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, NovaSeq X Plus).

Suitable for: Eukaryotic cell lines and tissues

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

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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
Filters and tubes	1 bag	RT
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 Immagin'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 eliminate 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. For specimens with very high translation levels (such as HeLa, HEK and CHO), 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://www.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 RPFs. 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. Ribosomes are thus purified by affinity purification and magnetic separation, and the ribosomes are extracted from the ribosomal complex. 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 of nuclease cleavage, LaceSeq™ exploits it to specifically target the RPFs and drive their preferential uptake into the NGS library.

After the initial selective ligation between the RPFs 3'P end and our Linker, a second specific intra-molecular ligation is initiated. The kinetic of the second ligation ensures lower incorporation of RNA contaminants. The original proof-of-concept of the technology was published on NAR in 2021 under the "circAID" name since the technology was originally developed for nanopore sequencing.

The structure of the LaceSeq™ Linker has also been optimized to support downstream processes of NGS sequencing and Data analysis by a sharp trimming of the 5'-end of the RPFs. 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 Gel free includes the Illumina Index Primer - UDI (48 rxns) plate (Cat. no. # UDI-0Z-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://www.immaginabiotech.com/ for a complete overview of our products & services and our proprietary technologies.

WORKFLOW OVERVIEW

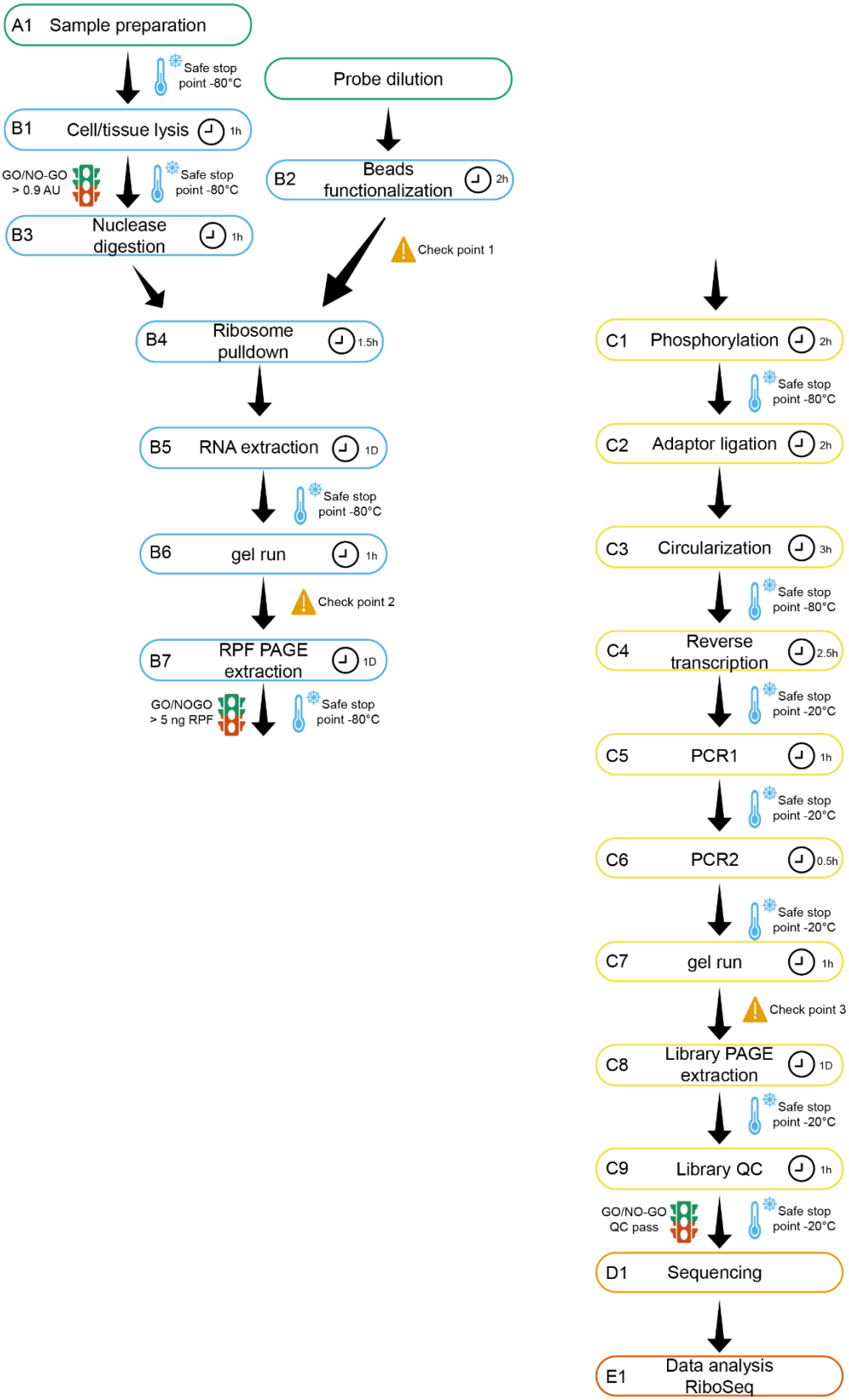


Fig.1 Overview of the ALL-IN-ONE RiboLace Pro Kit workflow. *In each box, steps are present in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures), GO/NO-GO conditions (traffic lights), and a checkpoint (caution 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 between 1 to 1.5 µg of RNA, and after PAGExt extraction, at least 5 ng to start with the preparation of the library. The protocol provides guidelines for different RPFs amount recovered after PAGExt 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.
- Before starting the experiment, please check if a newer version of the protocol is present by visiting our website.

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 Abs260nm) 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 RPFs pulldown.
- The second, is the amount of RNA retrieved after Ribosome & RPFs 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. For tissues (such as liver and brain) we suggest starting with 30 mg of material.

Given specimen-to-specimen variability, as a preliminary experiment, we suggest testing the lysis step on different sample amounts, recording the corresponding total A.U., and using it to fine-tune volumes and sample size during the real experiment (See Table 2 for lysis buffer volumes).

After RPF pulldown (end of day 1) we expect to obtain between 1 to 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 A.U. of your sample is measured using a spectrophotometer, most commonly a Nanodrop. Set the instrument to measure the absorbance at 260 nm (Abs260nm; usually Nucleic Acid function) and measure the absorbance of both your lysate and the Supplemented Lysis Buffer (SLB) using water as blank (see Before starting the experiment – Lysis Buffer Supplementing & Table 3). After recording the absorbance of both the SLB and the lysate, subtract the absorbance of the SLB to the lysate. The use of different lysis buffers is strongly discouraged because it may interfere with the efficiency of ribosome pull-down and with the A.U. calculation (some components may absorb at 260 nm).

Example:

- ☐ *Supplemented Lysis buffer SLB Abs260nm = 7 AU*
- ☐ *Specimen Abs260nm = 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	# IBT0033	50 µL
Cell	1 – 5 million cells	# IBT0033	150 µL
Cell	> 5 million cells	# IBT0033	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's 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 required volume of lysate 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: 0.9AU/0.01 AU/µL = 90 µL 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: 0.9AU/0.004 AU/µL = 225 µL 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)	# IBT0033	18 mL	-20°C	Bottle	--
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	Binding Buffer (BB)	# IBT0021	10 mL	4°C	Bottle	--
B2	RiboLace magnetic beads v2.1 (RmB v2.1)	# IBT0042	1.8 mL	4°C	Vial	clear
B2	OH Buffer (OH)	# IBT0051	10 mL	4°C	Bottle	--
B2	mPEG	# IBT0061	260 µL	-20°C	Vial	clear
B2/B5	Nuclease free water	Additionally Required Material				
B2*	RiboLace smart Probe (RsP)	# IBT0012	200 µL	-20°C	Vial	clear
B2	diluted RiboLace smart Probe (dRsP)	Dilute Aliquot from RsP		-80°C		
B2/B3/B4	Wash 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	15 µL	-20°C	Vial	clear
B3	SUPERaseIn	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/µL	RiboLock RNase Inhibitor 40 U/µL	Final Volume
N=1	267 µL	30 µL	1.5 µL	1.5 µL	300 µL
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. Please note that the Lysis Buffer # IBT0033 and the Wash buffer # IBT0071 contain 10 µg/mL of CHX while the Tissue Lysis Buffer # IBT0032 contains 100 µg/mL of CHX.

Adherent Cells lysis

☐ **B1.1a** Treat the cells with 10 µg/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 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** Either trypsinize the cells according to standard protocols for your cell lines of interest or directly scrape them in PBS/detaching solution. Please avoid using any detaching reagent containing EDTA which could damage the ribosomal integrity.

1) **MECHANICAL SCRAPING.** 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 PBS/detaching solution needed.

- 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.

2) **TRYPSINIZATION**. To ensure good lysis, follow these guidelines for trypsinization:

- Wash cells quickly with cold PBS containing CHX (20 µg/mL).
- Remove PBS and cover the cells in the desired amount of trypsin.
- Place at 37°C and wait until the cells start detaching, checking under the microscope.
- Stop trypsin action by adding desired amount of complete medium.
- Wash the plate thoroughly to remove all attached cells, then transfer everything into a tube for further processing.

☐ **B1.3a** Centrifuge cells at 950 x g (*this value can be adjusted according to the cell line used*) for 5 minutes at 4°C. Remove the media and wash the pellet with **cold PBS** containing CHX (20 µg/mL).

☐ **B1.4a** Centrifuge cell pellet at 950 × g (*this value can be adjusted according to the cell line used*) for 5 minutes at 4°C. Remove supernatant completely. **IMPORTANT: It is crucial to remove all the leftover PBS, to avoid sample dilution during lysis.**

☐ **B1.5a** Perform the lysis directly by adding the **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.2.2 - Input lysate preparation and quantification - & Table 2) to the cell pellet and resuspending with P200 pipette. Avoid creating bubbles.

☐ **B1.6a** Collect the cell lysate in a 1.5 mL microcentrifuge tube and pellet the cell debris and nuclei by centrifugation at 20,000 x g for 5 min at 4°C.

☐ **B1.7a** Transfer the supernatant to a new tube and keep it on ice for 20 min.

☐ **B1.8a** Check the absorbance of the cell lysate and the supplemented lysis buffer at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of NFW as blank. After recording the absorbance of both the SLB and the lysate, subtract the absorbance of the SLB to the lysate (for troubleshooting check A.3 A.U. calculation - Input lysate quantification). If the sample is not processed on the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing. Appendix 4 can be used to take notes of the volumes used for sample lysis and relative A260 absorption.

Suspension Cells lysis

☐ **B1.1b** Treat the cells with 10 µg/mL of **cycloheximide (CHX)** for 5 min at 37°C before lysis. CHX treatment is suggested 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 950 x g (*this value can be adjusted according to the cell line used*) for 5min at 4°C, remove the media, and wash with cold PBS containing CHX (20 µg/mL).
- ☐ **B1.3b** Collect and centrifuge at 950 x g (*this value can be adjusted according to the cell line used*) for 5 min at 4°C. Remove the supernatant completely. **IMPORTANT: It is crucial to remove all the leftover PBS, to avoid sample dilution during lysis.**
- ☐ **B1.4b** Resuspend cell pellet in **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.2.2 - Input lysate preparation and quantification - Table 2).
- ☐ **B1.5b** Perform the lysis directly by adding the complete **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.3 - Input lysate preparation and quantification - & Table 3) to the cell pellet and resuspending with P200 pipette. Avoid creating bubbles.
- ☐ **B1.6b** Pellet the nuclei and cell debris by centrifugation at 20,000 x 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 and the supplemented lysis buffer at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of NFW as blank. After recording the absorbance of both the SLB and the lysate, subtract the absorbance of the SLB to the lysate (for troubleshooting check A.3 A.U. calculation - Input lysate quantification). If the sample is not processed on the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing. Appendix 4 can be used to take notes of the volumes used for sample lysis and relative A260 absorption.

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 **Tissues Lysis Buffer** (not included - Immagina catalog no. #IBT0032; for resuspension volumes check the guidelines in section A.2.2 - Input lysate preparation and quantification -Table 2) supplemented as per instruction in the section “Before starting the experiment – Lysis Buffer Supplementing” & Table 3.
- ☐ **B1.3c** Centrifuge at max speed (20,000 × 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 (20,000 × g) at 4°C and collect the supernatant. Keep on ice for 20 min.
- ☐ **B1.5c** Check the absorbance of the cell lysate and the supplemented lysis buffer at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of NFW as blank. After recording the absorbance of both the SLB and the lysate, subtract the absorbance of the SLB to the lysate (for troubleshooting check A.3 A.U. calculation - Input lysate quantification). If the sample is not processed on the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing. Appendix 4 can be used to take notes of the volumes used for sample lysis and relative A260 absorption.

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 is concentrated and is NOT intended to be used without proper dilution. Before starting the experiment, as first time opening the kit, please thaw on ice the 200 µL of concentrated RiboLace smart probe (RsP) and add 800 µL of Binding 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 µL 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 µL 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 µL 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 µL 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 µL of **Binding 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 µL of volume of BB.
- ☐ **B2.7** Keep at least 2 µL 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 µL 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 Nuclease Digestion (STEP.B3).

☐ **B2.10** After the incubation, place the tube on a magnet and remove 3 µL of the supernatant (unbound probe) for the security checkpoint (see below). Keep the remaining volume in the vial.

☐ **B2.11** Add 7.5 µL 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 µL 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 µL of **Wash 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 µL of Wash 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 RPFs 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, please note that the concentration of the nuclease is critical for the outcome as using the incorrect quantity might lead to varying effects on the read length distribution. You can use Appendix 8 as a guide for the digestion process.

⚠ *If after sequencing you notice that the readlength distribution of your samples is not showing a major peak around 30-35 nt, you may consider increasing the Nux amount for digestion of your samples. Please refer to Appendix 8 for expected readlength distribution.*

- ☐ **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 Wash buffer (**WB**) to the final volume of 450 µL.
- ☐ **B3.2** Add 0.9 µL of **Nux Enhancer (NE)**.
- ☐ **B3.3** Dilute 2 µL of **Nuclease (Nux)** by adding 18 µL Wash Buffer (**WB**). Pipet up and down 5 times to mix well the diluted Nux solution (dNux). The 20 µL of dNux prepared will be enough to process 2 samples in one single experiment. If you plan to process only 1 sample, you can dilute 1 µL Nux by adding 9 µL Wash Buffer (WB)
- ☐ **B3.4** Digest the sample in a 1.5 mL tube for 45 min at 25 °C with 9 µL 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 µL of **SUPERaseIn** for 10 min on ice.

Step B4. RIBOSOMES PULLDOWN

Remove the Wash 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 a 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 Wash 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).

☐ **B4.7** 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 x 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 x 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).

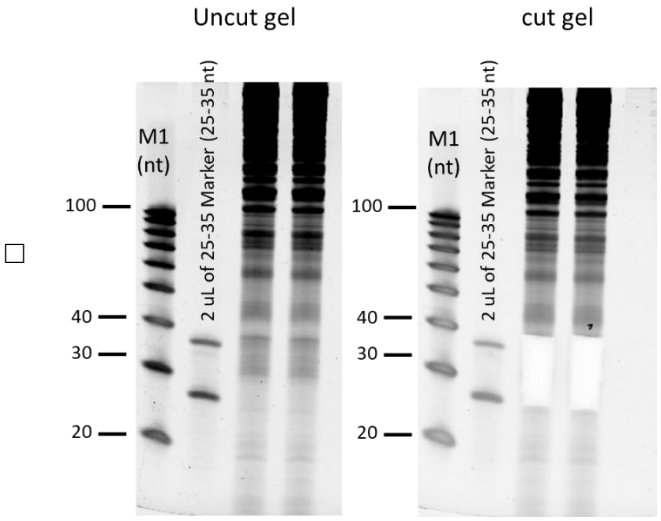


Fig.2 RNA extracted after pulldown run on 15% TBE Urea gel. *The RPFs are ~25-35 nt in length. The 25-35 Marker is a mix of two oligos 25 nt and 35 nt in length. It can be used as a size marker. Please note that the part that needs to be cut is between 25-35 depicted in the white rectangle on right gel.*

☐ **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 µL 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 1000 x g for 3 min at RT to remove the gel debris. Transfer the eluted solution to a new tube.

☐ **B7.6** Add 700 µL of isopropanol and 1.5 µL 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 20,000 x g for 30 min at 4°C.

☐ **B7.9** Remove the supernatant and wash the pellet once with 500 µL of 70% cold ethanol. Centrifuge for 5 min at 20,000 x g, 4°C.

☐ **B7.10** Remove the supernatant being careful to get rid of all ethanol residues. Resuspend the pellet in 12 µL TR buffer.

☐ **B7.11** Quantify the RPFs (2 µL) 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	**2 columns/sample**			
C1/C2/ C3/C5	Nuclease Free Water	Additionally Required Material				
C2	Buffer L2 (BL2)	#IBT0181	100 µL	-20°C	vial	Blue
C2	L2 enzyme (L2)	#IBT0191	15 µL	-20°C	vial	Blue
C2	MnCl2	#IBT0211	30 µL	-20°C	vial	Blue
C2	GTP	#IBT0201	30 µL	-20°C	vial	Blue
C2	Linker MC+ (0.1 µM) (MC+)	#IBT0222	50 µL	**-80°C**	vial	Blue
C3	Buffer L3 (BL3)	#IBT0231	100 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 L4 (PL4)	#IBT0262	50 µL	-20°C	vial	Green
C4	Buffer L4 (BL4)	#IBT0271	100 µL	-20°C	vial	Green
C4	L4 enzyme (L4)	#IBT0281	15 µL	-20°C	vial	Green
C4	DTT	#IBT0291	50 µL	-20°C	vial	Green
C4	dNTPs	#IBT0301	40 µL	-20°C	vial	Green
C4	AR Enzyme (AR)	#IBT0311	10 µL	-20°C	vial	Green
C5	L5 enzyme (L5)	#IBT0321	1.5 mL	-20°C	vial	clear
C5	Fw PCR1 (F1)	#IBT0331	50 µL	-20°C	vial	clear
C5	Rev PCR1 (R1)	#IBT0341	50 µL	-20°C	vial	clear
C5	Agencourt AMPure XP	Additionally Required Material				

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
C6	Illumina Index Primer - UDI (48 rxns)	UDI-0Z-48		-20°C	plate	
C6	NucleoSpin Gel and PCR CleanUp kit	Additionally Required Material				
C6	TR buffer (TR)	#IBT0351	500 µL	4°C	vial	Clear
C7	Agilent 2100 Bioanalyzer	Additionally Required Material				
C7	Agilent High Sensitivity DNA Kit	Additionally Required Material				
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 (BL1)	5µL
ATP (10 mM)	5 µL
L1	1 µL
RNA from Step B7.11	10 µL
H_2O	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 12,000 x g for 30 seconds, unless otherwise specified.

☐ **C1.4** Prepare adjusted Zymo 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. Discard the flow-through. To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 x g at RT. Discard the flow-through. 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 wait 1 minute at RT.

☐ **C1.12** Centrifuge and save the flow-through.

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

Step C2. ADAPTOR LIGATION

Follow these guidelines for selecting the appropriate linker volume:

☐ 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 C1.12)	6 µL	6 µL	6 µL	6 µL
Buffer L2 (BL2)	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 L2 (L2)	1 µL	1 µL	1 µL	1 µL
Linker MC 1µM	0.25 µL	0.5 µL	1 µL	2 µL
H_2O	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 12,000 x g for 30 seconds, unless otherwise specified.

☐ C2.5 Prepare adjusted Zymo RNA Binding Buffer by mixing 50 µL of buffer and 50 µL of ethanol (95-100%).

☐ C2.6 Add the 100 µL adjusted Zymo RNA Binding Buffer (from step C2.5) 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. Discard the flow-through. To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 x g at RT. Discard the flow-through. Carefully, transfer the column into a new RNase-free tube.

☐ C2.12 Add **9 µL** of **nuclease-free water** directly to the column matrix and wait 1 minute at RT.

☐ C2.13 Centrifuge and 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 µL ATP + 9 µL 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 C2.13)	**9 µL**
Buffer L3 (BL3)	2 µL
ATP (1mM)	1 µL
PEG8000*	8 µL
Enzyme L3	1 µL

*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 µL **nuclease-free water.**

☐ C3.4 Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 12,000 x g for 30 seconds, unless otherwise specified.

☐ C3.5 Prepare adjusted Zymo RNA Binding Buffer by mixing 50 µL of buffer and 50 µL of ethanol (95-100%).

☐ C3.6 Add the 100 µL adjusted Zymo RNA Binding Buffer (from step C3.5) 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 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ C3.9 Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

☐ C3.10 Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

☐ C3.11 Add 400 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through. To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 x g at RT. Discard the flow-through. Carefully, transfer the column into a new RNase-free tube.

☐ C3.12 Add 12 µL of **nuclease-free water** directly to the column matrix and wait 1 minute at RT.

☐ C3.13 Centrifuge and 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 C3.13)	**12 µL**
dNTPs	1 µL
Primer RT_T	1 µL

☐ **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 µL
DTT	1 µL
L4 enzyme	1 µL

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

⚠ **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_2O	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. Carefully transfer the supernatant into a new tube.

⚠ **SAFE STOPPING POINT (store at -20°C)**

Step C6. PCR AMPLIFICATION – PCR 2

The Illumina Index Primer - UDI (48 rxns) sequences can be found at this link. Please utilize one set of UDI for each reaction.

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

PCR1 (from Step C5.12)	49 µL
L5 enzyme (L5)	50 µL
Illumina Index Primer - UDI (48 rxns)	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	4°C	∞

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

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

⚠ **SAFE STOPPING POINT (store at -20°C)**

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 adapter dimers band!

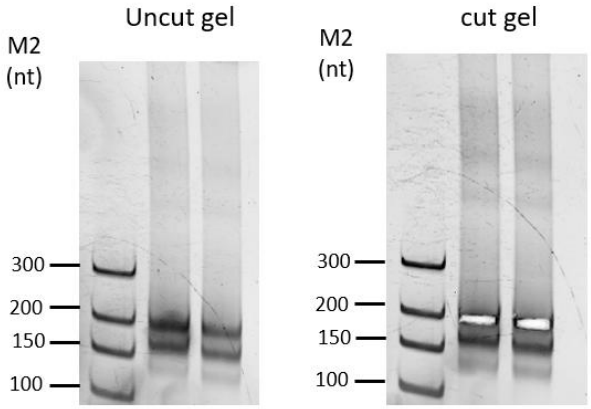


Fig.3 Example of library run on 10% TBE gel. The library is 200 nt length, while the adaptor dimer is 170 nt. Please note that the part that needs to be cut is exactly the band at 200 nt, leaving the whiskies out as depicted in the white rectangle on right gel. The sharper the cut, the lower the quantity 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 µL 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 µL of **Isopropanol** and 1.5 µL **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 µL of 70% cold ethanol. Centrifuge for 5 min at 20,000g, 4°C.

☐ **C8.9** Remove the supernatant and resuspend the pellet in 11 µL 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.

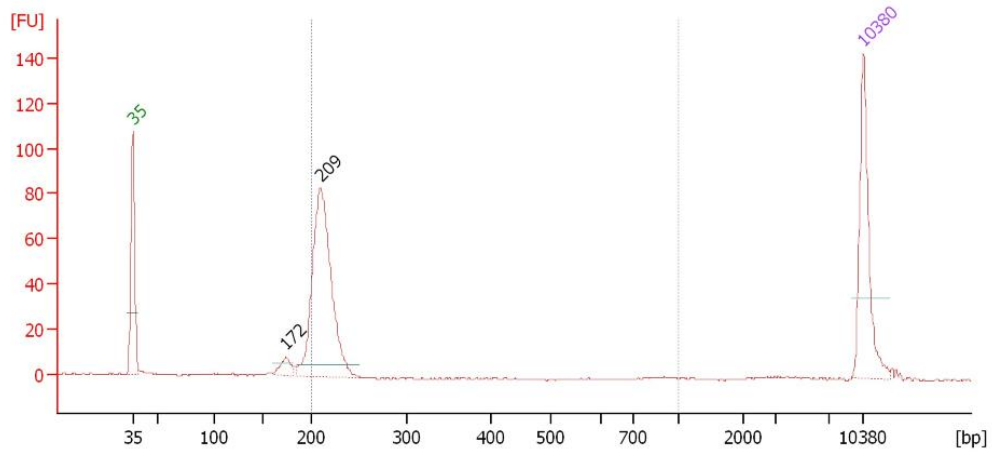


Figure 4. Example electropherogram libraries results. *Typical electropherogram for a library prepared with an immortalized cell line. The library was analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The electropherogram need to present at least one major peak between 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 NovaSeq X Plus: For best results, pool our libraries with at least 50% high-complexity libraries from other samples/projects to increase overall pool complexity. We do not recommend sequencing our libraries alone or as the majority of the pool due to insufficient library complexity.

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.

E. BIOIT GUIDELINES – do it yourself

Expected Illumina sequencing output.

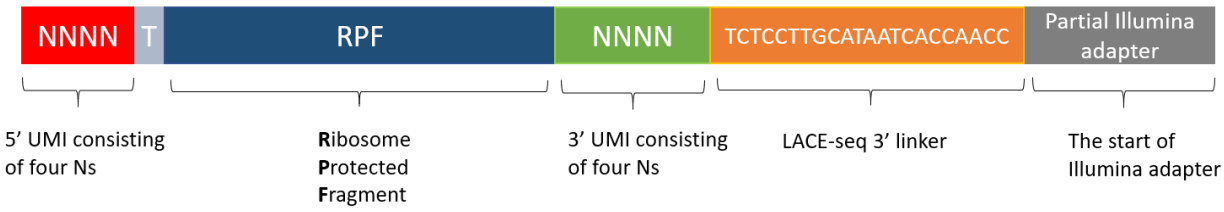


Figure 1. Expected Illumina sequencing output: example of a read generated.

Unique molecular identifiers (UMIs) are strings of random nucleotides that are attached to RPFs prior to PCR amplification and can be used to accurately detect PCR duplicates.

The T at position fifth precedes the start of the RPF. The sequence content of a high-quality library has a T peak in position fifth in 90-100% of the reads (Figure 2).

To check T peak use fastqc command:

- fastqc --outdir outputdir input.fastq

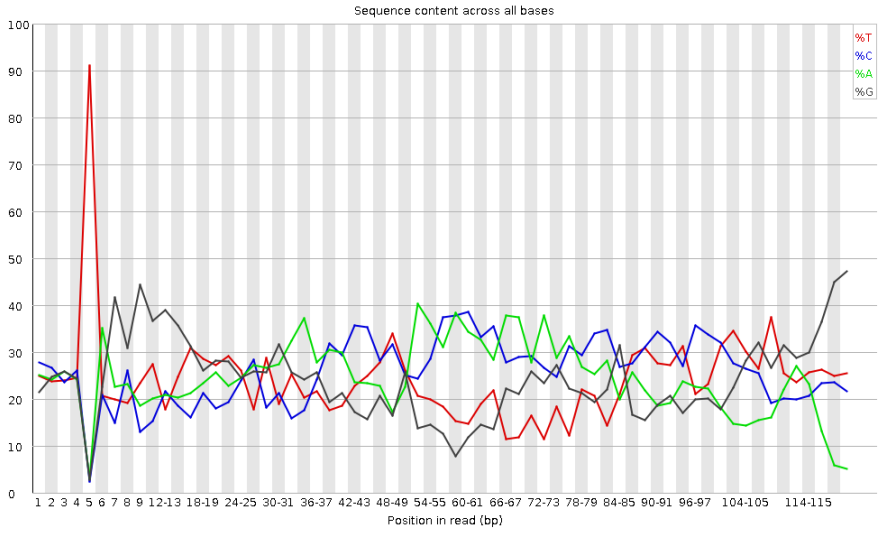


Figure 2. Sequence content across all bases graph. The sequence content of a high-quality library has a T peak in position five in 90-100% of the reads.

Workflow overview

There are 5 main steps in the analysis pipeline:

E1. Software installation

E2. Trimming/UMI extraction

E3. Filtering rRNA, tRNA and ncRNA

E4. STAR alignment

E5. RiboWaltz pipeline

Step E1. Software installation:

Information and guides to install the required tools. Though more recent versions of the programs will also be compatible with this pipeline, the workflow is intended to function with the versions listed:

- Dependencies
  - Trimming:
    - Cutadapt (https://cutadapt.readthedocs.io/en/stable/installation.html)
  - Quality Control:
    - Fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
  - Alignment:
    - bowtie2 (https://bowtie-bio.sourceforge.net/bowtie2/index.shtml)
    - STAR (https://github.com/alexdobin/STAR)
  - Utilities:
    - umi_tools (https://github.com/CGATOxford/UMI-tools)
    - samtools (https://www.htslib.org/)
  - Ribosomal Footprint Analysis:
    - RiboWaltz (R) (https://github.com/LabTranslationalArchitectomics/riboWaltz)
- Build Aligner Indexes
  - To build bowtie2 indexes fasta files of tRNAs, rRNAs and snRNAs or ncRNAs are necessary. You can find those files https://rnacentral.org/.
  - To build STAR index also gtf file is needed. And those files can be found at https://www.gencodegenes.org/ and https://www.ensembl.org

Once the tools have been installed, you will need to make sure that the UNIX environment variables are appropriately set. You can either add the location of the executables installed to your PATH variable or create a new directory called bin in your home directory, copy the executables to this location, and add the location of the bin directory to your PATH variable.

To change your PATH variable, enter (assuming bash shell):

```
> export PATH = <list of paths>:$PATH
```

Parameter	Definition
`PATH = <list of paths>:$PATH`	specify number of threads in computer for this job (Depends on the computer)

Step E2: UMI extraction/Trimming

Proper trimming of the reads is important for efficient mapping. Here we provide some guidance on the use of (E2.1) cutadapt (Martin M. 2011) to remove Linker MC+ (MC+), (E2.2) UMI-tools extract (Smith T. 2017) to move the UMI sequence from the read to the read name so that PCR duplicates can be removed after the alignment, (E2.3) cutadapt to remove the T preceding the RPF.

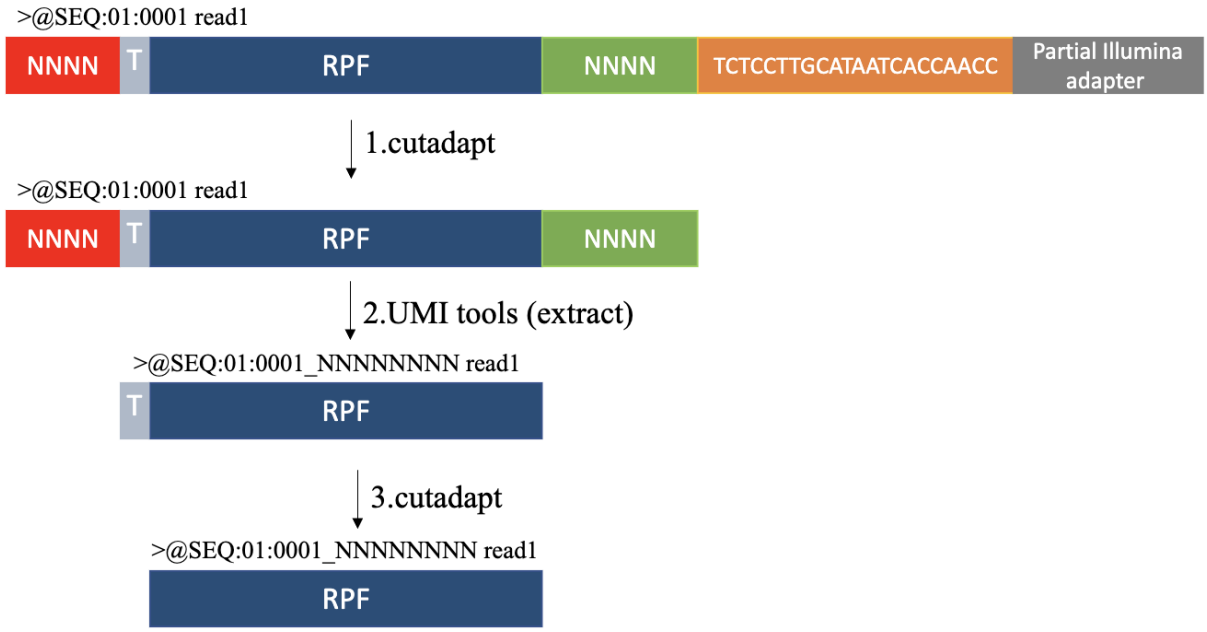


Figure 3. UMI extraction and trimming step schematic. The RPF extraction is done in 3 steps: linker removal, UMI extraction, T removal.

E2.1: cutadapt

First the Linker MC+ (MC+) is trimmed from the 3' end of each read and only reads longer than X+9 nt are retained, while shorter reads are discarded:

```
cutadapt --cores N --minimum-length X+9 -a TCTCCTTGCATAATCACCAACC --discard-untrimmed -
o trim.fastq input.fastq
```

Parameter	Definition
`--cores N`	specify number of threads in computer for this job (Depends on the computer)
`--minimum-length X+9`	Reads are retained if they are longer than X+9 nt, where X is the length of the RPF (usually X=20 for ribosome profiling analysis), and 9 is the sum of the lengths of the 5' and 3' UMIs
`-a TCTCCTTGCATAATCACCAACC`	Removal of the LACE-seq 3' linker and any sequence that may follow
`--discard-untrimmed`	Reads in which *no* adapter is found are discarded
`-o trim.fastq`	The output file name
`input.fastq`	The input file name

E2.2: UMI-tools (extract)

The sequence of the 5' and 3' UMIs are moved from the read sequence to the read name:

```
umi_tools extract -I trim.fastq --bc-pattern='^(?P<umi_1>.{4}).+(?P<umi_2>.{4})$' --
extract-method=regex -S extract.fastq --log=<umi_extract.log>
```

Parameter	Definition
`-I trim.fastq`	The input file name must be the same as the output file name in step1
`--bc-pattern='^(?P<umi_1>.{4}).+(?P<umi_2>.{4})$'`	extract the first 4 (5'UMI) and the last 4 bases (3'UMI) of each read
`--extract-method`	defines method for UMI extraction
`-S extract.fastq`	The output file name

NOTE: UMI-tools dedup can be used after alignment to remove duplicates based on the mapping coordinate and the UMI attached to the read name.

E2.3: cutadapt

The T preceding the RPF is then removed:

```
cutadapt --cores N -g ^T --discard-untrimmed -o trim2.fastq extract.fastq
```

Parameter	Definition
`--cores N`	specify number of threads in computer for this job (Depends on the computer)
`-g ^T`	Removal of the first T at the start of each read
`-o trim2.fastq`	The output file name
`extract.fastq`	The input file name must be the same as the output file name in step E2.2

Step E3: Filtering rRNA, tRNA and ncRNA

In order to remove and quantify ribosomal RNA (rRNA) content or other contaminants (tRNAs and snRNAs etc) in your sample prior to alignment to the genome, you can align the trimmed reads against specific contaminant sequences. The first step in removing contaminants is to create a FASTA formatted file containing contaminating sequences from your sample to align against, using the Bowtie aligner (Bowtie2-build https://bowtie-bio.sourceforge.net/bowtie2/index.shtml).

To build bowtie2 indexes fasta files of tRNAs, rRNAs and snRNAs or ncRNAs are necessary. You can find those files https://rnacentral.org/.

```
bowtie2-build --threads N - f <reference.fasta.file> <given_index_name>
```

Parameter	Definition
`--threads N`	specify number of threads in computer for this job (Depends on the computer)
`- f <reference.fasta.file> <given_index_name>`	f: specify fasta file location and name (Eg: /go/to/reference.fa) and given_index_name refers to the location and name of the indexes (Eg: /go/to/index/rRNA)

E3.1: removing rRNA contaminant

```
bowtie2 --threads N -N 1 --no-1mm-upfront -q <trimmed.fastq.gz> --
un=<norRNA.fastq.gz> -x <rRNA_bowtie_index>
```

Parameter	Definition
`--threads N`	specify number of threads in computer for this job (Depends on the computer)
`-N 1`	Number of allowed mismatches
`--no-1mm-upfront`	This option prevents Bowtie 2 from searching for 1-mismatch end-to-end alignments
`-q <trimmed.fastq.gz>`	Input filename
`--un=<norRNA.fastq.gz>`	output not aligned reads
`-x <rRNA_bowtie_index>`	Index file for alignment

E3.2: removing tRNA contaminant

```
bowtie2 --threads N -N 1 --no-1mm-upfront -q <norRNA.fastq.gz> --
un=<norRNA_notRNA.fastq.gz> -x <tRNA_bowtie_index>
```

Parameter	Definition
`--threads N`	specify number of threads in computer for this job (Depends on the computer)
`-N 1`	Number of allowed mismatches
`--no-1mm-upfront`	This option prevents Bowtie 2 from searching for 1-mismatch end-to-end alignments
`-q <trimmed.fastq.gz>`	Input filename
`--un=<norRNA_notRNA.fastq.gz>`	output not aligned reads
`-x <tRNA_bowtie_index>`	Index file for alignment

E3.3: removing ncRNA contaminant

```
bowtie2 --threads N -N 1 --no-1mm-upfront -q <norRNA_notRNA.fastq.gz> --
un=<norRNA_notRNA_noncRNA.fastq.gz> -x <ncRNA_bowtie_index>
```

Parameter	Definition
`--threads N`	specify number of threads in computer for this job (Depends on the computer)
`-N 1`	Number of allowed mismatches
`--no-1mm-upfront`	This option prevents Bowtie 2 from searching for 1-mismatch end-to-end alignments
`-q <trimmed.fastq.gz>`	Input filename
`--un=<norRNA_notRNA_noncRNA.fastq.gz>`	output not aligned reads
`-x <ncRNA_bowtie_index>`	Index file for alignment

Step E4: Sequence Alignment

The next step for analysis is to align the remaining reads to the genome using the STAR (https://github.com/alexdobin/STAR).

To build STAR index also gtf file is needed. And those files can be found at https://www.gencodegenes.org/ and https://www.ensembl.org

```
STAR --runMode genomeGenerate --runThreadN N --genomeDir <location_for_index> --
genomeFastaFiles <location_of_fasta_file> --genomeSAindexNbases <calculated size>
--sjdbGTFfile <location_of_gtf_file>
```

Parameter	Definition
`--runMode genomeGenerate`	option directs STAR to run genome indices generation job
`--runThreadN N`	specify number of threads in computer for this job (Depends on the computer)
`--genomeDir <location_for_index>`	location_for_index: refers to the location and name of the indexes
`--genomeFastaFiles <location_of_fasta_file>`	location_of_fasta_file: specifies one or more FASTA files with the genome reference sequences. The tabs are not allowed in chromosomes' names, and spaces are not recommended.
`--genomeSAindexNbases <calculated size>`	genomeSAindexNbases: can be find with; min (14, log2(GenomeLength)/2-1) for hg38 genome its min (14, log2(3272116950)/2-1) = 14
`--sjdbGTFfile <location_of_gtf_file>`	location_of_gtf_file: specifies the path to the file with annotated transcripts in the standard GTF format.

Step E5: RiboWaltz pipeline

For the RiboSeq Quality Metrics analysis you can use RiboWaltz, an R package that integrates quality controls of the ribosome profiling data, P-site identification for improved interpretation of positional information and a variety of graphical representations.

Use transcriptome BAM file and GTF annotation file to run riboWaltz (https://github.com/LabTranslationalArchitectomics/riboWaltz).

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.

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

Table 6. *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 staring solution of the diluted RiboLace smart probe (RsP) (collected in Step B2.7) allows an estimation of the binding efficiency.

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

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				

Table 7. *Sample beads functionalization summary*

App.7 Nuclease digestion checklist

Table 8 provides the output from Step Lysis B1 with the possibility, if needed, to list the amount of Wash Buffer to reach the reaction amount.

Sample number	Sample name	AU/µL	V for 0.9 AU (µL)	V of Wash Buffer up to 450 (µL)
1				
2				
3				
4				
5				
6				

Table 8. *Summary of the volumes to utilize for the dilution of lysate Step B3.*

Table 9 is intended as a guideline to follow for digesting the lysate.

Reagent	0.9 A.U	Needed in Step	Step-by-Step Checklist
Wash Buffer	____	B3.1	□ If needed, dilute the lysate calculated following Step A2.1 in Wash Buffer up to "___"
Nux Enhancer (NE)	0.9 µL	B3.2	□ Add 0.9 µL of NE to the lysate
Nux (Nux) + Wash Buffer		B3.3	□ Dilute 2 µL of Nux in 18 µL Wash Buffer to create diluted Nux (dNux)
Diluted Nux (dNux)	9 µL	B3.4	□ Add 9 µL of dNux to the lysate □ Incubate 45 min at 25°C
SUPERase•In	1.5 µL	B3.5	□ Stop the reaction by adding 1.5 µL of SUPERase•In into the lysate □ Incubate for 10 min on ice

Table 9. *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 0.3 AU as the starting material and Wash Buffer up to a final volume of 150 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
0.3 AU	0	NT
0.3 AU	0.3 µL	A
0.3 AU	3 µL	B
0.3 AU	30 µL	C

Table 10. *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 1.5 µL of SUPERaseIn for 10 min on ice.
- ☐ Add 300 µL of the Zymo RNA Binding Buffer (ZBB).
- ☐ Incubate the solution at RT for 5 min with shaking at 600 rpm.
- ☐ Add 450 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ Transfer 700 µL of the mixture to the Zymo-Spin™ Column and centrifuge for 30 seconds at 12,000 x g at RT. Discard the flow-through.
- ☐ Transfer the remaining volume of the mixture to the Zymo-Spin™ Column and centrifuge for 30 seconds at 12,000 x g at RT. Discard the flow-through.
- ☐ Add 400 µl RNA Prep Buffer to the column and centrifuge for 30 seconds at 12,000 x g at RT. Discard the flow-through.
- ☐ Add 700 µl RNA Wash Buffer to the column and centrifuge for 30 seconds at 12,000 x g at RT. Discard the flow-through.
- ☐ Add 400 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
- ☐ To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 x g at RT. Discard the flow-through. Carefully, transfer the column into a new RNase-free tube.
- ☐ Add **11 µL** of **Nuclease Free Water** directly to the column matrix and wait 1 minute.
- ☐ Centrifuge for 30 seconds at 12,000 x g at RT. The extracted RNA is present in the flow-through. Collect the flow through.

- ☐ With Nanodrop, measure the absorbance of each sample at 260 nm (set up the “nucleic acid” function of the Nanodrop), using 1 µL of Nuclease Free Water as blank.
- ☐ 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.

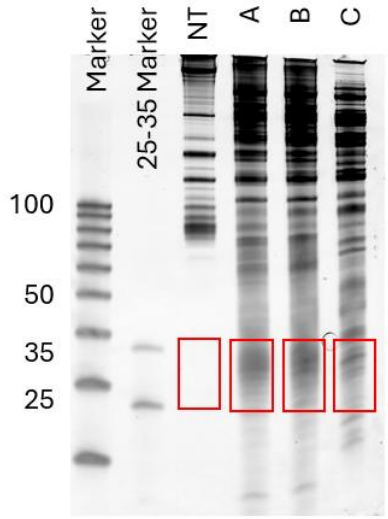


Fig.6 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel. In the red square the sizes are between 25-35 nt.

As depicted in the figure above, a non-digested (NT) sample does not present the typical enrichment of fragments at 25-35 nt (red square in Fig.6). Under-digested sample (A) does display an enrichment of RPFs but they are not well resolved, while the over-digested sample (C) should display a ladder-like pattern of bands below 50 nt and the disappearance of the signal on the top part of the gel. In this example, the quantity of Nux used in sample B needs to be utilized for all the reactions as it displays a sharper definition of peaks between 25-35 nt.

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 50 nt and the disappearance of the signal on the top part of the gel. 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.

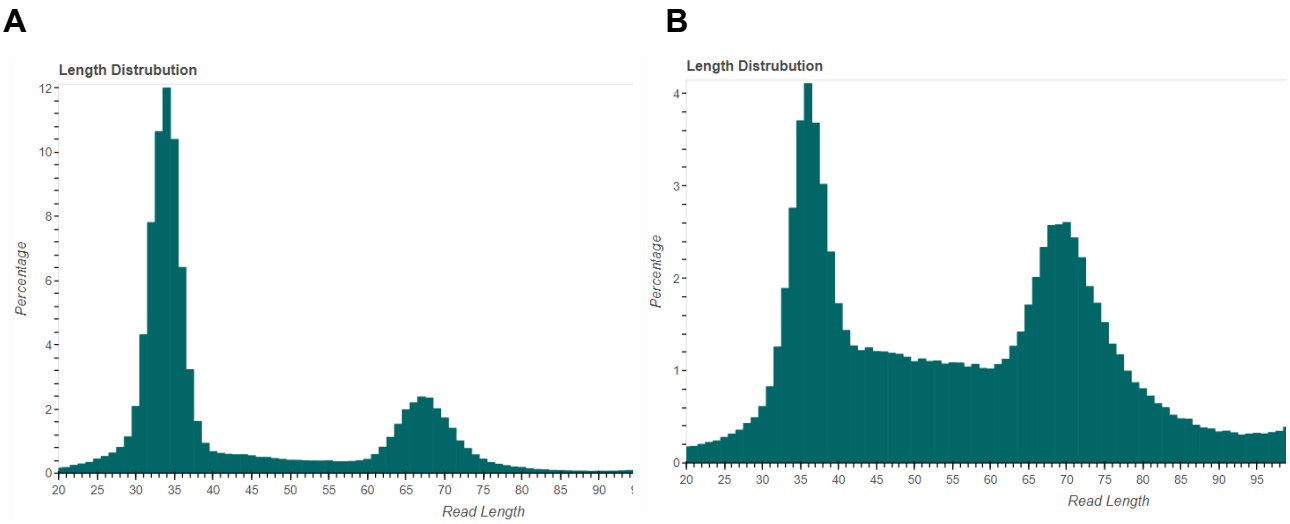


Fig.7 Example of read length distribution obtained by sequencing libraries originated from A) sample digested with the right amount of Nux or B) underdigested sample.

When the Nux is utilized correctly for the specimen of interest (Fig.7A), the resulted reads after analysis of the sequenced libraries, will display a high accumulation between 25-35 nt and between 60-70 nt. The percentage of reads not in these ranges, thus not belonging to RPFs will be limited, producing high-quality results. On the contrary, if a smaller amount of Nux than needed is used (Fig.7B), the digestion is not efficient, and the boundaries of the RNA protected by the ribosome are not sharp. From the 25-35 nt population, longer reads will be created, resulting in difficulties in downstream analysis and in-frame calculation.

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 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:

Lysate input / Reagent	Standard Amounts	30% increase	Needed in Step
	0.9 AU	1.2 AU	
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	1.2 AU	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 µM (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 µL of the 3P-RNA 1 µM (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.

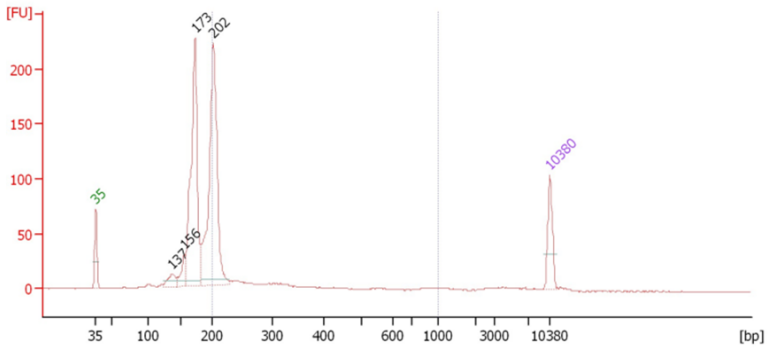


Figure 6. Example electropherogram libraries results before size selection. *Typical electropherogram for a library prepared with RPFs obtained from an immortalized cell line with RiboLace kit. The library was analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The electropherogram 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.*

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Notes: