BSA.
6. Multi-channel pipette.
7. 96-well plates.
8. Microplate spectrophotometer.

2.6 Western Blot

1. Gel casting, migration support, and Trans-Blot Turbo Transfer System (Bio-Rad).
2. Kit for hand casting 12% TGX stain-free polyacrylamide gels, with Resolver A, Resolver B, Stacker A, and Stacker B solutions (Bio-Rad).
3. 10% ammonium persulfate (APS).
4. Tetramethylethylenediamine (TEMED).
5. Absolute and 70% ethanol.

6. 4× loading dye.
7. β-Mercaptoethanol.
8. 10× TG Buffer: 30 g of Tris, 144 g of glycine, and 1 L of deionized water.
9. TGS Migration Buffer: 100 mL of 10× TG Buffer, 10 mL of 10% SDS, and 890 mL of deionized water.
10. Size marker: unstained protein standard.
11. Polyvinylidene fluoride membrane (PVDF) and extra thick Blot Filter Paper.
12. Transfer buffer: 40 mL of 5× Transfer buffer TURBO, 40 mL of absolute ethanol, and 120 mL of deionized water.
13. 10× TBS Solution: 24.2 g of Tris, 80 g of NaCl, 1 L of deionized water, pH 7.5 adjusted with HCl.
14. Blocking solution: 1× TBS with 5% (w/v) powdered milk.
15. 1× TBST solution: 0.1% (v/v) Tween-20 in 1× TBS.
16. TBST-Milk: 3–5% (w/v) powdered milk in 1× TBST.
17. Shaker or Lab Tube Roller.
18. Antibodies targeting the gene of interest and for normalization: mAb Rabbit IgG anti-EZH2 (clone D2C9, dilution 1:5000, Cell Signaling Technology) and mAb Rabbit IgG anti--β-Actin (clone 13E5, dilution 1/1000, Cell Signaling Technology) as primary antibodies; Goat anti-Rabbit IgG(H + L)-HRP with minimal reactivity to mouse and human proteins (dilution 1:5000, SouthernBiotech) as secondary antibody.
19. Dehybridization solution: 100 mM Glycine, 1% (w/v) SDS, 0.1% (v/v) Nonidet-P40.
20. ECL Chemiluminescent Substrate Reagent Kit with ECL Reagent A and ECL Reagent B.
21. Chemiluminescent imaging system.

3 Methods

3.1 Selection of the Out-of-Frame Exon to Target and SSO Design

1. Using ExonSkipper 1.0, enter gene name and select organism (e.g., human or mouse).
2. Out-of-frame exons (nt length not a multiple of 3) are indicated by an asterisk (*see* **Note 6**). Simulation of out-of-frame exon skipping determines the PTC position on the alternative transcript. Check that the PTC position will achieve strong NMD activation (i.e., PTC located ≥50–55 nt upstream of the last exon–exon junction).
3. ExonSkipper 1.0 uses MaxEntScan for splice site prediction. Check for the absence of strong donor (5′ss) or acceptor (3′ss)

cryptic splice sites in the vicinity of the targeted out-of-frame exon, as this could generate unwanted alternative transcripts and attenuate the desired SSO-mediated exon skipping (*see* **Note 7**).

4. Select a 50-nt sequence encompassing the 5′ss or 3′ss of the targeted out-of-frame exon (e.g., the 5′ss of *EZH2* exon 9). Usually take 25-nt length from the intron and 25-nt length from the exon.
5. Submit this 50-nt sequence to a supplier for the design of a steric blocker SSO. When vivo-morpholino SSO are designed on Gene Tools website, select "Splice Modifying (pre-mRNA target)" and the working temperature from the organism (e.g., 37 °C).
6. Once the SSO sequence has been obtained, it is important to check that it does not hybridize significantly with a transcribed sequence in the organism (use an alignment search tool such as Blast).

3.2 Treatment of Cells by SSO and RNA Extraction

1. In a 24-well plate, add 2 mL of cells (e.g., U266 or OCI-Ly3) at a density of 0.5–1 × 10^6 cells/mL in culture medium and incubate at 37 °C with 5% CO_2 in a humidified atmosphere. Make duplicate (or triplicate) wells per condition: non-treated (NT), control SSO (CTRL) and SSO to be studied (SSO: EZH2-exon9-5'ss SSO). EZH2-exon9–5′ss SSO) condition.
2. Treat the cells with SSO at a final concentration of 0.5–6 μM and incubate for 48 h (*see* **Note 8**).
3. Collect the cells (seeded at 0.5–1 × 10^6 cells/mL) and centrifuge at 300× *g* for 5 min.
4. Wash the cell pellet with 1× PBS and centrifuge at 300× *g* for 5 min.
5. Remove the supernatant and resuspend the pellet in 0.5–1 mL TRIzol. Shake immediately (*see* **Note 9**) and wait for 5 min at room temperature. Then, proceed to the next step, or store samples overnight at 4 °C or at −20 °C for long-term storage (up to one year).
6. Thaw RNA at room temperature (if necessary) and add 0.2 mL of chloroform per mL of TRIzol.
7. Shake vigorously for 2–3 min to mix and incubate at room temperature for 5 min.
8. Centrifuge at 12,000× *g* for 20 min at 4 °C.
9. Carefully transfer the clear upper phase to a new tube (*see* **Notes 10** and **11**).
10. Add 0.5 mL of isopropanol per mL of TRIzol. Vortex well and incubate at 4 °C for 10 min.

11. Centrifuge at 12,000–16,000× *g* for 10 min at 4 °C.
12. Gently remove supernatant using a pipette.
13. Wash the pellet with 1 mL of 75% cold ethanol per mL of TRIzol.
14. Vortex briefly to gently loosen the pellet.
15. Centrifuge at 7500× *g* for 5 min at 4 °C.
16. Carefully remove supernatant and let the RNA pellet to air-dry for 5 min.
17. Add 20–30 μL of RNase-free water and incubate at 55–60 °C for 10 min to dissolve the RNA pellet.
18. Measure RNA concentration and purity using NanoDrop spectrophotometer (*see* **Note 12**).
19. Proceed to reverse transcription (RT) or place RNA samples at −80 °C for long-term storage.

3.3 Reverse Transcription and PCR (RT-PCR)

1. In a 0.2 mL PCR tube, add 0.2–2 μg of RNA and adjust to 10 μL with RNase-free water. Add the same quantity of RNA to all samples for RT (RT+) and control (no reverse transcriptase: RT-) reactions (*see* **Note 13**).
2. Prepare a DNase Mix by adding 1.2 μL of 10× DNase I Buffer and 1 μL of 1 U/μL DNase I per sample.
3. Add 2.2 μL of the DNase Mix to the side of each tube containing RNA, and then briefly spin to mix.
4. Incubate for 15 min at room temperature.
5. Add 2 μL of EDTA to the side of the tube, and then briefly spin and incubate for 10 min at 65 °C.
6. Prepare a RT+ Mix by adding 2 μL of 10× RT Buffer, 2 μL of 10× Random Primers, 0.8 μL of dNTP Mix, and 1 μL of Reverse Transcriptase, per tube.
7. For the RT+ reaction, add 5.8 μL of RT+ Mix in each tube containing DNase-treated RNA samples (*see* Subheading 3.3, **step 5**).
8. Prepare a RT- Mix by adding 2 μL of 10x RT Buffer, 2 μL of 10× Random Primers, 0.8 μL of dNTP Mix, and 1 μL of RNase-free water, per tube.
9. For the RT- reaction, add 5.8 μL of RT- Mix in each tube containing DNase-treated RNA samples (*see* Subheading 3.3, **step 5**).
10. Place the tubes in a thermocycler and start the following program: 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C.

    Stop point: store cDNAs at 4 °C for a few days or at −20 °C for long-term storage.

11. Prepare a PCR reaction mixture by adding 1–2 µL of cDNA (or RNase-free water for the negative control), 2 µL of 10× Taq Reaction Buffer, 0.4 µL of 10 mM dNTP mix, 2 µL of 10 µM Forward primer, 2 µL of 10 µM Reverse primer, 0.2 µL of Taq DNA polymerase, and adjust to 20 µL with RNase-free water.
12. Place the PCR tubes in a thermocycler and run the following program: 94 °C for 2 min, 38 cycles (94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s), 70 °C for 10 min, and cool to 15 °C (*see* **Note 14**).

3.4 Gel Electrophoresis and Isolation of PCR Products

1. Prepare a 2% (w/v) agarose gel in 1× TBE supplemented with 2 µL of Midori Green per 100 mL of gel (*see* **Note 15**).
2. Add 4 µL of 6× Orange Dye loading buffer to the PCR products and load 20 µL of samples on the agarose gel.
3. Load 5 µL of 100 bp DNA ladder into one well to determine the size of PCR products.
4. Run gel electrophoresis at 120 V for ~45 min.
5. Visualize the amplification products under UV light (Fig. 2).
6. Use protective equipment against UV and finely cut PCR products from the gel using a scalpel (*see* **Note 16**).
7. Weigh the gel slice and transfer it to a 1.5 mL tube.
8. Add 200 µL of Buffer NTI per 100 mg of gel and incubate for 30–60 min at 50 °C. Vortex regularly for complete dissolution of agarose gel.
9. Transfer up to 700 µL of mixture containing the PCR product into a NucleoSpin column and place it in a collection tube (repeat this step in the same tube if your volume exceeds 700 µL).
10. Centrifuge for 30 s at 11,000× *g*. Then, empty the collection tube and replace the NucleoSpin column on it.
11. Add 700 µL of Buffer NT3 and centrifuge for 30 s at 11,000× *g*. Empty the collection tube and repeat this step.
12. Centrifuge for 1 min at 11,000× *g* to dry the silica membrane.
13. Place the NucleoSpin column in a new 1.5 mL tube and elute DNA by adding 20 µL of Buffer NE or nuclease-free water. Wait for 1 min and centrifuge for 1 min at 11,000× *g*.
14. Quantify eluted PCR products using a NanoDrop spectrophotometer.

    Stop point: store de purified PCR products at −20 °C until the next step.

3.5 Characterization of Alternative Transcripts by Sanger Sequencing

For further validation of SSO-mediated exon skipping use Sanger sequencing. This sequencing is also used to characterize the large alternative transcript in our case (Fig. 2). For each band to be sequenced, two PCR reactions are performed: one reaction using the "forward" primer and a second one with the "reverse" primer.

1. Prepare on ice a 1 μM solution by diluting forward and reverse PCR primers (*see* Subheading 2.2, **item 7**) with nuclease-free water.
2. For Sanger sequencing of purified PCR products, add 2.5 μL of 1 μM primer solution (forward or reverse), ~5 ng of the purified PCR product (*see* Subheading 3.4, **step 14**), 1.5 μL of BigDye Terminator v3.1 Ready Reaction Mix, 3 μL of 5× BigDye Buffer, and nuclease-free water up to 15 μL, per reaction (*see* **Note 17**).
3. Place the tubes in a thermocycler and run the following sequencing program: 96 °C for 1 min, 25 cycles (96 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min), 15 °C for 10 min.
4. For purification of sequencing reactions, distribute Sephadex powder into the wells of a 96-well filter plate.
5. Add 350 μL of nuclease-free water into each well and incubate for 3 h at room temperature or overnight at 4 °C.
6. Position a 96-well recovery plate beneath the filter plate and centrifuge at 1500× *g* for 7 min.
7. Discard the contents of the recovery plate.
8. Add 15 μL of sample (*see* Subheading 3.5, **step 3**) in each well of the filter plate.
9. Place another 96-well plate (sequencing plate) beneath the filter plate and centrifuge at 1500× *g* for 7 min.
10. Samples are sequenced on a DNA sequencer.
11. Analyze the sequence of alternative transcripts and check for the presence of a PTC, as good NMD substrates (Fig. 3).

3.6 Protein Extraction and Quantification

All steps for protein extraction are performed on ice.

1. Collect 0.5–1 × 10^6 cells and centrifuge 300× *g* for 5 min at 4 °C.
2. Wash the cell pellet with 1× PBS and centrifuge at 300× *g* for 5 min at 4 °C.
3. Prepare fresh RIPA lysis buffer, add 30 μL to the cell pellet, and vortex vigorously.
4. Incubate for 30 min, vortexing regularly.
5. Centrifuge at 10,000× *g* for 20 min at 4 °C.

6. Transfer supernatant containing proteins into a new tube and proceed to protein quantification using BCA Protein Assay Kit (*see* **Note 18**).
   Stop point: store protein extracts at −20 °C.
7. Reconstitute the colorimetric reagent from the BCA Protein Assay kit: 5 mL of reagent A and 100 µL of reagent B per one 96-well plate.
8. In the first row (A), add 97.5 µL of colorimetric reagent, and fill the wells of the subsequent rows with 50 µL of reagent. Use two columns for the standard curve and other columns according to the number of samples.
9. Add 50 µL of 1× PBS in two wells for blank measurements.
10. Add 2.5 µL of 2 mg/mL BSA to wells A1 and A2 for the standard curve, and 2.5 µL of protein samples (*see* Subheading 3.6, **step 6**) to the following wells in row A.
11. Pipette 50 µL in row A using a multi-channel pipette and make serial 1:2 dilutions (rows B–G).
12. Incubate for 30 min at 37 °C.
13. Measure absorbance at 562 nm using a microplate spectrophotometer and determine the protein concentration in each sample by reference to the standard curve.

3.7 Assessment of Protein Amounts by Western Blot

Western blot analysis is an essential technique for the identification and quantification of specific proteins. Here, it is used to assess the decrease in the protein targeted by the SSO-mediated knockdown approach (*see* **Note 19**). In addition, it could be a good way to control that there are no additional bands of lower molecular weight originating from an alternative transcript that would not be degraded by NMD (Fig. 4).

1. For electrophoresis, assemble the casting system and ensure tightness by placing little ethanol between the glass plates, and then remove the ethanol by flipping.
2. Prepare the migration gel by mixing 3.5 mL of Resolver A, 3.5 mL of Resolver B, 60 µL of 10% APS, and 6 µL of TEMED.
3. Pour the migration gel and cover it with one centimeter of 70% ethanol.
4. Prepare the stacking gel by combining 1.5 mL of Stacker A, 1.5 mL of Stacker B, 30 µL of 10% APS, and 6 µL of TEMED.
5. Pour the stacking gel until it reaches the top of the assembly and keep excess solution as a polymerize control.
6. Insert the comb and let it polymerize for 15–20 min. Then, gently remove the comb and place the assembly in your migration tank.

7. Prepare for each sample: 10–50 μg of protein extracts, 3 μL of 4× loading dye, 2.5% of β-mercaptoethanol, and RIPA lysis buffer up to 12 μL.
8. Heat the tubes at 95 °C for 5 min. Then, centrifuge the samples for a few seconds.
9. Add TGS Migration Buffer in the migration system until the gel is completely covered.
10. Load the samples into the migration gel. Also add 5 μL of size marker to a separate well.
11. Migrate at 120 V for ~45 min.
12. Activate a PVDF membrane in absolute ethanol for 10 min.
13. Rinse the gel in Transfer buffer.
14. Prepare a transfer cassette with stack of Transfer buffer-soaked Blot Filter Papers.
15. Transfer the proteins onto the activated PVDF membrane using Trans-Blot Turbo Transfer System with the following settings: 1.3 A, 25 V, 10 min.
16. After transfer, incubate the PVDF membrane in blocking solution for 1 h at room temperature under agitation, or overnight at 4 °C (*see* **Notes 20** and **21**).
17. Rinse the membrane in TBST.
18. Prepare the primary antibody solution in TBST-Milk (dilute according to the antibody datasheet).
19. Incubate the membrane for 1 h at room temperature (or overnight at 4 °C) under agitation.
20. Incubate the membrane for 5 min in TBST and repeat this step for two further washes.
21. If the primary antibody is coupled to HRP (Horseradish peroxidase), proceed directly to detection (*see* Subheading 3.7, **steps 23–26**), otherwise prepare the secondary antibody solution in TBST-Milk (usually diluted to 1/5000) and incubate the membrane for 1 h at room temperature under agitation.
22. Incubate the membrane for 5 min in TBST and repeat this step for two further washes.
23. Incubate the membrane for 5 min in 1× TBS before detection.
24. Prepare ECL Chemiluminescent Substrate: add 500 μL of ECL Reagent A and 500 μL of ECL Reagent B for one membrane.
25. Drain the membrane and place it on a plastic sheet. Then, distribute the ECL substrate onto the membrane.
26. Proceed to signal acquisition using a Chemiluminescent imaging system (Fig. 4).

27. If the detection of other proteins (e.g., housekeeping) requires membrane dehybridization (*see* **Note 22**), incubate the membrane in the dehybridization solution for 5–15 min, depending on the intensity of the first signal.
28. Perform a quick rinse in TBST to remove dehybridization solution and stop the reaction.
29. Incubate the membrane for 5 min in TBST and repeat this step for two further washes. Check that the membrane is properly dehybridized (*see* **Note 23**).
30. Repeat **steps 16–26** in Subheading 3.7 for the detection of other proteins.

4 Notes

1. U266 and OCI-Ly3 cells express significant levels of EZH2 but other cell lines or primary cells can be used.
2. It is important to note that the use of a culture medium with a high percentage of FBS should be avoided, as the proteins contained within FBS have the capacity to quench the SSO and thereby reduce its efficacy. For cell cultures requiring a high percentage of FBS, higher SSO concentrations may be required.
3. Vivo-morpholino ASOs (GeneTools) are phosphorodiamidate morpholino oligomer coupled with an octaguanidine dendrimer. These molecules are supplied as lyophilized powder in glass vessel with a metal seal on top. For ease of use, we recommend removing this seal and wrapping the lid with parafilm to prevent evaporation.
4. Controls other than the irrelevant SSO shown here can be used. It is possible to order a "scramble" control SSO which has a sequence of randomized nt compared to the SSO to be studied. Another control referred to as "sense SSO"in the opposite orientation as the SSO to be studied. It is essential to ensure that the control SSO cannot hybridize significantly with a transcribed genomic sequence, as this is a prerequisite for the SSO to be studied (*see* Subheading 3.1, **step 6**).
5. Choose PCR primers surrounding the exon of interest in order to detect simultaneously full-length and alternatively spliced mRNAs by RT-PCR.
6. Pay attention to exon number, as ExonSkipper 1.0 begins numbering from the first coding exon and does not account for non-coding exons in the 5′ untranslated region (5′ UTR).

7. This splice site prediction step could be optional because the use of cryptic splice site is often hard to predict and must be tested empirically.
8. Pre-treatment with SSO control is recommended to determine the appropriate concentration range (maximum concentration with limited cytotoxicity) for each cell line (*see* also Chap. 13). SSO treatments are generally performed during 24–72 h. For instance, primary B cells are usually treated with 2 μM SSO for 48 h.
9. Vortex or pipette up and down vigorously immediately to lyse the pellet as clumping and difficulty in resuspension may occur if this action is delayed.
10. The clear upper aqueous phase contains RNA and should be gently collected by tilting the tube at a 45-degree angle. Avoid withdrawing too quickly or too much to prevent contamination from the red phenol/chloroform layer or DNA at the interface.
11. (Optional) Add 5–10 μg of nuclease-free glycogen as a visual aid to see RNA pellets. This also serves as a carrier, helping precipitation of low RNA amounts without interfering with downstream applications.
12. A 260 nm/280 nm absorbance ratio >1.8 (ideally ~2) and a 260/230 ratio >1.8 are expected for RNA samples, indicating minimal protein or organic solvent contaminations.
13. Perform negative controls (RT-) to ensure that there is no contamination by genomic DNA.
14. In order to visualize alternative transcripts that are highly degraded by NMD and to check their sequence, we recommend using a high number of PCR cycles (38 cycles in this case).
15. A 2% agarose gel is recommended for good separation of small PCR products.
16. This step should be done quickly to reduce DNA damage caused by UV exposure.
17. Thaw BigDye Reagents at the last minute. Do not refreeze, but they can be stored at 4 °C for up to a month.
18. The BCA method relies on two successive chemical reactions: (1) the peptide bonds of the proteins reduce Cu^{2+} ions to Cu^{+}, and (2) the Cu^{+} ions react with bicinchoninic acid to form a colored complex. This complex has a maximum absorbance at 562 nm, enabling precise quantification.
19. SSO-mediated knockdown can also be assessed at the RNA level by RT-qPCR using primers that do not include the

targeted exon in order to analyze full-length and alternative (exon skipping) mRNAs in the same way.

20. Always use 1× TBS for the Western blot analysis of phosphorylated proteins. Do not use 1× PBS because phosphate ions can induce high background signal, decrease detection specificity, and potentially participate in protein dephosphorylation reactions.
21. For overnight incubation, it is preferable to use lab tube roller to limit volume loss due to evaporation.
22. Membrane dehybridization is necessary when different proteins with similar molecular weights need to be detected or when the first signal is too diffuse and may interfere with further detection.
23. To control membrane dehybridization, prepare ECL Chemiluminescent Substrate and repeat **steps 24–26** in Subheading 3.7. No signal should be detected when the membrane is correctly dehybridized.

Acknowledgments

This work was supported by grants from INCa (PLBIO2022-110, INCA_16734), ANR (2017-CE15-0024- 01), Ligue Contre le Cancer (comités Corrèze, Haute-Vienne), Fondation Française pour la Recherche contre le Myélome et les Gammapathies monoclonales (FFRMG), and Région Nouvelle-Aquitaine (AAP ESR 2020 and 2024).

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Bennett CF (2019) Therapeutic antisense oligonucleotides are coming of age. Annu Rev Med 70:307–321. https://doi.org/10.1146/annurev-med-041217-010829
2. Roberts TC, Langer R, Wood MJA (2020) Advances in oligonucleotide drug delivery. Nat Rev Drug Discov 19:673–694. https://doi.org/10.1038/s41573-020-0075-7
3. Zammarchi F, de Stanchina E, Bournazou E, Supakorndej T, Martires K, Riedel E, Corben AD, Bromberg JF, Cartegni L (2011) Antitumorigenic potential of STAT3 alternative splicing modulation. Proc Natl Acad Sci USA 108:17779–17784. https://doi.org/10.1073/pnas.1108482108
4. Marchalot A, Lambert J-M, Boyer F, Pollet J, Moreau J, Feuillard J, Faumont N, Delpy L (2020) Splice switching oligonucleotide mediated gene knockdown in B cells and plasma cells. 2020.09.18.302984
5. Lejeune F, Maquat LE (2005) Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells. Curr Opin Cell Biol 17:309–315. https://doi.org/10.1016/j.ceb.2005.03.002
6. Carrard J, Lejeune F (2023) Nonsense-mediated mRNA decay, a simplified view of a complex mechanism. BMB Rep 56:625–632. https://doi.org/10.5483/BMBRep.2023-0190

7. Haas M, Cherfa S, Nguyen L, Bourgoin M, Caron G, Dessauge E, Marchand T, Delpy L, Auberger P, Moreaux J, Jacquel A, Fest T (2025) PIM2 inhibition promotes MCL1 dependency in plasma cells involving integrated stress response-driven NOXA expression. Nat Commun 16:256. https://doi.org/10.1038/s41467-024-55572-5
8. Haas M, Caron G, Chatonnet F, Manenti S, Alaterre E, Devin J, Delaloy C, Bertolin G, Viel R, Pignarre A, Llamas-Gutierrez F, Marchalot A, Decaux O, Tarte K, Delpy L, Moreaux J, Fest T (2022) PIM2 kinase has a pivotal role in plasmablast generation and plasma cell survival, opening up novel treatment options in myeloma. Blood 139:2316–2337. https://doi.org/10.1182/blood.2021014011
9. Simon JA, Lange CA (2008) Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutat Res Fundam Mol Mech Mutagenesis 647:21–29. https://doi.org/10.1016/j.mrfmmm.2008.07.010
10. Liu Y, Yang Q (2023) The roles of EZH2 in cancer and its inhibitors. Med Oncol 40:167. https://doi.org/10.1007/s12032-023-02025-6



Chapter 17

Screening Methods for NMD Inhibitors or Readthrough Activators

Selma Nedjma and Fabrice Lejeune

Abstract

Nonsense mutations cause approximately 10% of genetic disease cases. Certain molecules can rescue the expression of genes carrying a nonsense mutation, either by activating readthrough of the premature termination codon or by inhibiting the mRNA surveillance mechanism called nonsense-mediated mRNA decay (NMD). In the first approach, a full-length protein will be synthesized, differing by at most one amino acid from the wild-type protein. In the second approach, a truncated protein will be synthesized, which sometimes retains partially or completely the function of the wild-type protein. Here we describe methods for identifying, by screening, molecules having the capacity either to inhibit NMD or to activate readthrough.

Key words Screening method, Nonsense-mediated mRNA decay (NMD), Readthrough, Small molecules

1 Introduction

Gene expression requires several steps, including transcription, maturation of the pre-messenger RNA into an mRNA, export of the latter to the cytoplasm, translation of the mRNA to a protein, maturation of the protein, and finally mRNA and protein degradation. All these steps require quality control, i.e., mechanisms controlling their proper execution [1]. These mechanisms play an extremely important role, as they guarantee the identity and integrity of the gene and of the message it carries.

Among the quality control mechanisms, nonsense-mediated mRNA decay (NMD) is responsible for checking reading frames in order to detect the presence of premature termination codons (PTCs) [2–6]. The presence of a PTC in an open-reading frame should lead to synthesis of a truncated protein which might have acquired activity deleterious to the cell or which very often will not be functional. This latter case might seem trivial, but it entails an

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4_17,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

energy cost that the cell prefers to avoid. Lastly, although the presence of a PTC may also lead to a truncated protein retaining partially or completely the activity of the wild-type protein, it leads very generally to silencing of the gene bearing the mutation because NMD very quickly degrades PTC-carrying mRNAs and unfortunately cannot distinguish this more favorable situation from the others. NMD can thus amplify the consequences of a PTC by degrading mRNA that could otherwise yield a (partially) functional protein.

Thus, nonsense mutations (point mutations transforming a sense codon into a translation-stopping nonsense codon) and PTC-creating frameshift mutations are among the most impactful, since they cause a gene to be silenced. It is therefore not surprising that PTCs are found in almost one-third of cases of genetic diseases and in many forms of cancer [7]. Yet there is currently no treatment available for patients suffering from a genetic disease caused by a PTC.

Several strategies have been developed to try to induce expression of PTC-carrying genes. A first strategy involves inhibiting NMD, in the hope that from a PTC-bearing gene of interest, a protein retaining the function of the wild-type protein, totally or partially, will be synthesized [8]. A second strategy mainly targets nonsense mutations because it involves forcing the ribosome, during translation, to incorporate an amino acid when it reaches a PTC. This mechanism is called readthrough [9, 10]. The result of readthrough is synthesis of a full-length protein differing by at most one amino acid from the wild-type protein [9, 10].

It is among small molecules that we find the most NMD inhibitors and readthrough activators. One advantage of small molecules over other biological molecules is that they can be simpler and cheaper to synthesize than gene therapy tools or antibodies, for example. Secondly, very often small molecules need no additive to enter cells. Thirdly, derivatives can be synthesized to optimize physicochemical properties such as solubility and stability. One can thus constitute a family of compounds sharing a common biological activity, notably as fuel for structure-function studies.

To identify NMD inhibitors or readthrough activators, different screening systems have been developed. We describe here the screening systems that we use, with their advantages and the different steps required for screening and for validating the selected molecules [11, 12].

1.1 Principle of the Screening System to Identify NMD Inhibitors

When building a screening system, several parameters must be considered. First of all, the test must be as simple as possible, i.e., the number of steps should be limited with a view to automating the test. The procedure must also make it possible to select molecules having the desired activity, with as few false positives as possible. Lastly, the background noise must be as low and

homogeneous as possible in order to maximize test sensitivity, as the objective is to be able to select candidate molecules on the basis of a large signal-to-noise ratio.

In our case, the idea of a screening system is to correlate enzymatic activity or fluorescence with NMD inhibition efficiency. Our choice was therefore to use a single transfection construct with a read-out based on luciferase activity measurement, as systems using fluorescence can often encounter problems related to cell autofluorescence generating high background noise. This is all the more problematic as it is difficult to create a construct whose mRNA will be 100% degraded by NMD, and as non-degraded mRNA can generate a signal.

We present here the system we use and its advantages, but it is possible to use any construct linking enzymatic activity or fluorescence to NMD inhibition. To correlate luciferase activity with NMD inhibition efficiency, it was necessary to subject luciferase mRNA to NMD. The simplest way to do this would have been to introduce a PTC into the luciferase cDNA upstream of a splicing event, but such a system would not have been suitable because inhibition of NMD would have led to synthesis of a truncated, probably nonfunctional luciferase protein. A way to induce NMD of a wild-type mRNA is to create a situation similar to regulation of the expression of splicing factor SRSF2 [13], by introducing a splicing event in the 3'UTR part of the mRNA that we wish to subject to NMD. With such a construct, the higher the luciferase activity, the greater the inhibition of NMD [11]. However, this screening system as such could select an obvious category of false positives: inhibitors of splicing of the intron located in the 3'UTR (Fig. 1a). Therefore, in an attempt to limit selection of such false positives, we introduced the same intron also into the open-reading frame encoding luciferase. Consequently, a universal splicing inhibitor or an inhibitor recognizing a possible intronic sequence silencing would cause synthesis of an aberrant, non-functional protein. Hence, such a molecule would not be selected by the screening procedure.

1.2 Principle of the Screening System to Identify Readthrough Activators

Several screening systems have been described by different laboratories. They are very often based on use of a construct carrying a cDNA encoding either an enzyme (luciferase, for example) or a fluorescent protein, interrupted by a PTC, or carrying two cDNAs in phase, separated by a stop codon (Fig. 1b). In the latter system, the two cDNAs encode different enzymes or fluorescent proteins; the second open-reading frame makes it possible to detect readthrough and the first open-reading frame is for standardizing the measurement of the second, notably to eliminate the transfection bias. Both types of screening system have the advantage of analyzing only readthrough to the exclusion of NMD, as the absence of splicing downstream of a stop codon makes the mRNA immune to

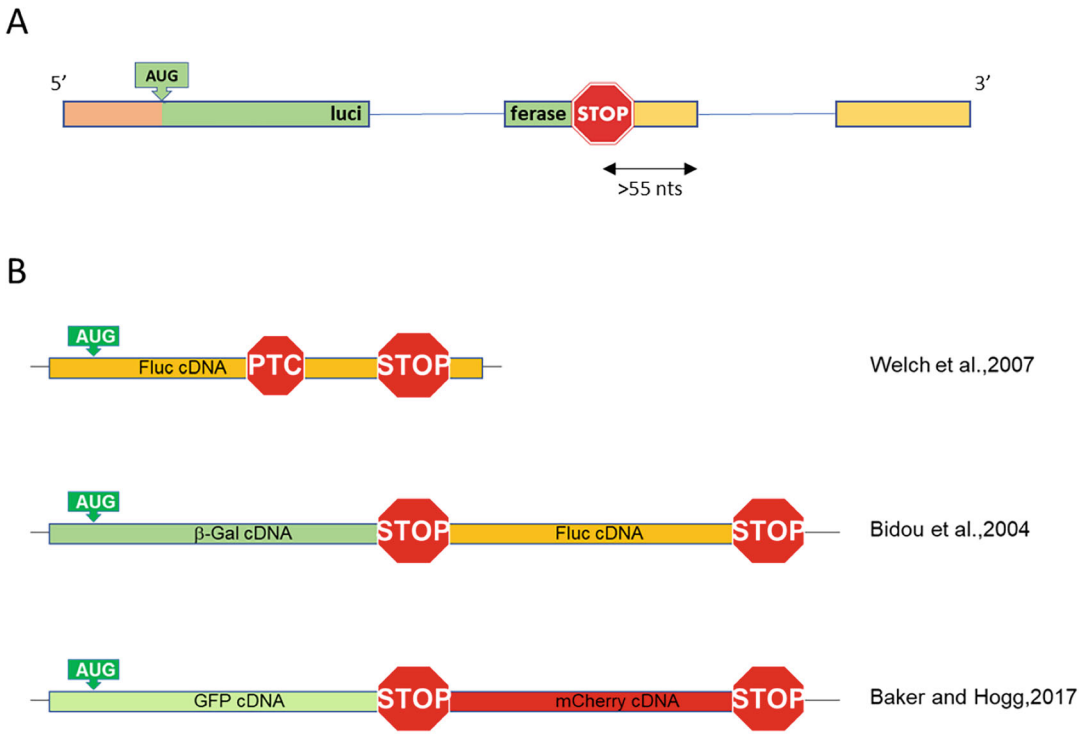


Fig. 1 Screening strategies for identifying correctors of nonsense mutations. (**a**) Representation of a possible construct for identifying NMD inhibitors by screening. An intron (horizontal blue line) was introduced into the 3′ UTR more than 55 nucleotides downstream of the natural termination codon. The same intron was also introduced in the open-reading frame. (**b**) Schematic representations of several constructs used to identify readthrough molecules by screening. At the top, the cDNA encoding firefly luciferase is interrupted by a PTC. Luciferase activity will be measured only if PTC readthrough occurs [17]. In the middle, two in-frame cDNAs encoding two enzymatic activities are separated by a stop codon. The first enzymatic activity is for normalization (which notably eliminates transfection bias) and the second activity will be detected only if readthrough occurs [18]. At the bottom, the principle is similar to that of the previous construct, except that the enzymes have been replaced with fluorescent proteins [19]. It is possible to use in phase a cDNA coding for a fluorescent protein and a cDNA coding for an enzyme, separated by a stop codon [20], and either of these two cDNAs can be placed first

NMD [14]. Yet when looking for molecules liable to be of therapeutic interest, it is important to mimic as closely as possible the situation encountered in the patient. This is why we have chosen to build a screening system that reproduces as faithfully as possible the situation found in human cells. As more than 90% of our genes are transcribed into multi-exonic pre-messenger RNA, the presence of a PTC most often results in the activation of NMD, which very significantly reduces the quantity of substrate mRNA available for readthrough. It is therefore very important to set up a screening system that reproduces this situation in order to select only molecules that will be capable of inducing detectable expression of the mutant gene even when NMD is active.

Our screening system is thus based on a construct carrying the cDNA coding for firefly luciferase interrupted by an intron, with one of the three stop codons (UGA, UAG, or UAA) located more than 55 nucleotides upstream of this intron. We checked that the corresponding mRNA is subject to NMD in order to ensure that the construct reproduces the fate of an mRNA carrying a PTC in a human cell [15]. A new screening system, also including the concept of NMD, has recently been described: it successfully identified as a readthrough inducer a small molecule inhibiting the eRF1 subunit of the translation termination complex [16].

A final point worth discussing is the fact that some molecules are reported to correct only one or two stop codon(s). Furthermore, readthrough efficiency depends on various parameters, including the identity of the stop codon. Therefore, in order not to fail to select a molecule that promotes readthrough of only one type of stop codon, it is important to run the screen on all three stop codons. Yet to avoid screening each chemical library three times, which would triple the screening cost, it is advisable to perform the initial screen on all three stop codons at once, using a mixture of cells expressing constructs with different stop codons, and to carry out validation on separate stop codons.

2 Materials

2.1 Plastic and Equipment

1. 10-cm-diameter dish.
2. Plates: the screening procedure described here is done in white 96-well plate with a transparent bottom and treated for cell culture (Corning) (*see* **Note 1**). Thanks to the transparent bottom, it is possible to check the cell morphology at all steps of the procedure.
3. Cell culture incubator at 37 °C with 5% CO_2.
4. Multi-channel electronic pipet.
5. Vortex.
6. Microplate luminometer.

2.2 Cell Culture, Library Preparation, and Assessment

1. U-2 OS cells: we chose to use U-2 OS (HTB-96 ATCC) cells for several reasons. These are human cells which transfect very well (as efficiently as HEK293 cells) and which have the advantage of being much more adherent to plastic than HEK293 cells.
2. Culture medium: cells were cultured in DMEM (Gibco) containing GlutaMAX and supplemented with 10% fetal bovine serum (Eurobio Scientific) and 1% ZellShield (Minerva Biolabs).

3. Trypsin: the 0.5% trypsin solution used to detach the cells was prepared from a 2.5% trypsin solution (Gibco) diluted in PBS, with EDTA (BDH Laboratory Supplies) added to reach a final concentration of 1 mM.
4. Dimethylsulfoxide (DMSO).
5. Libraries: the libraries of compounds to be screened can be pure molecules or plants, microorganisms, animals, or fungi extracts. The libraries can be targeted against a particular activity such as phosphorylation, binding, or ion channel for example, or nonspecific. The advantage of targeted libraries is to provide information on the mode of action of the molecule. Very generally, the molecules or extracts are resuspended in DMSO. The molecule concentration should be 5 mM or higher and the extract concentration should be 10 mg/mL or higher. We will describe the use of libraries in the 96-well format but the screening systems described here are also suitable for the 384-well format (*see* **Notes 2** and **3**).
6. Transfection: we selected JetOptimus (Polyplus) as a transfection reagent for its transfection efficiency and its low impact on cell viability. It is then possible to perform the screen with transitory transfected cells as described in the following procedure or to use stably transfected cells (*see* **Note 4**).
7. Luciferase substrate: we opted for Steadylite Plus (Revvity). This luciferase substrate contains a lysis buffer allowing one step to lyse cells and detect luciferase activity. This reagent is added directly to the well containing the cells and the culture medium. There is therefore no step to remove the culture supernatant or possibly wash the cells before lysing them.
8. G418: 10 mg/mL G418 disulfate solution.

3 Methods

3.1 Identification of NMD Inhibitors

1. Prepare a 10-cm-diameter dish containing 6×10^6 U-2 OS cells and incubate it at 37 °C under 5% CO_2.
2. Prepare one or more 96-well plates containing the molecules to be tested, each at a concentration 10 times higher than the concentration at which it is to be tested on the cells. Molecule dilutions in the wells must be made in the cell culture medium and not in DMSO. Be sure to include, on these plates, control wells containing DMSO at the same concentration as for the wells containing molecule as a negative control (if the molecules are taken up in DMSO) and a positive control. As a positive control, a reported NMD inhibitor can be used at its effective concentration. It is possible to use any NMD inhibitor, as long as it is not a translation inhibitor (such as

cycloheximide), since the screening system requires functional translation to synthesize firefly luciferase. The plate is then stored at −20 °C until its use (*see* **step 5**).

3. The day after, using JetOptimus according to the manufacturer protocol, transfect six million U-2 OS cells with 1 μg of the construct expressing firefly luciferase and then bring back cells to 37 °C and 5% of CO_2.
4. 24 h after the transfection, distribute 50 μL of transfected cells per well into a 96-well plate described in Subheading 2.1, **item 2** using a multi-channel electronic pipet. A 10-cm dish of confluent transfected cells allows preparing up to five 96-well plates.
5. Transfer 5.5 μL from each well of the 10 times concentrated molecule plate obtained in **step 2** to a well containing 50 μL cell using a multi-channel pipet.
6. Vortex the plates shortly.
7. The plates containing both cells and molecules are then stored in an incubator at 37 °C under 5% CO_2.
8. 24 h after, add 50 μL Steadylite Plus to each well containing cells and a molecule using a multi-channel pipet before taking two readings of the plate on a luminometer. The wells are read over 1–10 s.
9. Analyze the results. A molecule can be considered a potential NMD inhibitor if the luciferase activity measured in the corresponding test well reaches at least three times the luciferase activity measured in the well(s) containing DMSO (Fig. 2a).

3.2 Identification of Readthrough Activators

1. Prepare three 10-cm-diameter dishes (one for each stop codon), each containing six million U-2 OS cells, and incubate them at 37 °C under 5% CO_2.
2. Prepare one or more 96-well plates containing the molecules to be tested, each at a concentration 10 times higher than the concentration at which it is to be tested. Molecule dilutions in the wells must be made in the cell culture medium and not in DMSO. Be sure to include, on these plates, control wells containing DMSO at the same concentration as in the wells containing molecule (negative control), if the molecules are taken up in DMSO, and a positive control. As a positive control, G418 at 10 mg/mL can be used to reach 1 mg/mL final concentration. G418 is easy to use and stable and can read through UGA, UAG, and to a lesser extent UAA. The plate is then stored at −20 °C until its use (*see* **step 6**).
3. The day after, using JetOptimus according to the manufacturer protocol, transfect the U-2 OS cells in each dish with 1 μg

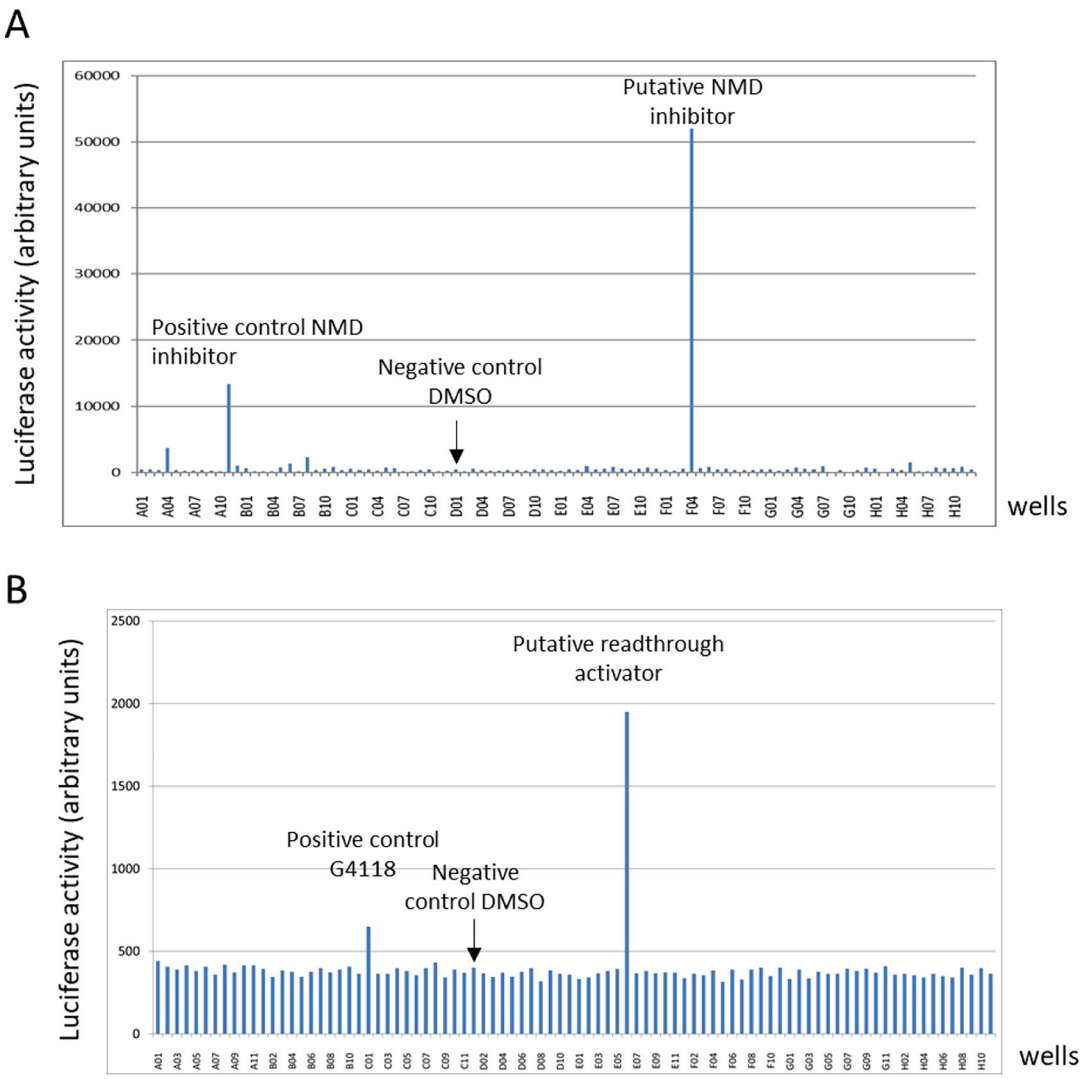


Fig. 2 Example of results obtained by screening. (**a**) Results from the screening to identifying NMD inhibitors with one molecule having potential NMD-inhibiting activity much higher than that of the positive control. (**b**) Results from the screening to identifying readthrough activators with one molecule having a potential readthrough activity. In this example, screening was carried out on the construct carrying the UAA premature stop codon

firefly-luciferase-expressing construct carrying one of the three premature termination codons.

4. Use trypsin to collect from the three dishes the cells transfected on **step 3** and mix together the collected cells in a 50-mL reagent reservoir.
5. Distribute 50 μL per well of the mixture of transfected cells into 96-well plates using a multi-channel electronic pipet.

A 10-cm dish of confluent cells allows preparing up to five 96-well plates.

6. Transfer 5.5 μL from each well of the concentrated molecule plate obtained in **step 2** to a well containing 50 μL transfected cells in the 96-well plates.
7. Vortex the plates containing both cells and molecules and then store them in an incubator at 37 °C under 5% CO_2.
8. The day after, to each well containing the cells and molecules, add 50 μL Steadylite Plus before taking two readings of the plate on the luminometer. The wells are read over 1–10 s.
9. Analyze the results. A molecule can be considered a potential readthrough activator if the measured luciferase activity reaches that observed with G418 (Fig. 2b).

4 Notes

1. White multi-well plates are used when the test involves luciferase, but a black plate with a transparent bottom and treated for cell culture can be used if the test involves fluorescence.
2. Miniaturizing the screening format: screening using in 96-well plates is suitable for the analysis of hundreds or even a few thousand molecules. When the size of the chemical library to be tested reaches several tens of thousands of molecules or more, switching to 384-well or even 1536-well plates becomes necessary. This makes it necessary to miniaturize the procedure by validating the possibility of detecting NMD-inhibiting or readthrough-activating activity in a smaller volume and hence with fewer cells. The two screening systems described above are compatible with the 384-well format (the 1536-well format has not yet been tested).
3. Automation: the procedures described above can be performed with multi-channel pipettes, but when the size of the chemical library reaches several tens of thousands of molecules to be tested or more, it may be advantageous to automate the procedure. Some facilities can automate the entire procedure from cell distribution to reading results, but the steps of days 0–2 remain quite complex to automate. It is important to consider automation when designing the screen, in order to involve the fewest possible steps and to limit medium changes and transfers from one support to another. In the two procedures described above, for example, there is no washing step and the luciferase substrate used contains a concentrated lysis buffer which makes it possible to avoid aspirating the culture medium before adding the luciferase substrate.

4. The two procedures described above can be simplified by obtaining stably transfected cell lines. All procedures are therefore shortened by one day, allowing more runs to be carried out in the same week and making it easier to automate screening.

Acknowledgments

SN was supported by funding from Université de Lille. FL was supported by funding from Agence Nationale de la Recherche (PEPR Biotherapy—projet RNAvac—France 2030), Inca (PLBIO21-147), La Ligue contre le cancer, Vaincre la mucoviscidose (Di-T-CAP projet ClassFirstTheraRead), the Association Française contre les Myopathies, the Fondation Maladies Rares, and the Rotary club de Linselles. The Canther Laboratory is part of the ONCOLille institute. This work was supported by a grant from Contrat de Plan Etat-Région CPER Cancer 2015–2020.

Competing Interest Statement The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

References

1. Isken O, Maquat LE (2007) Quality control of eukaryotic mRNA: safeguarding cells from abnormal mRNA function. Genes Dev 21(15):1833–1856
2. Sun L, Mailliot J, Schaffitzel C (2023) Nonsense-mediated mRNA decay factor functions in human health and disease. Biomedicines 11(3)
3. Carrard J, Lejeune F (2023) Nonsense-mediated mRNA decay, a simplified view of a complex mechanism. BMB Rep 56:625
4. Yi Z, Sanjeev M, Singh G (2021) The branched nature of the nonsense-mediated mRNA decay pathway. Trends Genet 37(2):143–159
5. Lloyd JPB (2018) The evolution and diversity of the nonsense-mediated mRNA decay pathway. F1000Res 7:1299
6. Silva AL, Romao L (2009) The mammalian nonsense-mediated mRNA decay pathway: to decay or not to decay! Which players make the decision? FEBS Lett 583(3):499–505
7. Frischmeyer PA, Dietz HC (1999) Nonsense-mediated mRNA decay in health and disease. Hum Mol Genet 8(10):1893–1900
8. Benslimane N, Loret C, Chazelas P, Favreau F, Faye PA, Lejeune F, Lia AS (2024) Read-through activators and nonsense-mediated mRNA decay inhibitor molecules: real potential in many genetic diseases harboring premature termination codons. Pharmaceuticals (Basel) 17(3)
9. Palma M, Lejeune F (2021) Deciphering the molecular mechanism of stop codon read-through. Biol Rev Camb Philos Soc 96(1): 310–329
10. Martins-Dias P, Romao L (2021) Nonsense suppression therapies in human genetic diseases. Cell Mol Life Sci 78(10):4677–4701
11. Carrard J, Ratajczak F, Elsens J, Leroy C, Kong R, Geoffroy L, Comte A, Fournet G, Joseph B, Li X, Moebs-Sanchez S, Lejeune F (2023) Identifying potent nonsense-mediated mRNA decay inhibitors with a novel screening system. Biomedicines 11(10)
12. Trzaska C, Amand S, Bailly C, Leroy C, Marchand V, Duvernois-Berthet E, Saliou JM, Benhabiles H, Werkmeister E, Chassat T, Guilbert R, Hannebique D, Mouray A, Copin MC, Moreau PA, Adriaenssens E, Kulozik A, Westhof E, Tulasne D, Motorin Y, Rebuffat S, Lejeune F (2020) 2,6-Diaminopurine as a highly potent corrector of UGA nonsense mutations. Nat Commun 11(1):1509

13. Sureau A, Gattoni R, Dooghe Y, Stevenin J, Soret J (2001) SC35 autoregulates its expression by promoting splicing events that destabilize its mRNAs. EMBO J 20(7):1785–1796
14. Lejeune F, Maquat LE (2005) Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells. Curr Opin Cell Biol 17(3):309–315
15. Benhabiles H, Gonzalez-Hilarion S, Amand S, Bailly C, Prevotat A, Reix P, Hubert D, Adriaenssens E, Rebuffat S, Tulasne D, Lejeune F (2017) Optimized approach for the identification of highly efficient correctors of nonsense mutations in human diseases. PLoS One 12(11):e0187930
16. Sharma J, Du M, Wong E, Mutyam V, Li Y, Chen J, Wangen J, Thrasher K, Fu L, Peng N, Tang L, Liu K, Mathew B, Bostwick RJ, Augelli-Szafran CE, Bihler H, Liang F, Mahiou J, Saltz J, Rab A, Hong J, Sorscher EJ, Mendenhall EM, Coppola CJ, Keeling KM, Green R, Mense M, Suto MJ, Rowe SM, Bedwell DM (2021) A small molecule that induces translational readthrough of CFTR nonsense mutations by eRF1 depletion. Nat Commun 12(1):4358
17. Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P, Paushkin S, Patel M, Trotta CR, Hwang S, Wilde RG, Karp G, Takasugi J, Chen G, Jones S, Ren H, Moon YC, Corson D, Turpoff AA, Campbell JA, Conn MM, Khan A, Almstead NG, Hedrick J, Mollin A, Risher N, Weetall M, Yeh S, Branstrom AA, Colacino JM, Babiak J, Ju WD, Hirawat S, Northcutt VJ, Miller LL, Spatrick P, He F, Kawana M, Feng H, Jacobson A, Peltz SW, Sweeney HL (2007) PTC124 targets genetic disorders caused by nonsense mutations. Nature 447(7140):87–91
18. Bidou L, Hatin I, Perez N, Allamand V, Panthier JJ, Rousset JP (2004) Premature stop codons involved in muscular dystrophies show a broad spectrum of readthrough efficiencies in response to gentamicin treatment. Gene Ther 11(7):619–627
19. Baker SL, Hogg JR (2017) A system for coordinated analysis of translational readthrough and nonsense-mediated mRNA decay. PLoS One 12(3):e0173980
20. Gurzeler LA, Link M, Ibig Y, Schmidt I, Galuba O, Schoenbett J, Gasser-Didierlaurant-C, Parker CN, Mao X, Bitsch F, Schirle M, Couttet P, Sigoillot F, Ziegelmuller J, Uldry AC, Teodorowicz W, Schmiedeberg N, Muhlemann O, Reinhardt J (2023) Drug-induced eRF1 degradation promotes readthrough and reveals a new branch of ribosome quality control. Cell Rep 42(9):113159

INDEX

A

Alternative polyadenylation (APA) 40, 185–187
Antisense oligonucleotide (ASO) 171–183, 185–197, 237, 249
Antisense transcripts 17, 18, 22, 24
Asymmetric paired-end sequencing 86
AT-rich interaction domain 3A (ARID3A) 172, 173, 176

B

Bacterial transformation 163, 164
Biotinylated probe 39–47
B lymphocytes 83, 84, 103

C

Cancer 25–36, 63, 121–139, 150, 172, 201, 238, 256
Cell culture 4–7, 11, 54, 56, 142, 144, 159, 164, 167, 174, 182, 188, 190, 191, 195, 220, 222, 224, 225, 239, 249, 258, 260–262
Cell transfection 156, 159, 160, 167
Crosslinking immunoprecipitation and sequencing (CLIP-seq) 52

D

Diagnosis 25, 27, 122
DNA extraction 66, 68, 70–72, 80
Dorsal root ganglion 217–233

E

3'-end RNA isoforms 185–197
Enhancer of Zeste Homolog 2 (EZH2) 238–241, 244, 249
Epitranscriptomics 40, 201
Exon skipping 122, 124, 171–183, 238, 239, 243, 244, 247, 252

F

Filtration 67, 68, 70, 78, 79, 142, 143, 145–147, 149
Formalin-fixed paraffin-embedded (FFPE) tissue 123, 135
Fusion transcripts 27–30, 35, 36, 121, 122, 134

G

Gene expression 3, 4, 116, 141, 180, 186, 189, 201, 227, 232, 237–252, 255
Gene fusions 25–36, 121–139
Gene silencing 256

H

Homologous recombination-mediated cloning 157, 163
Hybrid minigene 156, 157

I

Illumina 29, 33, 34, 67, 84–86, 90–92, 94, 96, 98, 104, 108, 109, 114–118, 124–126, 133, 136, 139, 222, 228, 229
Immunoglobulin (Ig) 46, 172, 185, 186
Immunoglobulin repertoire 83–101, 103–118
Ion Torrent 67

K

Knockdown 237–252

L

Library preparation 52, 53, 55, 56, 60, 74, 78, 81, 97, 99, 124, 138, 150, 228, 258

M

Mass spectrometry (MS) 40, 199–212
Messenger RNA (mRNA) 30, 40, 51, 52, 86, 157, 172–174, 182, 183, 185–187, 189, 197, 201, 204–206, 212, 218, 219, 227, 238, 240, 255–259
Metagenomics 65–68, 74
mRNA regulation 217, 218, 232
Multiple reaction monitoring (MRM) 202, 207, 208

Laurent Delpy (ed.), *RNA Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 2962,
https://doi.org/10.1007/978-1-0716-4726-4,

© The Editor(s) (if applicable) and The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2025

N

Next generation sequencing (NGS) 29, 33–36, 84, 122, 124, 201, 222, 228
Nociceptors 217
Nonsense-mediated mRNA decay (NMD) 172, 183, 218, 219, 221, 225–227, 231–232, 237–252, 255–264
Non-small cell lung cancer (NSCLC) 121, 122, 124, 138
Nuclear bodies RNAs 142

O

Oligo(dT) 4–6, 9, 43, 84, 106, 125, 133, 160, 168, 188

P

Paired-end sequencing 86, 104, 105, 116
Phase separation 17, 39, 141
Polyadenylation 185–188, 194, 195, 237
Protein:RNA interactions 51–62

Q

Quantitative polymerase chain reaction (qPCR) 3–12, 15–24, 98, 122, 125, 133, 136, 150, 176, 180–182, 189, 193–196, 219, 227, 228, 233

R

5'RACE (Rapid Amplification of cDNA Ends) 84, 85, 88, 89, 92, 93, 103–118
Random primers 4, 5, 9, 160, 168, 175, 178, 240, 245
Readthrough 255–264
Repertoire sequencing (RepSeq) 84
Reverse transcription 3–12, 16–18, 20, 21, 23, 26, 31, 32, 35, 52, 55, 59, 84, 85, 88, 89, 92, 104, 106, 107, 112, 138, 149, 160, 168, 175, 178, 189, 193–195, 197, 221, 226, 240, 245
RNA 4, 16, 26, 39, 51, 84, 106, 122, 142, 155, 171, 186, 199, 218, 237, 255
RNA-based therapeutics 122
RNA binding proteins (RBPs) 39, 40, 51–55, 59–62
RNA capture 39–41, 45, 46
RNA extraction 4–6, 8, 11, 26, 31, 42, 43, 88, 92, 106, 110, 125, 135, 137, 156, 160, 167, 170, 174, 177, 189, 191, 196, 209, 225, 226, 228, 239, 244
RNA isolation 16–19, 21, 22, 40, 42, 58, 59, 88
RNA modifications 39, 40, 199–212
RNA purification 5, 110, 148, 189, 195, 221, 226
RNA sequencing (RNASeq) 3, 122, 124, 137, 155, 219
RNA splicing 28, 35, 155, 157, 237
RNA translation 51, 201, 237, 255

S

Screening methods 122, 255–264
Sensory neurons 217, 231, 232
Small molecules 218, 219, 256, 259
Splice-switching oligonucleotide (SSO) 238–241, 243, 244, 249, 251
16S rRNA sequencing 65–81
Strand-specific 15–24

T

Transcript enrichment 40
Transcript isolation 39–47
Translocation 27, 35, 136
Truncated protein 171–183, 255, 256

U

Unique molecular identifiers (UMI) 30, 34, 36, 84–89, 98, 99, 104–107, 112, 116

V

Variable region 15, 65–67, 70–73, 84, 88, 104

W

Water samples 65–81
Wet lab process 121–139