

ALL-IN-ONE RiboLace Pro

A RiboSeq workflow (sample to NGS library) that provides deeper insights.

Product	Catalog no	Rxns.
ALL-IN-ONE RiboLace Pro	RS0XL-12	12

Shipping: Blue Ice and Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: ALL-IN-ONE RiboLace Pro contains all reagents to perform ribosome profiling from cells/tissues to the final Next Generation Sequencing library with the RNA size selection and PAGE-gel extraction steps. The kit includes components for 12 reactions, and it is suitable for Illumina platforms (MiSeq, NovaSeq 6000, NextSeq1000/2000).

Suitable for: Eukaryotic cell lines and tissues

For Research Use Only. Not Intended for Diagnostic or Therapeutic Use.

Table of Contents

Kit storage info..... 4

Additionally Required Materials 4

INTRODUCTION..... 5

 RiboLace™ Technology (Section B):..... 5

 LaceSeq™ Technology (Section C): 5

WORKFLOW OVERVIEW..... 7

 Optimal Workflow Recommendations 8

A. SAMPLE PREPARATION 9

 A.1 Sample Amounts Recommendations..... 9

 A.2 AU Calculation - Input lysate Quantification 9

 A.2.1 Measure Lysate AU 9

 A.2.2 Lysis Volume Selection..... 10

 A.3 Calculate the volume of lysate needed for the pulldown..... 10

B. ACTIVE RIBOSOME PULLDOWN 11

 Step B1. CELL LYSIS..... 12

 ⚠ Before starting the experiment – Lysis Buffer Supplementing 12

 Adherent Cells lysis 12

 Suspension Cells lysis 13

 Tissues lysis 14

 Step B2. BEADS FUNCTIONALIZATION 15

 ⚠ First-time opening of the kit – RiboLace Smart Probe (RsP) dilution and aliquoting..... 15

 Step B3. NUCLEASE DIGESTION 17

 Step B4. RIBOSOMES PULLDOWN..... 18

 Step B5. RNA EXTRACTION..... 19

 Step B6. GEL RUN..... 20

 Step B7. PAGE EXTRACTION OF THE RPF 22

C. RPF’s LIBRARY PREPARATION 23

 Step C1. 5’ PHOSPHORYLATION 24

 Step C2. ADAPTOR LIGATION 25

 Step C3. CIRCULARIZATION..... 27

 Step C4. REVERSE TRANSCRIPTION 28

 Step C5. PCR AMPLIFICATION – PCR 1 29

 Step C6. PCR AMPLIFICATION – PCR 2 31

 Step C7. GEL RUN..... 32

 Step C8. LIBRARY EXTRACTION 33

 Step C9. LIBRARY QUALITY CHECK 34

D. SEQUENCING 35

Step D1. HOW TO SEQUENCE – DO IT YOURSELF 35

Step D2. HOW TO SEQUENCE – OUTSOURCE TO IMMAGINA 36

APPENDIX..... 37

App.1 Guidelines for sample input amount optimization..... 37

App.2 Alternative CHX removal protocol..... 38

App.3 Lysis buffer supplementation issues..... 39

App.4 Sample Lysis output summary table..... 40

App.5 Beads functionalization checklist 41

App.6 Check proper beads functionalization (for Step B2 – Beads functionalization) 42

App.7 Nuclease digestion checklist..... 43

App.8 Optional Nuclease optimization (for Step B3 – Nuclease Digestion) 44

App.9 Library Preparation: Low Amount of Starting Material – Troubleshooting Guidelines..... 46

App.10 Library Preparation: Positive control..... 47

Kit storage info

	Qty.	Storage
4°C components	1 box	4°C
-20°C components	1 box	-20°C
-80°C components	1 bag	-80°C
iUDIs plate	1 box	-20°C

Table 1. Kit composition (in boxes and bag) and storage temperature.

Additionally Required Materials

- PBS
- Sodium deoxycholate 10% solution in DNase/RNase-free water
- Cycloheximide (Sigma-Aldrich, catalog no. C4859-1ML)
- DNase I (Thermo Scientific catalog no. 89836)
- RiboLock RNase Inhibitor (Thermo Scientific catalog no. EO0381)
- SUPERaseIn (Invitrogen, catalog no. AM2696)
- RNase-free water
- Nanodrop ND-1000 UV-VIS Spectrophotometer
- Microcentrifuge and non-stick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- Automatic wheel (rotator)
- Magnetic stand for 1.5mL tube
- Acid-phenol:chloroform (Ambion catalog no. AM9720)
- GlycoBlue (Ambion catalog no. AM9515)
- Isopropanol (Sigma catalog no. 278475)
- 15% TBE-Urea polyacrylamide gel (e.g. BioRad catalog no. 450-6053 or Thermo Scientific catalog no. EC6885BOX)
- Gel Loading Buffer II (Denaturing PAGE) (Thermo Scientific catalog no. AM8546G)
- Ultra-low range molecular weight marker (i.e., Thermo Scientific catalog no. 10597012 or similar)
- SYBR Gold (Thermo Scientific, catalog no. S11494)
- Qubit Fluorometer
- Qubit™ microRNA Assay Kit
- RNA Clean & Concentrator™-5 (Zymo catalog. no. R1015 or R1016)
- AMPure XP for PCR Purification (Beckman Coulter catalog no. A63881)
- PCR Clean-up column kit (i.e., NucleoSpin® Macherey-Nagel catalog no 740609)
- Agilent 2100 Bioanalyzer
- Agilent High Sensitivity DNA Kit (Agilent Tech. catalog no. 5067-4626)
- 10% TBE polyacrylamide gel (e.g. Thermo Scientific catalog no. EC6275BOX)
- DNA Gel Loading Dye (e.g., Thermo Scientific catalog no. R0611)

INTRODUCTION

The ALL-IN-ONE RiboLace Pro kit is a complete RiboSeq solution combining IMMAGINA's RiboLace™ and PAGExt technologies for the isolation of active Ribosome Protected Fragments (RPFs) with the LaceSeq™ technology for the preparation of efficient and precise NGS libraries of small RNA fragments. This method retains the gel purification, but eliminates the need of bulky equipment, or intricate manipulations such as ultracentrifugation. Furthermore, it demonstrates robustness by enhancing the RPF incorporation into the library.

The kit is suitable for eukaryotic primary, and immortalized cell lines (either freshly harvested or flash-frozen) and tissue (for tissues please purchase the dedicated Tissue Lysis Buffer Cat. no. #IBT0032),

The lowest starting material depends on the specimen type and its translational state and ranges between 0.5 and 1 million cells. For specimens with very high translation levels, it is possible to go as low as 300,000 cells. For lower input samples please contact us at techsupport@immaginabiotech.com.

IMMAGINA's mission is to develop unique and smart enabling technologies to break down the walls in complex Translational studies. Please visit our website <https://immaginabiotech.com/> for a complete overview of our products and services and our proprietary technologies.

RiboLace™ Technology (Section B):

At the heart of the method lies a proprietary puromycin derivative, called 3P, that retains the ability to interact with active ribosomes while being covalently linked to a biotin molecule. The samples are first exposed to cycloheximide to clamp ribosomes on the mRNA fragments (recommended step), then lysate and nuclease digested to produce the RPF. In parallel, magnetic beads are functionalized with the 3P, and once the digestion is over, they are added to the reaction mix to pull down the active ribosomal complex. Eventually, the RPFs are purified by the ribosomal complex by simple differential elution. The original proof-of-concept of the technology was published on [Cell Report in 2018](#).

LaceSeq™ Technology (Section C):

LaceSeq™, an Immagina proprietary technology, has been developed to elevate both the efficiency and simplicity of library preparation. The nucleases commonly used for the generation of the RPFs, leave a phosphate at the 3' extremity of the digested RNA fragments. Unlike the standard library preparation protocols that remove this molecular signature, LaceSeq™ exploits it to specifically target the RPFs and drive their preferential uptake into the NGS library.

After the initial selective ligation between the RPF 3'P end and our Linker, a second specific intramolecular ligation is initiated. The kinetics of the second ligation ensures lower incorporation of RNA contaminants. The original proof-of-concept of the technology was published on [NAR in 2021](#) with the circAID name.

The structure of the LaceSeq™ Linker has also been optimized to support downstream processes of NGS sequencing and Data analysis. Specific Unique Molecular Identifiers (UMI) are present in the linker, allowing the identification of PCR duplication products. Illumina adapters and Unique Dual Indexes (iUDIs) required for multiplexing are added after circularization and Reverse Transcription via a two-step PCR amplification.

It's important to note that the ALL-IN-ONE RiboLace Pro includes the iUDIs plate and that we offer four different sets of iUDIs primers, 12 Forward and 12 Reverse per each set (Cat. no. #UDI0Z1-12, Z13-24, Z25-36 or Z37-48).

IMMAGINA's mission is to develop unique and smart enabling technologies to break down the walls in translational studies. Please visit our website <https://immaginabiotech.com/> for a complete overview of our products & services and our proprietary technologies.

WORKFLOW OVERVIEW

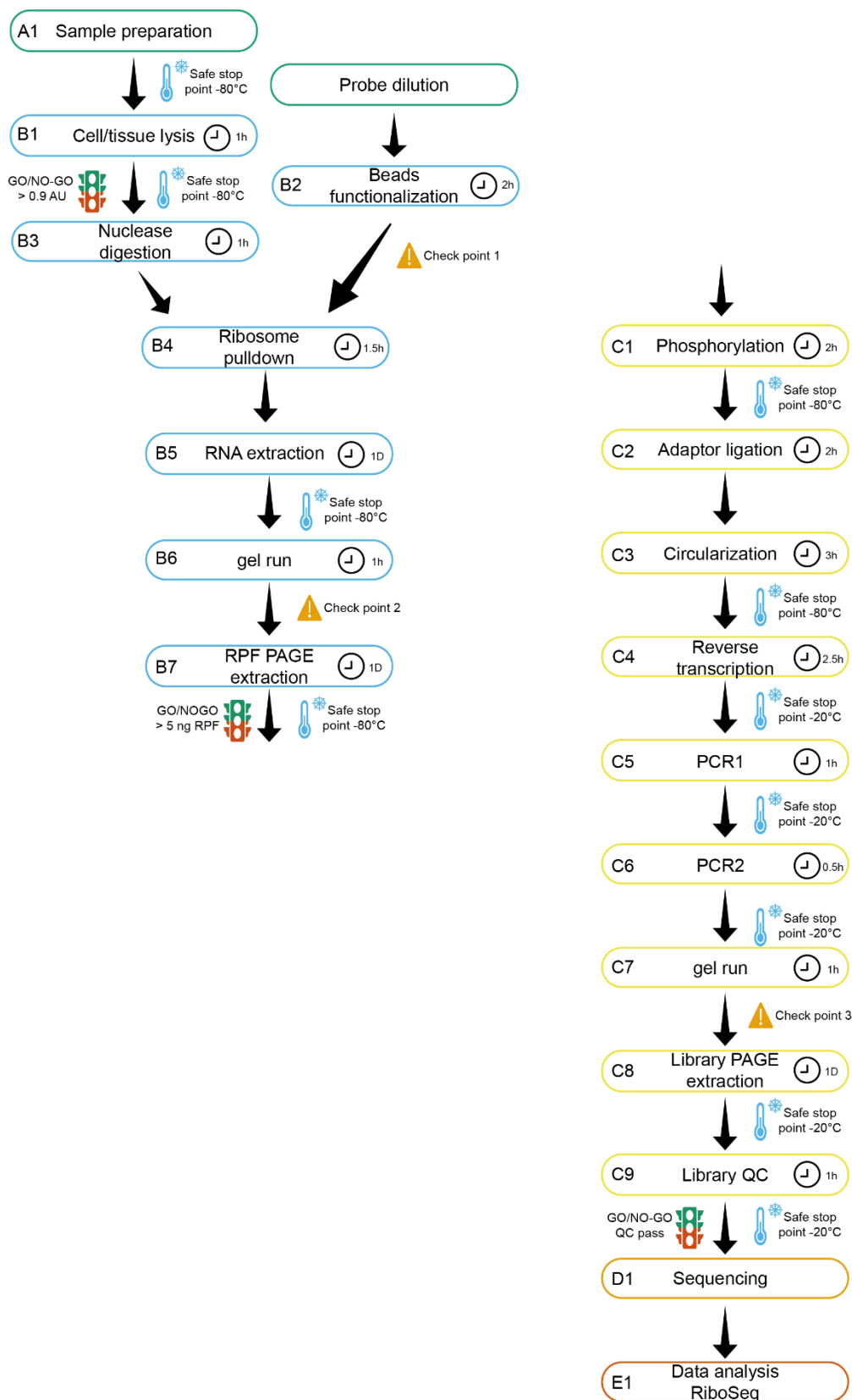



Fig.1 Overview of the ALL-IN-ONE RiboLace Pro Kit workflow. In each box, steps are present in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures), GO/NO-GO conditions (traffic lights), and a checkpoint (caution signal) are placed right at the bottom of the corresponding steps.

Optimal Workflow Recommendations

-  Please avoid running more than 6 samples in parallel. Longer manipulation time may introduce an unwanted variability between the first and the last samples.
- Allocate at least 5-7 days for the completion of the entire workflow.
- If possible, please perform a preliminary lysis experiment to set the lysis volume following the suggested AU operational range (see section A3).
- The Beads Functionalization and the Nuclease Digestion (Steps B2 and B3) can be performed in parallel, to shorten the protocol length.
- This protocol has been optimized to perform the RPF pulldown and the library preparation starting with 0.9 AU (Abs260nm) of lysate, and at least 5 ng of RPF after pulldown and PAGE extraction. Crucially, if the sample amount does not allow it to reach 0.9 AU, it is still possible but not advisable, to lower the lysate input down to 0.6 AU without the need of modifying the kit stoichiometry.
- Starting with 0.9 AU of specimen lysate after RPF pulldown we expect to obtain at least 1.5 µg of RNA, and after PAGE extraction, at least 5 ng to start with the preparation of the library. The protocol provides guidelines for different RPFs amount recovered after PAGE extraction, from 5 to 40 ng.
- A set of tables is available in the Appendixes to allow for a step-by-step approach while running the experiment. To improve user experience, we suggest printing them and having them available during the actual manipulations.
- A positive control reaction for library preparation is present in the kit, please check Appendix 10 for more details.

A. SAMPLE PREPARATION

A.1 Sample Amounts Recommendations

The amount of Ribosome Protected Fragments (RPF) that can be isolated from a sample is strongly affected by its translational state and must be considered when programming experiments with the IMMAGINA ALL-IN-ONE RiboLace Pro kit. For instance, two lysates similarly concentrated (i.e., similar Abs_{260nm}) but from different cell types or specimens (e.g. human vs mouse, brain vs liver, or immortalized vs primary), or with different treatments (e.g. drugs and transfection reagents) could have completely different amounts of translating ribosomes, leading to opposite outcomes.

While it is not possible to provide a minimal sample size as a defined number of cells or weight of tissue, two indicators can be used as a checkpoint and go/no-go at two convenient stages during the protocol:

- The first is the total AU after cell lysis (Step B1), and before the RPF pulldown.
- The second, is the amount of RNA retrieved after Ribosome & RPF pulldown (Step B5).

As a general indicator 5 million non-treated cells, coming from an immortalized line (such as HeLa, HEK, CHO, and K562) at 70 to 80% confluence represent a comfortable starting point.

Given specimen-to-specimen variability, as a preliminary experiment, we suggest testing the lysis step on different sample amounts, recording the corresponding total AU, and using it to fine-tune volumes and sample size during the real experiment. Suggested quantities are 0.3, 1.5, and 5 M cells, and for tissues, less than 10 mg and more than 10 mg (See Table 2 for lysis buffer volumes).

After RPF pulldown (end of day 1) we expect to obtain at least 1.5 µg of RNA to run on gel. After RPF extraction from gel, we expect at least 5 ng of RPFs to start with the preparation of the library.

A.2 AU Calculation - Input lysate Quantification

A.2.1 Measure Lysate AU

Cells and tissues should be lysed following Step B1 a, b, or c instructions depending on your specimen type. The AU of your sample is measured using a spectrophotometer, most commonly a Nanodrop. Set the instrument so to measure the Abs at 260 nm (usually Nucleic Acid function) and measure the absorbance of your lysate using the Supplemented Lysis Buffer (SLB) as blank (see Before starting the experiment – Lysis Buffer Supplementing & Table 3). The use of different lysis buffers is strongly discouraged because it may interfere with the efficiency of ribosome pull-down and with the AU calculation (some components may absorb at 260 nm).

If the instrument does not allow to use of the SLB as blank, please use water instead, then record the absorbance of both the SLB and the lysate and subtract the absorbance of the SLB to the lysate.

Example:

- Supplemented Lysis buffer SLB Abs_{260nm} = 7 AU*
- Specimen Abs_{260nm} = 17 AU*
- Absorbance value of lysate = 17 – 7 = 10 AU*

A.2.2 Lysis Volume Selection

It is important to lysate the specimen in an appropriate volume to obtain a lysate with an optimal range of Abs at 260 between 7 to 15 AU. It is still possible to use the kit with an AU as low as 2 and as high as 30. Lower or higher values may affect the efficiency and reproducibility of the kit since using smaller amounts or using more diluted lysate could cause quantification and/or pipetting errors.

The resuspension values suggested in Table 2, should set you within the optimal AU range. For instance, starting with 5 million immortalized cells lysed in 300 µL of lysis buffer an absorbance between 7 to 15 AU is expected after blank subtraction. Feel free to modify those resuspension values according to your sample behavior.

Specimen	Quantity	Lysis buffer	Volume of supplemented LB (µL)
Cell	0.3 – 1 million	# IBT0031	50 µL
Cell	1 – 5 million cells	# IBT0031	150 µL
Cell	> 5 million cells	# IBT0031	300 µL
Tissue	< 10 mg	# IBT0032	500 µL
Tissue	> 10 mg	# IBT0032	800 µL

Table 2. The quantity of lysis buffer depends on specimen amount.

A.3 Calculate the volume of lysate needed for the pulldown

The absorbance of your sample depends on your sample characteristics (type of cell/tissue and amount) and the volume in which it has been resuspended. Given this volume dependence, it is possible to consider the AU read out as a concentration, and we can decide arbitrarily to set it as AU/mL.

To calculate the volume of lysate to utilize to pipet 0.9 AU, follow the examples below.

Example 1: Nanodrop absorbance value of lysate at 260 nm = 10 AU.

This means that, arbitrarily, we set the absorbance of the lysate at 10 AU/ml, which is divided by 1000 µL/mL to get the concentration per µL = 0.01AU/µL.

- To start with 0.9 AU use: $0.9\text{AU}/0.01\text{ AU}/\mu\text{L} = 90\ \mu\text{L}$ of lysate









Example 2: Nanodrop absorbance value of lysate at 260 nm = 4 AU.

This means that, arbitrarily, we set the absorbance of the lysate at 4 AU/ml (=0.004 AU/µl).

- To start with 0.9 AU use: $0.9\text{AU}/0.004\text{ AU}/\mu\text{L} = 225\ \mu\text{L}$ of lysate

B. ACTIVE RIBOSOME PULLDOWN

ALL-IN-ONE RiboLace Pro components and additional required materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
B1	Lysis buffer (LB)	# IBT0031	4 x 1.3 mL	-20°C	Vial	 clear
B1	SDC 10%	Additionally Required Material				
B1	DNase I	Additionally Required Material				
B1	RiboLock RNase Inhibitor	Additionally Required Material				
B1	Cycloheximide (CHX)	Additionally Required Material				
B1	PBS	Additionally Required Material				
B2	B-Buffer (BB)	# IBT0021	10 mL	4°C	Bottle	--
B2	RiboLace magnetic beads (RmB) v2-1	# IBT0042	1.8 mL	4°C	Vial	 clear
B2	OH-buffer (OH)	# IBT0051	5 mL	4°C	Bottle	--
B2	mPEG	# IBT0061	120 µL	-20°C	Vial	 clear
B2/B5	Nuclease free water	Additionally Required Material				
B2*	RiboLace smart probe (RsP)	# IBT00112	200 µL	-20°C	Vial	 clear
B2	diluted RiboLace smart probe (dRsP)	Dilute Aliquot from RsP		-80°C		
B2/B3/B4	W-buffer (WB)	# IBT0071	2 x 25 mL	4°C	Bottle	--
B3	Nuclease (Nux)	# IBT0091	21 µL	-20°C	Vial	 clear
B3	Diluted Nuclease (dNux)	Dilute Aliquot from Nux				
B3	Nux Enhancer (NE)	# IBT0081	13 µL	-20°C	Vial	 clear
B3	SuperRNase In	Additionally Required Material				
B5	Proteinase K	# IBT0111	130 µL	-20°C	Vial	 clear
B5	SDS 10%	# IBT0121	600 µL	4°C	Vial	 clear
B5	Acid Phenol: Chloroform:Isoamyl Alcohol	Additionally Required Material				
B5	Isopropanol	Additionally Required Material				
B5	GlycoBlue	Additionally Required Material				
B5	70% cold ethanol	Additionally Required Material				

Step B1. CELL LYSIS

Before starting the experiment – Lysis Buffer Supplementing

To ensure optimal reproducibility, for both cell and tissue lysis buffer, we recommend producing a fresh Supplemented Lysis Buffer (SLB) aliquot for each new experiment, right before proceeding with the Lysis Step. Working on ice, combine the SLB by following Table 3 instructions and multiply the volumes according to the number of samples being processed (N). please combine the different reagents following the left-to-right order.

	Lysis buffer (LB)	Sodium deoxycholate (SDC) 10% (W/V)	DNase I 1 U/ μ L	RiboLock RNase Inhibitor 40 U/ μ L	Final Volume
N=1	267 μ L	30 μ L	1.5 μ L	1.5 μ L	300 μ L
N= _____	_____	_____	_____	_____	_____

Table 3. Recipe for the supplementation of the provided lysis buffer or tissues lysis buffer.

The SLB final concentration is Sodium deoxycholate (1%), DNase I (5U/mL), and RiboLock RNase Inhibitor (200 U/mL).

Please if the SLB appears as a whiteish and cloudy solution, do not proceed and check Appendix 3.

Adherent Cells lysis

- B1.1a** Treat the cells with 10 μ g/mL of **cycloheximide (CHX)** for 5 min at 37°C before lysis. We recommend using cells at 70-80% confluence. CHX treatment is suggested – but it is not mandatory - to increase the efficiency of the ribosomes' affinity purification. CHX treatment could induce the accumulation of ribosomes within the first 10 codons. Should you not wish to add CHX check Appendix 2 for the alternative protocol.
- B1.2a** After incubation, place the cells on ice and wash them quickly with **cold PBS** containing CHX (20 μ g/mL).
- B1.3a** Remove all residual PBS with P200 pipette. **All the PBS must be removed before proceeding with the lysis to avoid diluting the lysis buffer.**
- B1.4a** Perform the lysis directly adding the complete **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.3 - Input lysate preparation and quantification - & Table 3) to each cell dish and scrape vigorously. Mechanical scraping helps the downstream processing by disrupting the cell membrane and releasing the cellular contents, including ribosomes.

To ensure good lysis, follow these guidelines for mechanical scraping:

- Before scraping, make sure you are working in a sterile environment using appropriate aseptic techniques.
 - Prepare your sample by adding the necessary lysis buffer or solution as per the protocol.
 - Using a suitable tool such as a cell scraper, spatula, or pipette tip, gently scrape the surface of the cell culture dish or tissue to dislodge the cells.
 - Apply consistent but gentle pressure to ensure thorough scraping while avoiding excessive force that may introduce debris.
 - Scrape in a systematic manner, covering the entire surface area to ensure an even distribution of lysed cells.
 - Continue scraping until you observe the desired level of cell detachment and release of cellular material.
 - Transfer the lysate to a suitable collection vessel, such as a microcentrifuge tube, for further processing or analysis.
- **B1.5a** Collect the cell lysate in a 1.5 mL microcentrifuge tube and pellet the cell debris and nuclei by centrifugation at 20,000 g for 5 min at 4°C.
 - **B1.6a** Transfer the supernatant to a new tube and keep it on ice for 20 min.
 - **B1.7a** Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 AU calculation - Input lysate quantification). If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing.

Suspension Cells lysis

- **B1.1b** Treat the cells with 10 µg/mL of **cycloheximide (CHX)** for 5 min at 37°C before lysis, should you not wish to add CHX check Appendix 2 for the alternative protocol. We recommend using cells at 70-80% confluence. CHX treatment is suggested – but it is not mandatory - to increase the efficiency of the ribosomes’ affinity purification. CHX treatment could induce the accumulation of ribosomes within the first 10 codons. Should you not wish to add CHX check Appendix 2 for the alternative protocol.
- **B1.2b** Collect the cells and centrifuge at 950g for 5min at 4°C, remove the media, and wash with **cold PBS** containing CHX (20 µg/mL).
- **B1.3b** Collect and centrifuge at 950g for 5 min at 4°C. Remove the supernatant completely.
- **B1.4b** Resuspend cell pellet in **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.3 - Input lysate preparation and quantification - & Table 3).
- **B1.5b** Lysate cells by passing them through a G26 needle ~20 times (please note that if the volume is below 50 µL, using the syringe will lead to the loss of specimen, as a possibility you could pipette up and down ~20 times avoiding creating bubbles).

- **B1.6b** Pellet the nuclei and cell debris by centrifugation at 20000 g for 5 min at 4°C.
- **B1.7b** Transfer the supernatant to a new tube. Leave on ice for 20 min.
- **B1.8b** Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 AU calculation - Input lysate quantification). If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing.

Tissues lysis

- **B1.1c** Pulverize the tissue under liquid nitrogen with mortar and pestle. Recover the powder in a 1.5 mL tube.
- **B1.2c** Resuspend with 800 µL of **Tissues Lysis Buffer** (not included - IMMAGINA catalog no. #IBT0032) supplemented as per instruction in the section “Before starting the experiment – Lysis Buffer Supplementing” & Table 3. Please note that both Tissues Lysis buffer and W-buffer contain CHX (20 µg/mL).
- **B1.3c** Centrifuge at max speed (20000 g) for 2 min at 4°C to remove tissue and membrane debris and collect the supernatant.
- **B1.4c** Centrifuge again the supernatant for 5 min at max speed (20000 g) at 4°C and collect the supernatant. Keep on ice for 20 min.
- **B1.5c** Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 AU calculation - Input lysate quantification). If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing.

Step B2. BEADS FUNCTIONALIZATION

⚠ DO NOT LET THE BEADS DRY OUT AT ANY POINT!

⚠ First-time opening of the kit – RiboLace Smart Probe (RsP) dilution and aliquoting.

The RiboLace smart probe (RsP) present in the kit are concentrated and are NOT intended to be used without proper dilution. Before starting the experiment, as first time opening the kit, please thaw on ice the 200 μ L of concentrated RiboLace smart probe (RsP) and add 800 μ L of B-buffer (4°C, you can keep it on ice during the procedure) to create the diluted RiboLace smart probe (dRsP). To avoid more than two freeze-thaw cycles, we suggest aliquoting the diluted probe and storing the solution at -80°C in ready-to-use aliquots. For simplicity, we suggest making 166 μ L aliquots as this approach allows you to conduct two experiments per aliquot with a lysate input of 0.9 AU per experiment.

Beads functionalization steps:

The amount of beads that need to be functionalized per experiment depends on the number of samples it is composed of and on the concentration of the lysate of the samples (expressed in AU). For clarity, the following steps refer to one reaction at the canonical concentration of 0.9 AU per sample. For multiple samples, it is possible to functionalize beads for more than one reaction in one single tube (within its capacity). To ensure an effortless and thorough process we suggest you print the checklist in Appendix 5, fill it with your specific volumes, and mark each completed step during the manipulation.

- B2.1** Remove the **RiboLace magnetic beads (RmB)** from 4°C and place the tube at RT for at least 30 min.
- B2.2** Vortex the RiboLace magnetic beads (RmB) tube thoroughly for > 30 sec.
- B2.3** Put 144 μ L of RiboLace magnetic beads (RmB) in a new 1.5 mL tube. Place the tube on a magnet to separate the RmB. Visually inspect that all the beads are attached to the magnet and remove the supernatant.
- B2.4** Remove the tube from the magnet and wash the RmB with 270 μ L of **OH-buffer (OH)** for 5 min shaking at 1,400 rpm at RT. Place back the tube and the magnet and remove the supernatant.
- B2.5** Wash with 1000 μ L of **nuclease-free water** by shaking for 2 min at 1,400 rpm at RT, place the tube on the magnet, and remove the supernatant. If RmB are binding to the plastic tube, you can add Triton X-100 to a final concentration of 0.1%.
- B2.6** Wash the RmB with 270 μ L of **B-buffer (BB)**, shaking for 3 min at 1,400 rpm at RT. Place the tube on the magnet for at least 1 minute and remove the supernatant. If RmB are binding to the plastic tube, you can add Triton X-100 to a final concentration of 0.1%. Repeat the wash once again with the same 270 μ L of volume of BB.
- B2.7** Keep at least 2 μ L of diluted RiboLace smart probe (**dRsP**, see “**First Time Opening – RiboLace Smart Probe (RsP) dilution and aliquoting**” above) for security checkpoint (see grey box below).

- **B2.8** Resuspend the RmB beads with 81 μ L of diluted RiboLace smart probe (**dRsP**).
- **B2.9** Incubate for 1h at RT in a shaker at 1,400 rpm. Do not allow beads to sediment.

During the incubation, we suggest starting the Nuclease Digestion (STEP.B3).

- **B2.10** After the incubation, place the tube on a magnet and remove 3 μ L of the supernatant (unbound probe) for the security checkpoint (see below). Keep the remaining volume in the vial.
- **B2.11** Add 7.5 μ L of **mPEG** to the tube and mix in a shaker at 1,400 rpm at RT for 15 min. Do not allow the beads to precipitate.
- **B2.12** Place the tube on a magnet for 2–3 min, discard the supernatant and wash 1000 μ L of **nuclease-free water**, for 2 min with shaking at 1,400 rpm at RT. Put Back on the magnet and remove the supernatant.
- **B2.13** Wash the functionalized RmB beads two times with 1000 μ L of **W-buffer (WB)** for 2 min with shaking at 1,400 rpm at RT. After the first wash, put the tube on the magnet to remove the supernatant before adding the solution. After the second wash, place the tube on the magnet and remove completely the supernatant.
- **B2.14** Resuspend the functionalized RmB beads with 100 μ L of W-buffer (**WB**).
- **B2.15** If the beads were functionalized for more than one reaction, equally divide the functionalized beads in individual tubes according to the (N) number of samples you are processing.

The beads are now functionalized and ready to be placed in contact with the digested lysate. To avoid drying the beads, please, **remove the WB buffer just before adding the digested lysate** (End of **Step B3**, Beginning of Step B4).

 **Security Check Point**

You can check for proper bead functionalization by following the instructions in Appendix 6. This step is optional, and it is useful to validate the proper execution of the above-mentioned functionalization steps.

Step B3. NUCLEASE DIGESTION

The Ribosome Protected Fragments are generated during the Nuclease Digestion step (ribosome footprinting). The suggested Nuclease amount and digestion timing are well-suited for most organisms and tissues. Nonetheless, should you need to perform a titration curve to assess the proper quantity of Nux to add to your (**non-conventional**) sample, you can check the guidelines in Appendix 8 - Optional Nuclease Optimization.

- B3.1** Start with a total volume of lysate corresponding to 0.9 A.U. (260 nm) (see Section A2.3 for calculation) diluted in W-buffer (**WB**) to the final volume of 450 μ L.
- B3.2** Add 0.9 μ L of **Nux Enhancer (NE)**.
- B3.3** Dilute 1.5 μ L of **Nuclease (Nux)** by adding 98.5 μ L W-buffer (**WB**). Pipet up and down 5 times to mix well the diluted Nux solution (dNux).
- B3.4** Digest the sample in a 1.5 mL tube for 45 min at 25 °C with 4.5 μ L of the diluted Nuclease (**dNux**) prepared before. Trash the remaining diluted Nux solution, for experiments performed on other days, prepare fresh diluted Nux.
- B3.5** Stop digestion with 1.5 μ L of **SUPERaseIn** for 10 min on ice.

Step B4. RIBOSOMES PULLDOWN

Remove the W-buffer (WB) from **Step B2.14** only immediately before adding the cell lysate!

- B4.1** Add the **digested cell lysate** to the functionalized beads (to avoid dilution, discard the supernatant of the beads before adding the cell lysate) and mix well.
- B4.2** Incubate for 70 min, on a wheel in slow motion (3-10 rpm) at 4°C.
- B4.3** Remove the tubes from the wheel. **DO NOT CENTRIFUGATE** but allow the entire solution with the beads to settle at the bottom of the tube. If residual solution is present on the lid, pull down the beads by gently flicking down the tube by hand 2 or 3 times. Place the tubes on ice. Place the magnet in an ice bucket before putting the tubes on it.
- B4.4** Keep working on ice and separate the beads with a magnet.
- DO NOT REMOVE THE BEADS FROM THE MAGNET and NEVER TOUCH THE BEADS IN THE NEXT WASHING STEPS.**
- B4.5** Remove the supernatant. Carefully wash the beads twice with 1000 µL W-buffer (**WB**). Do not remove the samples from the magnet. Carefully add the WB on the opposite side of the Eppendorf to where the beads are present. Carefully remove the supernatant without disturbing the beads.
- B4.6** Remove completely the W-buffer (WB) before removing the beads from the magnet and resuspend them in 400 µL of W-buffer (WB).
- B4.6** Transfer the bead suspension to a new nuclease-free 1.5 mL tube.

⚠ Your ribosomes are attached to the beads now, do NOT discard them!




Step B5. RNA EXTRACTION

 **It is important to use the ACID phenol:chloroform to avoid DNA contamination.**

- B5.1** Add 40 μL **SDS 10%** (**SDS**) and 10 μL **Proteinase K (K)** to the bead's suspension and incubate at 37 °C for 75 min.
- B5.2** Add 450 μL **Acid Phenol:Chloroform:Isoamyl Alcohol**.
- B5.3** Vortex and centrifugate at 14,000 x g for 5 min at 4°C.
- B5.4** If there is no phase separation, add 20 μL **NaCl 2M** and repeat the centrifugation.
- B5.5** Keep the aqueous phase and transfer it into a new vial.
- B5.6** Add 1000 μL **Isopropanol** and 2 μL **GlycoBlue**
- B5.7** Mix and incubate at RT for 3 min, then store at -80°C for:
 - at least 2 hours (fast procedure)
 - overnight (safe procedure, recommended for better recovery yields)
- B5.8** Pellet the RNA by centrifugation (20,000 g) for 30 min at 4°C.
- B5.9** Remove the supernatant and wash the pellet once with 1000 μL 70% cold ethanol. Centrifuge for 5 min at 20,000 g, 4°C.
- B5.10** Remove the supernatant and resuspend the pellet in 12 μL of **Nuclease Free Water**.

Step B6. GEL RUN

ALL-IN-ONE RiboLace Pro kit components and additional required materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
B6	15% TBE-Urea polyacrylamide gel	Additionally Required Material				
B6	Gel Loading Buffer II	Additionally Required Material				
B6	25-35 Marker (25-35 M)	# IBT0131	15 µL	-80°C	Vial	 clear
B6	Marker 1 (M1)	# IBT0401	13 µL	-80°C	Vial	 Yellow
B6	SYBR Gold	Additionally Required Material				
B7	Filter tubes	# IBT0391	24 pcs	RT	Bag	
B7	Pierced tubes	# IBT0371	24 pcs	RT	bag	
B7	RNA extraction buffer (REB)	# IBT0381	15 mL	4°C	Bottle	
B7	TR buffer	#IBT0351	500 µL	4°C	Vial	 Yellow
B7	Isopropanol	Additionally Required Material				
B7	GlycoBlue	Additionally Required Material				
B7	Qubit™ microRNA Assay Kit	Additionally Required Material				

NOTE: RNA extraction buffer (REB) contains SDS, thus a cloudy solution might appear when stored at 4°C. If cloudy, before using the solution, warm it at RT and mix it until the solution is clear.

The RNA recovered at the end of Step B5 contains the ribosome-protected fragments (RPFs) that need to be purified after RiboLace pulldown. 1 µL of this RNA can be quantified by Nanodrop and at least 1.5 µg of RNA should be extracted. All the extracted RNA should be run on gel, depending on the retrieved quantity, it should be run in one or multiple lanes. Please run up to 2 µg of extracted RNA in one single lane. E.g., if 3 µg are extracted after pulldown, please divide the sample in two lanes. In case the RPFs are not visible on the gel, please contact our tech support (techsupport@immaginabiotech.com).

- B6.1** Pre-run the gel at 200 V for 30 min in TBE prepared with nuclease-free water. Clean well the gel wells with a syringe to remove UREA residuals before loading the samples.
- B6.2** Prepare samples: add 10 µL of Gel Loading Buffer II to 10 µL of RNA (1:1 volume).

- B6.3** Prepare 25-35 Marker: mix 2 μL of 25-35 Marker, 3 μL nuclease-free water, and 5 μL Gel Loading Buffer II. And prepare Marker 1: mix 1 μL of Marker 1, 4 μL nuclease-free water, and 5 μL Gel Loading Buffer II.
- B6.4** Use also an ultra-low range molecular weight marker as a reference.
- B6.5** Load the samples, the 25-35 Marker and the Marker 1 on 15% TBE-urea polyacrylamide gel and run the gel at 200V until the bromophenol blue band reaches the bottom of the gel (about 50 min to 1 hour).
- B6.6** Stain the gel for 5 minutes with SYBR Gold in TBE and visualize the RNA using a UV-Transilluminator and proceed with the PAGE gel extraction.

Step B7. PAGE EXTRACTION OF THE RPF

- **B7.1** After gel visualization, please note that a signal between 25 nt and 35 nt should be visible. Bands present in this area belong to the 80S ribosome-protected fragments (RPFs) and their presence in the gel indicates a proper ribosome pulldown (Figure 2).

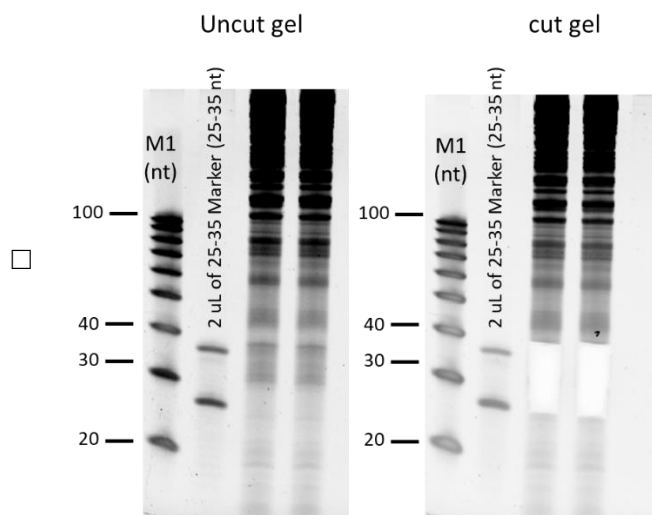




Fig.2 RNA extracted after pulldown run on 15% TBE Urea gel. The RPFs are ~25-35 nt in length. The 25-35 Marker is a mix of two oligos 25 nt and 35 nt in length. It can be used as a size marker. Please note that the part that needs to be cut is between 25-35 depicted in the white rectangle on right gel.

- **B7.2** RPFs need to be extracted from gel by size selection of the RPF between 25-nt and 35-nt according to the marker M1 and 25-35 Marker (white rectangle removed from Fig.2 right).
- **B7.3** Place each gel slice in a pierced tube (provided) and nest the pierced small tube inside a 1.5-ml RNase-free Microcentrifuge tube (provided). Spin at maximum speed for 3 min at RT. If part of the gel pieces is still in the pierced tube, repeat the spinning. In any case, transfer carefully any remaining gel debris from the pierced tube, discard the pierced tube and keep the 1.5 ml microcentrifuge tube.
- **B7.4** Add 400 μ L of **RNA Extraction Buffer (REB)**, close the vial with the provided cap and incubate the tubes for 1 hour at - 80°C. Thaw the tubes at RT and then place the samples on a wheel in slow motion (3-10 rpm), at RT overnight.
- **B7.5** With a 1 mL cut-tip, add the gel slurry to the provided filter tube and spin at 1,000g for 3 min at RT to remove the gel debris. Transfer the eluted solution to a new tube.
- **B7.6** Add 700 μ L of isopropanol and 1.5 μ L GlycoBlue to the eluted sample.
- **B7.7** Store at - 80°C for 2h (fast procedure) or overnight (safe procedure).
- **B7.8** Thaw the samples on ice and pellet the RNA by centrifugation at 20,000g for 30 min at 4°C.
- **B7.9** Remove the supernatant and wash the pellet once with 500 μ L of 70% cold ethanol. Centrifuge for 5 min at 20,000g, 4°C.
- **B7.10** Remove the supernatant being careful to get rid of all ethanol residues. Resuspend the pellet in 12 μ L TR buffer.
- **B7.11** Quantify the RPFs (2 μ L) using a Qubit™ microRNA Assay Kit.

C. RPF's LIBRARY PREPARATION

ALL-IN-ONE RiboLace Pro components and additional required materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color	
C1	Buffer BL1 (BL1)	#IBT0151	100 µL	-20°C	vial		Red
C1	L1 enzyme (L1)	#IBT0161	15 µL	-20°C	vial		Red
C1	ATP 10 mM	#IBT0171	100 µL	-20°C	vial		Red
C1/C2/C3	RNA Clean & Concentrator™-5	Additionally Required Material					
C1/C2/C3/C5	Nuclease Free Water	Additionally Required Material					
C2	Buffer L2 (BL2)	#IBT0181	60 µL	-20°C	vial		Blue
C2	L2 enzyme (L2)	#IBT0191	15 µL	-20°C	vial		Blue
C2	MnCl ₂	#IBT0211	30 µL	-20°C	vial		Blue
C2	GTP	#IBT0201	20 µL	-20°C	vial		Blue
C2	Linker MC (1 µM) (MC)	#IBT0221	30 µL	-80°C	vial		Blue
C3	Buffer L3 (BL3)	#IBT0231	50 µL	-20°C	vial		Yellow
C3	L3 Enzyme (L3)	#IBT0241	15 µL	-20°C	vial		Yellow
C3	PEG 8000 (PEG)	#IBT0251	300 µL	-20°C	vial		Yellow
C4	Primer RT_T (RT_T)	#IBT0261	20 µL	-20°C	vial		Green
C4	Buffer L4 (BL4)	#IBT0271	75 µL	-20°C	vial		Green
C4	L4 enzyme (L4)	#IBT0281	15 µL	-20°C	vial		Green
C4	dNTPs	#IBT0301	20 µL	-20°C	vial		Green
C4	DTT	#IBT0291	20 µL	-20°C	vial		Green
C5	L5 enzyme (L5)	#IBT0321	1.5 mL	-20°C	vial		clear
C5	Fw PCR1 (F1)	#IBT0331	20 µL	-20°C	vial		clear
C5	Rev PCR1 (R1)	#IBT0341	20 µL	-20°C	vial		clear
C6	TR buffer (TR)	#IBT0351	0.5 mL	4°C	vial		clear
C1*	3P-RNA 1 µM (RNA)	#IBT0361	20 µL	-80°C	vial		clear

* 3P-RNA 1 µM (RNA) is utilized as control RNA sample for the library preparation (see Appendix.10)

Step C1. 5' PHOSPHORYLATION

- **C1.1** Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

Buffer L1	5µL
ATP (10 mM)	5 µL
L1	1 µL
RNA from step 5.12	10 µL
H ₂ O	29 µL

- **C1.2** Incubate the reaction for 1h at 37 °C in a thermal cycler.
- **C1.3** Purify the reaction through the **RNA Clean & Concentrator™-5 kit**. Perform all steps at room temperature and centrifugation at 10,000-16,000 g for 30 seconds, unless otherwise specified.
- **C1.4** Prepare adjusted RNA Binding Buffer by mixing N x 50 µL of buffer and N x 50 µL of ethanol (95-100%).
- **C1.5** Add 100 µL adjusted RNA Binding Buffer (from step C1.4) to each sample and mix.
- **C1.6** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**
- **C1.7** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. **Discard the flow-through.**
- **C1.8** Add 400 µL RNA Prep Buffer to the column and centrifuge. **Discard the flow-through.**
- **C1.9** Add 700 µL RNA Wash Buffer to the column and centrifuge. **Discard the flow-through.**
- **C1.10** Add 400 µL RNA Wash Buffer to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer. Carefully, transfer the column into a new RNase-free tube.
- **C1.11** Add 7 µL of **nuclease-free water** directly to the column matrix and centrifuge. **Save the flow-through.**

 **SAFE STOPPING POINT** (store at -80°C)

Step C2. ADAPTOR LIGATION

NOTE: When dealing with RPF quantities falling between two predefined categories (e.g., if you have 13 ng), follow these guidelines for selecting the appropriate linker volume:

- If your RPF quantity is between the lower and upper limits of a category (e.g., 10 ng and 20 ng), choose the lower value of the linker (in the 13-ng example, use the linker for 10 ng, which is 0.5 µL).
- When working with intermediate quantities (e.g., 7 ng or 14 ng), opt for a linker volume below the midpoint. For instance, use 0.25 µL for 7 ng and 0.5 µL for 14 ng.
- Once your RPF quantity reaches or exceeds the halfway point of a category (e.g., 9 ng or 18 ng), consider utilizing the upper limit of the linker volume. For example, use 0.5 µL for 9 ng and 1 µL for 18 ng.

These guidelines will help ensure precise and consistent linker volume selection based on your RPF quantities.

- **C2.1** Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

	RPFs amount (25 – 35 nt)			
	5-7 ng	8-14 ng	15-24 ng	25-40 ng
RNA (from Step 7)	6 µL	6 µL	6 µL	6 µL
Buffer BA	1 µL	1 µL	1 µL	1 µL
GTP	0.5 µL	0.5 µL	0.5 µL	0.5 µL
MnCl ₂	0.6 µL	0.6 µL	0.6 µL	0.6 µL
Enzyme Mix A	1 µL	1 µL	1 µL	1 µL
Linker MC 1µM	0.25 µL	0.5 µL	1 µL	2 µL
H ₂ O	0.75 µL	0.5 µL	-	-

- **C2.2** Incubate the reaction for 1h at 37 °C in a thermal cycler.
- **C2.3** Add 40 µL **nuclease-free water**.
- **C2.4** Purify the reaction through the **RNA Clean & Concentrator™-5 kit**. Perform all steps at room temperature and centrifugation at 10,000-16,000 x g for 30 seconds, unless otherwise specified.
- **C2.5** Prepare adjusted RNA Binding Buffer by mixing 50 µL of buffer and 50 µL of ethanol (95-100%).
- **C2.6** Add the 100 µL adjusted RNA Binding Buffer (from step 7.5) to the sample and mix.
- **C2.7** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**
- **C2.8** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

- C2.9** Add 400 µL RNA Prep Buffer to the column and centrifuge. **Discard the flow-through.**
- C2.10** Add 700 µL RNA Wash Buffer to the column and centrifuge. **Discard the flow-through.**
- C2.11** Add 400 µL RNA Wash Buffer to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer.
- C2.12** Carefully, transfer the column into a RNase-free tube.
- C2.13** Add 9 µL of **nuclease-free water** directly to the column matrix and centrifuge. **Save the flow-through.**

Step C3. CIRCULARIZATION

- C3.1** Prepare a 1 mM **ATP** solution by diluting the ATP stock in **nuclease-free water** (e.g. 1 μ L ATP + 9 μ L nuclease-free water). Pipet up and down to mix well the solution. Assemble the following reaction in a 0.2 mL nuclease-free PCR tube:

RNA (from Step 7)	9 μL
Buffer L3	2 μ L
ATP (1mM)	1 μ L
PEG8000*	8 μ L
Enzyme L3	1 μ L

*Please note that PEG8000 is a very viscous solution. Carefully pipette and check that the right amount is in your tip. Possibly add it as the first reagent in the Eppendorf.

- C3.2** Incubate the reaction for 2h at 25 °C in a thermal cycler.
- C3.3** Add 30 μ L **nuclease-free water**.
- C3.4** Purify the reaction through the **RNA Clean & Concentrator™-5 kit**. Perform all steps at room temperature and centrifugation at 10,000-16,000 g for 30 seconds, unless otherwise specified.
- C3.5** Prepare adjusted RNA Binding Buffer by mixing 50 μ L of buffer and 50 μ L of ethanol (95-100%).
- C3.6** Add the 100 μ L adjusted RNA Binding Buffer (from step 8.5) to the sample and mix.
- C3.7** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**
- C3.8** Add 150 μ L of ethanol and mix. Transfer the mixture to a new column and centrifuge. **Discard the flow-through.**
- C3.9** Add 400 μ L RNA Prep Buffer to the column and centrifuge. **Discard the flow-through.**
- C3.10** Add 700 μ L RNA Wash Buffer to the column and centrifuge. **Discard the flow-through.**
- C3.11** Add 400 μ L RNA Wash Buffer to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer.
- C3.12** Carefully, transfer the column into a RNase-free tube.
- C3.13** Add 12 μ L of **nuclease-free water** directly to the column matrix and centrifuge. **Save the flow-through.**

 **SAFE STOPPING POINT** (store at -80°C)

Step C4. REVERSE TRANSCRIPTION


- C4.1** For the generation of single-strand cDNA, combine the following reagents:

Circular RNA (from step 8)	12 μ L
dNTPs	1 μ L
Primer RT_T	1 μ L

- C4.2** Incubate the circular RNA-primer mix at 70°C for 5 minutes and then transfer on ice for at least 1 minute.
- C4.3** Add the following reagents to the annealed RNA-primer mix:

Buffer L4	4 μ L
DTT	1 μ L
L4 enzyme	1 μ L

- C4.4** Incubate for 40 min at 50 °C, then heat-inactivate for 10 min at 80 °C.

 **SAFE STOPPING POINT:** for convenience, samples can be left overnight in the thermal cycler at 4°C, or at –20 °C for **one week**.

Step C5. PCR AMPLIFICATION – PCR 1

- **Note:** the cycles of Step C5 – PCR 1 and Step C6 – PCR 2 depend on the starting RPFs that you used in Step C1. The number of cycles is important for avoiding overamplification, please refer to the following table for the correct number of cycles to utilize.

Table 4 Number of cycles of PCR to use in Step C5 (PCR1) and C6 (PCR2)

	RPFs amount (25 – 35 nt)					
	5 ng	6-9 ng	10-14 ng	15-19	20-34 ng	35-40 ng
PCR 1 cycles	9	9	8	7	7	6
PCR 2 cycles	7	6	6	6	5	5

- **C5.1** Combine the following reagents (for reaction) in a final volume of 100 µL:

cDNA (from step C4.4)	20 µL
L5 enzyme (L5)	50 µL
F1	0.8 µL
R1	0.8 µL
H ₂ O	28.4 µL

- **C5.2** Place the tube(s) in a thermal cycler with a heated lid and run the following program:

Step	Temperature	Time
Initial denaturation	98°C	1 min
6-9 Cycles*	98°C	30 secs
	61°C	30 secs
	72°C	10 secs
	Hold	4°C

*Please refers to Table 4 for correct number of cycles.

- **C5.3** Transfer the reaction mix into a new 1.5 mL tube.
- **C5.4** Purify the PCR reaction by adding 160 µL of **Agencourt AMPure XP** beads (**Warm the beads at Room Temperature before use**) to each sample and mix well by pipetting the entire volume up and down at least 10 times.
- **C5.5** Incubate at room temperature for 5 minutes to let the library bind to the beads.
- **C5.6** Place the tubes on the magnetic rack until the solution is completely clear. While the tubes are still sitting on the magnetic separation device, discard the supernatant with a pipette.
- **C5.7** Keep the tubes on the magnetic rack. Wash the beads by adding 300 µL of 75% ethanol to each sample without disturbing the beads.

- C5.8** Wait for 30 seconds and use a pipette to carefully remove the supernatant containing contaminants. The library will remain bound to the beads during the washing process.
- C5.9** Repeat the washing step with 75% ethanol once, keeping the beads on the magnet.
- C5.10** Let the beads pellet dry on the magnetic rack at room temperature for ~2–4 minutes. **Avoid to over dry the beads (pellet cracked) as this will significantly decrease elution efficiency.**
- C5.11** Remove the tubes from the magnetic rack and add 50 µL of nuclease-free water to cover the pellet. Mix thoroughly by pipetting up and down to ensure complete bead dispersion. Incubate at room temperature for at least 3 minutes to rehydrate.
- C5.12** Place the sample tubes on the magnetic rack for 2 minutes or longer until the solution is completely clear.

Step C6. PCR AMPLIFICATION – PCR 2

- **C6.1** Combine the following reagents for reaction (final volume 100 µL):

PCR1 (from step C5.12)	49 µL
L5 enzyme (L5)	50 µL
LACEseq UDIs (10 µM)	1 µL

- **C6.2** Place the tube(s) in a thermal cycler with a heated lid and run the following program:



Step	Temperature	Time
Initial denaturation	98°C	1 min
5-7 Cycles*	98°C	30 secs
	60°C	30 secs
	72°C	10 secs
Hold	30 secs	∞

*Please refers to Table 4 for correct number of cycles.

- **C6.3** Purify the PCR reaction by using NucleoSpin Gel and PCR CleanUp kit (or equivalent) and following the manufacturer's standard protocol (Section 5.1 of the manual). Elute each sample in 21 µl of TR buffer (TR).

Step C7. GEL RUN

ALL-IN-ONE RiboLace Pro kit components and additional required materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
C7	10% TBE polyacrylamide gel	Additionally Required Material				
C7	DNA loading dye	Additionally Required Material				
C7	Marker 2 (M2)	# IBT0421	13 µL	-80°C	Vial	 Yellow
C7	SYBR Gold	Additionally Required Material				
C8	Filter tubes	# IBT0391	24 pcs	RT	Bag	
C8	Pierced tubes	# IBT0371	24 pcs	RT	bag	
C8	DNA extraction buffer (DEB)	# IBT0411	15 mL	4°C	Bottle	
C8	TR buffer	#IBT0351	500 µL	4°C	Vial	 Yellow
C8	Isopropanol	Additionally Required Material				
C8	GlycoBlue	Additionally Required Material				

- C7.1 Prepare samples:** add 4 µL of 6x DNA loading dye to 20 µL of cleaned-up PCR (from Step 12.3).
- C7.2 Prepare M2 marker:** mix 1 µL M2, 9 µL nuclease-free water and 2 µL of 6xDNA loading dye.
- C7.3 Load the samples** in two separate lanes, while Marker 2 in one lane on 10% TBE polyacrylamide gel. Run the gel for 50 min at 200V. If the loading dye contains xylene cyanol, run the gel till the xylene cyanol reaches the bottom of the gel.
- C7.4 Stain the gel** for 5 minutes with a solution made of 10 mL of TBE and 1.5 µL of SYBR Gold and visualize the RNA using a UV-Transilluminator.

Step C8. LIBRARY EXTRACTION

- **C8.1** Excise the library band at ~ 200- nt according to M2 (see Figure 3); take care not to excise the ~170 nt adaptor dimers band!

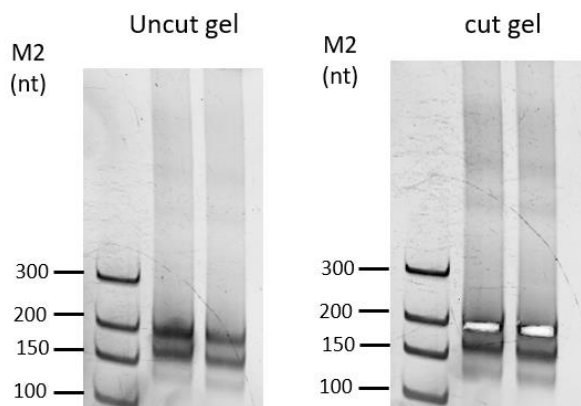


Fig.3 Example of library run on 10% TBE gel. The library is 200 nt length, while the adaptor dimer is 170 nt. Please note that the part that needs to be cut is exactly the band at 200 nt, leaving the whiskies out as depicted in the white rectangle on right gel. The sharper the cut, the lower the quantity of adaptor dimer that remains in the final library.

- **C8.2** Place each gel slice in a pierced tube (provided) and nest the pierced small tube inside a 1.5-ml RNase-free Microcentrifuge tube (provided). Spin at maximum speed for 3 min at RT. If part of the gel pieces is still in the pierced tube, repeat the spinning. In any case, transfer carefully any remaining gel debris from the pierced tube, discard the pierced tube and keep the 1.5 ml microcentrifuge tube.
- **C8.3** Add 400 μ L of **DEB (DNA Extraction Buffer)**, close the vial with the provided cap, incubate the tubes for 1 hour at -80°C . Thaw the tubes at RT and then place the samples on a wheel in slow motion (3-10 rpm), at RT overnight.
- **C8.4** With a 1 mL cut-tip, transfer the liquid and gel slurry into a spin filter (provided) and spin at 1,000g for 3 min at RT to remove the gel debris. Transfer the eluted solution to a new 1.5 ml tube.
- **C8.5** Add 700 μ L of **Isopropanol** and 1.5 μ L **GlycoBlue** to the eluted sample.
- **C8.6** Store at -80°C for 2h (fast procedure) or overnight (safe procedure).
- **C8.7** Thaw the samples on ice and pellet the DNA by centrifugation (20,000g) for 30 min at 4°C .
- **C8.8** Remove the supernatant and wash the pellet once with 500 μ L of 70% cold ethanol. Centrifuge for 5 min at 20,000g, 4°C .
- **C8.9** Remove the supernatant and resuspend the pellet in 11 μ L TR buffer. Proceed with Library Quality Check

Step C9. LIBRARY QUALITY CHECK

- **C9.1** Evaluate each size selected library by **Agilent 2100 Bioanalyzer** using the **Agilent High Sensitivity DNA Kit**.
- **C9.2** Use the library profile results to determine whether each sample is suitable for sequencing. Successful library production should yield a major peak at ~200-220 bp (see Fig. 4). Additional peaks might be observed at about 170-190 bp that originate from adapter dimers. If the peak areas are higher than 50% of the principal 200 bp peak, you need to purify again the libraries from gel before proceeding with sequencing.
- **C9.3** Perform a qPCR analysis using **P5 and P7 primers** on each library for highly accurate library quantification.

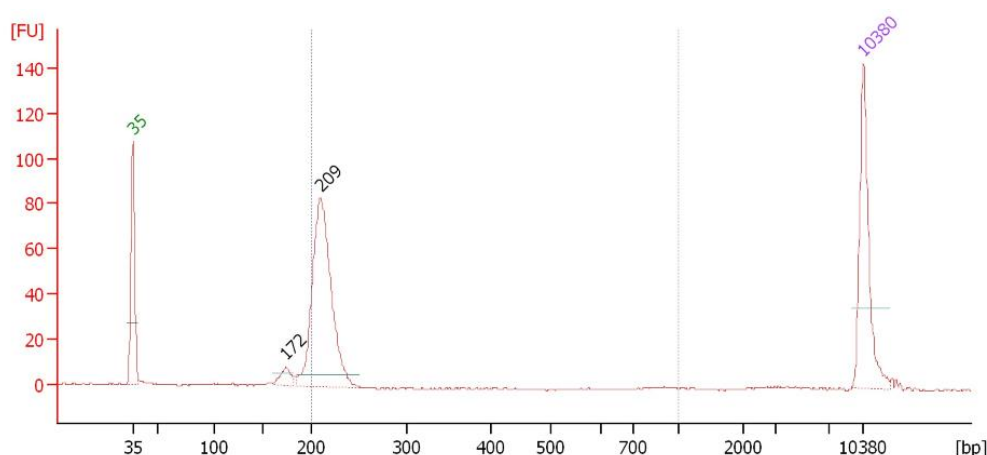


Figure 4. Example electropherogram libraries results. Typical electropherogram for a library prepared with an immortalized cell line. The library was analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The electropherogram need to present at least one major peak between 200 to 220. Tin this example, the peak at 209 bp corresponds to the size of RPFs, while the peaks at 172 bp correspond with the size of adaptor dimers.

D. SEQUENCING

Step D1. HOW TO SEQUENCE – DO IT YOURSELF

The libraries produced are suitable for Illumina platforms, we strongly suggest the use of sequencing platforms based on pattern flow cells such as MiSeq, NovaSeq 6000, and NextSeq 1000/2000. Although compatible, we do not advise the use of open-flow cells such as NextSeq 550, which in our experience yield less satisfactory sequencing runs.

Sequencing lengths shorter than 100 bp should be avoided, although theoretically sufficient, shorter read length may lead to loss of reads during computational analysis. We suggest Single-End sequencing run with depth between 100 and 120 Million reads/sample, and if you would like to observe rare translational events, such as uORF, and ribosome readthrough, to increase the depth to 200 M reads/sample.

To visualize RPFs deriving from the footprint of ribosome disome and trisome the sequencing length must be increased to at least 150 bp or 200bp.

In common practice, Single-End sequencing is less frequently used than Pair-End sequencing, and some facilities are more prone to performing PE sequencing. It is possible to sequence our library in PE (with a length of 150bp) but with a depth of 200 - 240 Million reads, this is because only the Forward Reads are retained for the Data analysis.

For Novaseq 6000 using the XP protocol, we suggest an entry concentration of the pool of 470 pM, while for standard protocol of 700 pM. In general, we prefer adding a 3.5% quantity of PhiX.

For MiSeq the loading concentration of the library pool should be 12 pM, while for NextSeq 1000/2000 we suggest loading the library at 500 pM, with a 10% spike-in of PhiX.

Step D2. HOW TO SEQUENCE – OUTSOURCE TO IMMAGINA

Supporting the whole RiboSeq workflow requirements, IMMAGINA provides sequencing services for RiboSeq NGS libraries, RNAseq libraries, and the required downstream data analysis:

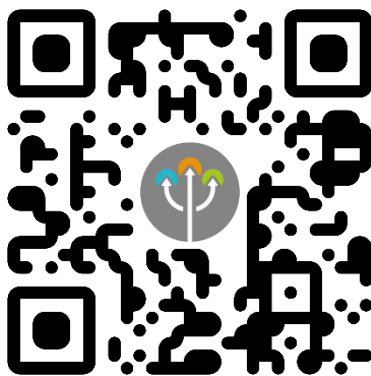
- a basic package for the trimming of the sequencing data, the alignment of the reads and the counts,
- a package for the calculation of the Translation Efficiency,
- a Premium package for the development of ad-hoc analysis.

You can contact us at info@immaginabiotech.com to discuss your needs in detail.

APPENDIX

App.1 Guidelines for sample input amount optimization

For guidelines supporting any custom protocol optimization, please refer to the RiboLace Starter kit protocol available on our website, or by clicking or scanning the following QR code.



Click or scan me to access the RiboLace Starter protocol.

App.2 Alternative CHX removal protocol

It is worth mentioning that CHX treatment could lead to the accumulation of ribosomes within the first 10 codons. Therefore, if you decide to use CHX treatment, be aware of this potential effect on ribosome distribution along the CDS (coding sequence). CHX treatment is recommended, but not mandatory, to enhance the efficiency of ribosome affinity purification. If you choose to avoid CHX treatment, it is crucial to ensure the prompt and proper flash freezing of the sample. Flash freezing helps to preserve the sample's integrity and minimize potential degradation. To achieve this, follow these steps:

- After collecting the sample (e.g., detaching or pelleting the cells), transfer it to a suitable container or tube.
- Pellet the cells and remove the media.
- Wash with cold PBS and remove completely the liquid.
- Place the container in a liquid nitrogen bath or use a dry ice and ethanol mixture for rapid freezing.
- Ensure that the sample is fully submerged in the liquid nitrogen or surrounded by the dry ice mixture to facilitate rapid cooling.
- Allow the sample to freeze rapidly for a few minutes until it reaches a fully frozen state.
- Once the sample is completely frozen, store it at -80°C or in a cryogenic storage system to maintain its stability until further processing.
- Once ready to perform the experiment, defrost the cell pellet in ice and proceed with treating the sample from step 1.4b (lysing the pellet cells in supplemented lysis buffer).

App.3 Lysis buffer supplementation issues

Please check if, after adding Sodium deoxycholate a whiteish and cloudy solution appears. If so, please do not proceed with the lysis of the sample and toss the supplemented LB. Subsequently, warm up the SDC at RT and add it to a new aliquot of the not-supplemented LB. If the whiteish and cloudy solution persists, please contact our tech support (techsupport@imaginabiotech.com).

App.4 Sample Lysis output summary table

The following table allows for recording the amount of sample and the relative amount obtained after the Cell Lysis Step - B1.

Sample number	Sample name	Amount utilized (n° cells or mg of tissue)	SLB V utilized (µL)	AU/mL (after blank subtraction)	AU/µL	V for 0.9 AU (µL)
1						
2						
3						
4						
5						
6						

Table 4. Sample Lysis Output Summary

App.5 Beads functionalization checklist

Reagent	N=1 0.9 A.U	N=___ 0.9 A.U	Needed in Step	Step-by-Step Checklist
			B2.1	<input type="checkbox"/> Place RmB v2-1 at RT for 30 min
			B2.2	<input type="checkbox"/> Vortex 30'
RiboLace magnetic beads (RmB)	144 µL	___	B2.3	<input type="checkbox"/> Add "___" Beads in 1.5 / 2 mL Tube <input type="checkbox"/> Place on magnet <input type="checkbox"/> REMOVE Supernatant
OH-buffer (OH)	270 µL	___	B2.4	<input type="checkbox"/> Remove from magnet <input type="checkbox"/> Wash OH Buffer: add "___" µL <input type="checkbox"/> Shake 5min 1400 rpm RT <input type="checkbox"/> Place on magnet <input type="checkbox"/> REMOVE Supernatant
Nuclease-free water	1000 µL	1000 µL	B2.5	<input type="checkbox"/> Remove from magnet <input type="checkbox"/> Wash N.F. Water: add 1000 µL <input type="checkbox"/> Shake 2min 1400 rpm RT <input type="checkbox"/> Place on magnet <input type="checkbox"/> REMOVE Supernatant
B-Buffer (BB)	270 µL	___	B2.6	<input type="checkbox"/> Remove from magnet <input type="checkbox"/> Wash B Buffer: add "___" µL <input type="checkbox"/> Shake 3min 1400 rpm RT <input type="checkbox"/> Place on magnet <input type="checkbox"/> REMOVE Supernatant <input type="checkbox"/> Repeat wash 2nd time
			B2.7	<input type="checkbox"/> Store 2 µL of diluted RsP for control
Diluted RiboLace Smart Probe (RsP)	81 µL	___	B2.8	<input type="checkbox"/> Remove from magnet <input type="checkbox"/> Resuspend in diluted RsP: "___" µL
			B2.9	<input type="checkbox"/> Incubate 1h shaking 1400 rpm RT
At this point, you can start the Nuclease digestion (step B3) in parallel				
			B2.10	<input type="checkbox"/> Place on magnet <input type="checkbox"/> Store 3 µL of Supernatant for control <input type="checkbox"/> Remove from magnet
mPEG	7.5 µL	___	B2.11	<input type="checkbox"/> Add mPEG "___" µL <input type="checkbox"/> Incubate 15min shaking 1400 rpm RT <input type="checkbox"/> Place on magnet
Nuclease-free water	1000 µL	1000 µL	B2.12	<input type="checkbox"/> REMOVE Supernatant <input type="checkbox"/> Remove from magnet <input type="checkbox"/> Wash with N.F. water: add 1000 µL <input type="checkbox"/> Shake 2 min 1400 rpm RT <input type="checkbox"/> Place on magnet
W-buffer (WB)	1000 µL	1000 µL	B2.13	<input type="checkbox"/> REMOVE Supernatant <input type="checkbox"/> Remove from magnet <input type="checkbox"/> Wash with W buffer 1000 µL <input type="checkbox"/> Shake 2 min 1400 rpm RT <input type="checkbox"/> Place on magnet
W-buffer (WB)	100 µL	___	B2.14	<input type="checkbox"/> REMOVE Supernatant <input type="checkbox"/> Resuspend in "___" µL w-Buffer
			B2.15	<input type="checkbox"/> Aliquot in 105 µL of equal volumes in N tubes

Table 5. Components' volumes to use for the Bead Functionalization Step B2. N = number of reactions. The table is intended as a guideline to follow when dealing with non-standard bead amounts and multiple samples.

App.6 Check proper beads functionalization (for Step B2 – Beads functionalization)

Comparing the difference in the absorbance measured at A 270 nm (Nanodrop ND-1000) for the unbound probe (collected in Step B2.10) and the starting solution of the diluted RiboLace smart probe (RsP) (collected in Step B2.7) allows an estimation of the binding efficiency.

$$\text{reduction in \%} = \left(1 - \frac{\text{Step B2.7 } A_{270}}{\text{Step B2.10 } A_{270}}\right) * 100$$

Between 10% and 50% absorbance reduction in the unbound probe compared to the starting solution is expected. If the decrease in absorbance is not observed, please incubate beads for up to 2 hours and check again the absorbance.

Sample number	Sample name	AU 270 Before Step B2.7	AU 270 After Step B2.10	Reduction %
1				
2				
3				
4				
5				
6				

Table 6. *Sample beads functionalization summary*

App.7 Nuclease digestion checklist

Table 7 provides the output from Step Lysis B1 with the possibility, if needed, to list the amount of W-buffer to reach the reaction amount.

Sample number	Sample name	AU/ μ L	V for 0.9 AU (μ L)	V of W-Buffer up to 450 (μ L)
1				
2				
3				
4				
5				
6				

Table 7. Summary of the volumes to utilize for the dilution of lysate Step B3.

Table 8 is intended as a guideline to follow for digesting the lysate.

Reagent	0.9 A.U	Needed in Step	Step-by-Step Checklist
W-Buffer	_____	B3.1	<input type="checkbox"/> If needed, dilute the lysate calculated following Step A2.1 in W-Buffer up to "_____"
Nux Enhancer (NE)	0.9 μ L	B3.2	<input type="checkbox"/> Add 0.9 μ L of NE to the lysate
Nux (Nux) + W-Buffer		B3.3	<input type="checkbox"/> Dilute 1.5 μ L of Nux in 98.5 μ L W-buffer to create diluted Nux (dNux)
Diluted Nux (dNux)	4.5 μ L	B3.4	<input type="checkbox"/> Add 4.5 μ L of dNux to the lysate <input type="checkbox"/> Incubate 45 min at 25°C
SUPERase•In	1.5 μ L	B3.5	<input type="checkbox"/> Stop the reaction by adding 1.5 μ L of SUPERase•In into the lysate <input type="checkbox"/> Incubate for 10 min on ice

Table 8. Components' volumes to use for the Digestion of lysate Step B3. The table is intended as a guideline to follow when dealing with non-standard nuclease amounts.

App.8 Optional Nuclease optimization (for Step B3 – Nuclease Digestion)

The quantity of Nuclease (Nux) to utilize for lysing the sample could be optimized before proceeding with the pulldown. The kit contains a concentrated vial of Nux (#IBT0091) that is intended to be diluted before use, and that needs to be added to the lysate sample in a fixed quantity, depending on the amount of AU as starting material. This quantity is suitable for most cell lines; however, it can be modulated depending on the needs and type of specimen. To optimize this quantity, after lysing the sample in Step B1, start with 0.4 AU as the starting material and W-buffer up to a final volume of 300 µL. Perform a titration assay, by adding to each reaction different quantities of Nux, below is an example:

Starting lysate	Quantity of diluted Nux (Step. 3.3)	Sample
0.4 AU	0	NT
0.4 AU	0.4 µL (AU x 1)	A
0.4 AU	2 µL (AU x 5)	B
0.4 AU	20 µL (AU x 50)	C

Table 9. Components' volumes to use for the Nuclease Optimization of lysate. The table is intended as a guideline to follow when dealing with non-standard samples that require ad hoc digestion.

- Digest the samples for 45 min at 25°C.
- Stop digestion with 1 µL of SUPERaseIn for 10 min on ice.
- Add 30 µL SDS 10% and 5 µL of Proteinase K and incubate at 37°C for 75 min.
- Add 310 µL of Acid Phenol:Chloroform:Isoamyl Alcohol.
- Vortex and centrifugate at 14,000 x g for 5 min.
- Keep the aqueous phase and transfer it into a new vial.
- Add 500 µL Isopropanol and 2 µL GlycoBlue.
- Mix and incubate an RT for 3 min, then store at -80°C for 2 hours.
- Pellet the RNA by centrifugation (20000g) for 30 min at 4°C.
- Remove the supernatant and wash the pellet once with 70% cold ethanol. Centrifuge for 5 min at 20000g, 4°C.
- Resuspend the pellet in 10 µL of Nuclease Free Water.
- Extracted RNA needs to be run on a 15% TBE-urea gel.
- Pre-run the gel at 200 V for 30 min in TBE prepared with nuclease-free water. Clean well the gel wells with a syringe to remove UREA residuals before loading the samples.
- Prepare samples: add Gel Loading Buffer II to 1.5 µg of RNA (1:1 volume).
- Use an ultra-low range molecular weight marker as reference.
- Load the samples and the Marker on 15% TBE-urea polyacrylamide gel and run the gel for 1 h at 200V until the bromophenol blue band reaches the bottom of the gel.

- Stain the gel for 5 minutes with SYBR Gold in TBE and visualize the RNA using a UV-Transilluminator.

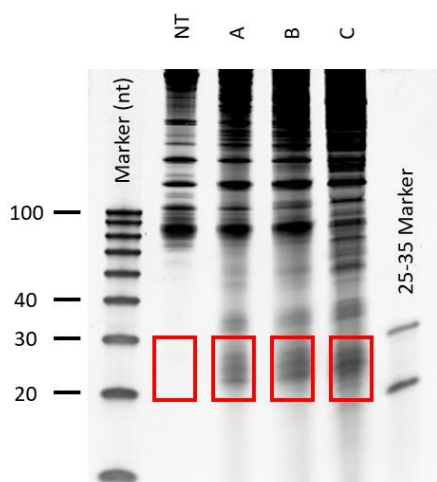


Fig.5 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel. In the red square the sizes between 25-35 nt.

As depicted in the figure above, not digested sample does not present the typical enrichment of fragments at 25-35 nt (red square in Fig.6). Under-digested sample (A) does not display a high enrichment of RPFs, while the over-digested sample (C) shows a smear of signal on the gel. In this example, the quantity of Nux used in sample B (AU x 5) needs to be utilized for all the reactions.

By conducting the titration assay, you can determine the ideal amount of Nux required for efficient lysis of your sample according to your specific needs and specimen characteristics. Furthermore, if nucleic acid from your non-digested (NT) sample is degraded, you may observe a ladder-like pattern of bands below 40 nt. In such cases, it is advisable to restart the experiment since the poor quality of the sample can significantly impact the results. It is crucial to ensure that the sample's integrity is maintained for reliable and accurate data during the Ribo-seq experiment.

App.9 Library Preparation: Low Amount of Starting Material – Troubleshooting Guidelines

Day one of this protocol ends with the pull-down or the RNA fraction containing the RPF fragments that need to be extracted from gel (end of section B) to be used for the production of the NGS libraries (beginning of section C). To properly enter the library preparation part of the protocol you should have at least 1.5 µg of pull-down RNA and consequently 5 ng of extracted RPF, this amount is required to compensate for the losses occurring during the multiple purification steps required to produce a library that can be sequenced.

In the case of low RNA extraction after pull-down, the suggested approach is to repeat the pull-down with an increased amount of AU by either loading a larger amount of lysate (if available with enough volume and concentration) or restarting the workflow from the beginning with a higher amount of starting material and rescale the volume of reagents for the pull-down accordingly. Please note that this will reduce the number of pull-down reactions that are possible to perform with the kit.

Example:

Reagent	Lysate input	Standard Amounts	30% increase	Needed in Step
		0.9 AU	1.2AU	
RiboLace magnetic beads (RmB) v2-1		144 µL	187 µL	B2.3
OH-buffer (OH)		270 µL	351 µL	B2.4
Nuclease-free water		1000 µL	1000 µL	B2.5
B-Buffer (BB)		270 µL	351 µL	B2.6
Diluted RiboLace Smart Probe (RsP)		80 µL	104 µL	B2.8
mPEG		7.5 µL	10 µL	B2.11
Nuclease-free water		1000 µL	1000 µL	B2.12
W-buffer (WB)		1000 µL	1000 µL	B2.13
W-buffer (WB)		105 µL	105 µL	B2.14
Lysate		0.9 AU	1.2AU	B3.1

For further optimization, you can download the RiboLace Starter Protocol for a complete guide to the optimization of the reaction volumes (see Appendix 1).

App.10 Library Preparation: Positive control

In the kit is present a positive control reaction for library preparation. The 3P-RNA 1 μ M (RNA) is an exogenous RNA fragment of known sequence, with a length comparable to the RPF's and a 3'P extremity. Mimicking an RPF molecule allows to carry on the multiple reaction and purification steps required for the production of the library.

Please quantify 1 μ L of the 3P-RNA 1 μ M (RNA) using a Qubit™ microRNA Assay Kit and use a volume equal to 10 ng of it to start the library preparation from Step C1. 5' PHOSPHORYLATION.

During gel run in Step C8. LIBRARY EXTRACTION, two bands of similar quantity should be visible as depicted in the image (Fig.3 page 33), while after extraction of the library, only the sharp peak between 200-220 should be recorded via Agilent 2100 Bioanalyzer (Fig.4 page 34).

In any case, we recommended to evaluate the library by Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit both before and after gel run to check the presence of the library and the adaptor dimer content.

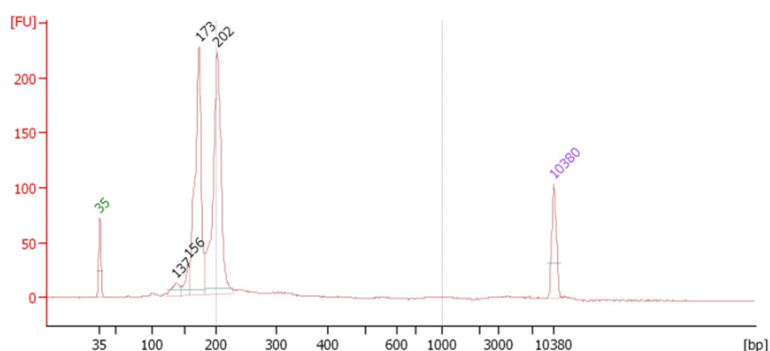


Figure 6. Example electropherogram libraries results before size selection. Typical electropherogram for a library prepared with RPFs obtained from an immortalized cell line with RiboLace kit. The library was analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The electropherogram presents a peak between 200 to 220. In this example, the peak at 202 bp corresponds to the size of RPFs, while the peaks at 173 bp correspond with the size of adaptor dimers.

Contacts



Info

info@immaginabiotech.com

Sale support (quoting, ordering, and order status update)

orders@immaginabiotech.com

Technical service (technical inquiries and quality complaints)

techsupport@immaginabiotech.com



Viale Dell'industria, 47, 38057, Pergine Valsugana (TN), ITALY



www.immaginabiotech.com



+39 04611787270

Notes:
