RiboLace Starter kit

Fast and flexible solution to isolate active ribosomes for ribosome profiling experiments.

Product	Catalog no	Rxns.
RiboLace Starter kit	# RL00S-04	4

Shipping: Blue Ice and Dry ice

Storage Conditions: store components according to the storage conditions reported on the labels, and on Page 6 of this manual.

Shelf Life: 12 months

Description: The RiboLace Starter kit contains all reagents to isolate active ribosomes by affinity purification and magnetic separation. Thanks to the adjustable protocol, this product is suggested for the de novo experimental designs when using specimens of unknown translational activity, to get acquainted with the technology, and when in need of further customizing of the protocol (reagents volumes and number of reactions optimization).

The kit is compatible with the PAGExt kit for RPF gel extraction (Cat. no. #KGE00-12) and with the LACEseq kit for NGS RiboSeq library preparation (LACEseq Cat. no. #LS001-12).

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

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
- Thermoshaker for 1.5 ml tubes.
- Microcentrifuge and non-stick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- Automatic wheel (rotator)
- Magnetic stand for 1.5 mL tube
- Acid-phenol:chloroform (Ambion catalog no. AM9720) or RNA Clean & Concentrator™-5 (Zymo catalog. no. R1015 or R1016)
- 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)

INTRODUCTION

The RiboLace Starter kit relies on a proprietary puromycin derivative, called 3P, that retains the ability to intercalate within the catalytic site of the active ribosome (typical of the puromycin) while being covalently linked to a biotin molecule. The samples are at first exposed to cycloheximide to clamp ribosomes on the translating RNA fragments (recommended step), and then lysate and nuclease digested to produce the RPF embedded inside translating ribosomes. 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 by rapid and simple magnetic separation.

The overall goal of this Kit is to provide researchers with an easy-to-access solution to test the RiboLace™ technology for the isolation of actively translating ribosomes, develop de novo experimental designs when using specimens of unknown translational activity, and (if needed) to act as an affordable sandbox for the further customization of the RiboLace protocol. 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 & services and our proprietary technologies.

WORKFLOW OVERVIEW

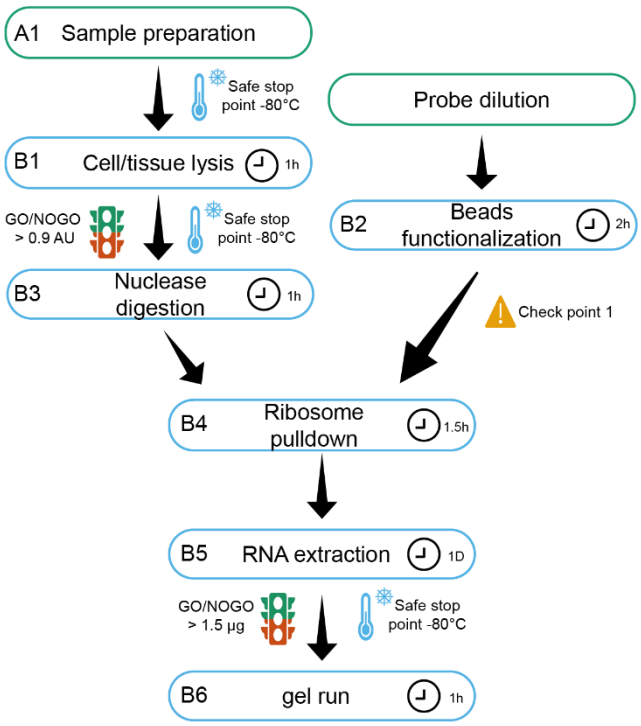


Fig.1 Overview of the RiboLace Starter Kit workflow. *For each box, the step name and number are shown in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures), GO/NO/GO conditions (traffic lights), and a checkpoint (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 1 day 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 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 starting with 0.9 AU (Abs260nm) of lysate. However, due to the adjustability of the protocol, it is possible to tailor the reagents to any input amount between 0.1 and 1.2 AU. For samples between 0.6-0.9 AU, you do not need to modify the reagents suggested in the protocol, for alternative concentrations please refer to the Appendixes.
- Starting with 0.9 AU of sample lysate, we expect to obtain between 0.5 to 1.5 µg of RNA after RPF pulldown. The choice of increasing or decreasing the input amount depends on the RNA recovery. Please refer to the Appendixes for protocol modification guidelines.
- 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.
- 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 RPFs that can be isolated from a sample is strongly affected by its translational state and must be considered when programming experiments with the IMMAGINA RiboLace Starter 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).

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

RiboLace Starter kit 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)	# IBT0011	76 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 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 76 µL of concentrated RiboLace smart probe (RsP) and add 324 µL 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 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 alternative sample concentrations please refer to the table in Appendix 5 that provides you with a summary of all the reagents adjusted volumes. 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 6 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 7. 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. **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 10 - 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 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 Wash buffer (WB) before removing the beads from the magnet and resuspend them in 400 µL of Wash 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 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**.

At this stage, the extracted RNA contains a mix of RPFs and other RNA (e.g. ribosomal RNA). The following section B6 explains how to run the pull-down RNA on a polyacrylamide gel to visually inspect the quality of the extract (e.g. rule out an eventual degradation) or further isolate the RPF via gel cutting of the corresponding bands. Our PAGExt kit (Cat. no #KGE00-12), has been specifically developed for this PAGE Purification step and it is available as a standalone kit, or as a component of the ALL-IN-ONE RiboLacePro kit (Cat. No # RL00P-12).

OPTIONAL STEP B6: PAGE VISUALIZATION OF THE RPF AFTER RIBOSOME PULLDOWN (END OF STEP B5)

RiboLace Starter kit components and additional required materials needed in this part:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
B6.1	15% TBE-Urea polyacrylamide gel	Additionally Required Material				
B6.3-4-5	Gel Loading Buffer II	Additionally Required Material				
B6.4	25-35 Marker (25-35 M)	# IBT0131	15 µL	-80°C	Vial	clear
B6.5	Ultra-low range molecular weight marker	Additionally Required Material				
B6.7	SYBR Gold	Additionally Required Material				

The RNA recovered at the end of Step B5 contains the ribosome-protected fragments (RPFs) that are purified after RiboLace pulldown. This RNA can be quantified by Nanodrop and run on a 15% TBE-urea gel following the protocol below to check the presence of RPFs. In case the RPFs are not visible on the gel, please contact our tech support (techsupport@immaginabiotech.com).

- ☐ **B6.1** The extracted RNA could be run on a 15% TBE-urea gel.
- ☐ **B6.2** 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.
- ☐ **B6.3** Prepare samples: add Gel Loading Buffer II to X µg of RNA (1:1 volume).
- ☐ **B6.4** Prepare 25-35 Marker: mix 2 µL of 25-35 Marker, 3 µL nuclease-free water, and 5 µL Gel Loading Buffer II.
- ☐ **B6.5** Use also an ultra-low range molecular weight marker as a reference.
- ☐ **B6.6** Load the samples and the 25-35 Marker 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).
- ☐ **B6.7** Stain the gel for 5 minutes with SYBR Gold in TBE and visualize the RNA using a UV-Transilluminator.
- ☐ **B6.8** Please note that a signal between 25 nt and 35 nt should be visible. Bands present in the red square belong to the 80S ribosome-protected fragments (RPFs) and their presence in the gel indicates a proper ribosome pulldown (Figure 2).
- ☐ **B6.9** RPFs can be extracted from gel by using the PAGE Gel Extraction Kit (Cat. no #KGE00-12).

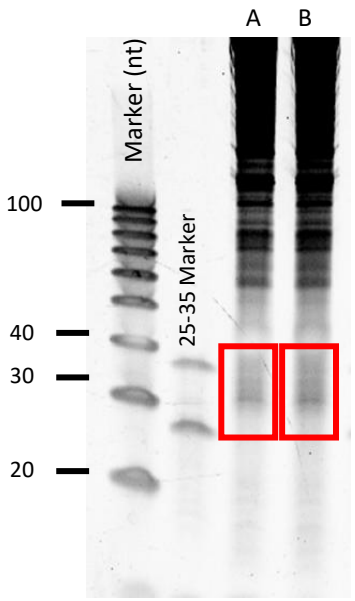


Fig.2 Example of RNA extracted after pulldown run on 15% TBE-Urea gel. The red boxes in lines A and B contain the bands relative to the RPF and corresponding to a length between 25 and 35. For proper RPF isolation via PAGE extraction please retrieve the gel portion within the red box.

APPENDIX

App.1 Guidelines for Sample Input Amount Optimization

While it is not possible to know the behavior of a sample upfront (in terms of successful RNA pull-down, satisfactory RPF isolation, and/or subsequent NGS library preparation) it is possible, to recursively optimize the protocol based on previous experience. One of the goals of the RiboLace Starter kit is to provide a cost-affordable solution to fine-tune the amount of input material and number of pull-down reactions doable with a single kit.

Usually (but not always!) obtaining 1.5 µg of RNA after pull-down allows to have enough RPFs to carry on with the production with an NGS library for RiboSequencing (the most common research pursuit when using our RiboLace family of kits), and it is thus a good operational marker to aim for during protocol optimization.

Let's imagine that the Practical Example 1 reported below, represents our known experimental setup. In this case, we are in a relatively favorable situation where we could aim for:

- the reduction of the Initial Sample Input, (scaling the corresponding amount of SLB) that could be useful if the sample is difficult to acquire, or
- we could decrease the amount of Lysate Input to e.g. 0.4 AU (= 40 µL) and be able to carry on more pulldown reactions, by scaling the number of beads required for the pull-down.

Practical Example 1

Initial Sample Input	5 million cells A
Supplemented lysis buffer (Table 1) volume	300 µL
Abs 260 nm read by Nanodrop	10 AU
Lysate Input	0.9 AU = 90 µL of lysate
RNA extracted after pull-down	5 µg
Downstream Process Status (e.g. Library prep)	Success

Conversely, let's consider Practical Example 2. In this unfavorable situation, we could increase to 150 µL the Lysate Input (corresponding to 1.2 AU- realistically the Lysate Upper Input Limit for the kit) and scale up accordingly the amount of beads required for the pull-down. This will allow us to increase the amount of RNA retrieved after pull-down to the detriment of the total number of reactions doable with the kit.

Practical Example 2

Initial Sample Input	1 million cells B
Supplemented lysis buffer (Table 1) volume	150 µL
Abs 260 nm read by Nanodrop	8 AU
Lysate Input	0.9 AU = 113 µL of lysate
RNA extracted after pull-down	0.4 µg
Downstream Process Status (e.g. Library prep)	FAIL

Please remember that Lysate Concentration (in AU) and amount of RNA after pull-down are indicators that are ultimately dependent on the Translational Activity of the sample, most commonly an unknown variable. Changing the operational conditions is thus an endeavor that we usually advise against, unless in the case of well-known routine experiments, or when in the case of “difficult” samples (difficult as in low Initial Sample Amount or Translational Activity).

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 __ AU (µL)
1						
2						
3						
4						

Table 4. *Sample Lysis Output Summary*

App.5 Beads functionalization (Step B2) alternative reagents amount

The protocol is intended for pulldown with 0.9 AU of input material per reaction. Starting with the suggested amount the kit allows performing 4 reactions. If needed, the user can decide to modify the input material to satisfy specific experimental requirements. An increase in the amount of input material used will lead to a decrease in the total amount of reactions available and vice versa. The table below (Tab 5), shows the volumes of reagents used for the Beads functionalization (Step B2) in relation to the amount of input material (expressed in AU). We suggest limiting the optimization of the protocol with input material ranging from 0.1 and 1.2 corresponding to 12 or 3 reactions respectively.

Reagent	0.1 < AU < 0.3 (12 reactions)	0.31 < AU < 0.6 (6 reactions)	0.61 < AU < 0.9 (4 reactions)	0.91 < AU < 1.2 (3 reactions)	Needed in Step
RiboLace magnetic beads (RmB) v2-0	48 µL	96 µL	144 µL	192 µL	B2.3
OH-buffer (OH)	90 µL	180 µL	270 µL	360 µL	B2.4
Nuclease-free water	1000 µL	1000 µL	1000 µL	1000 µL	B2.5
B-Buffer (BB)	90 µL	180 µL	270 µL	360 µL	B2.6
Diluted RiboLace Smart Probe (RsP)	27 µL	54 µL	81 µL	108 µL	B2.8
mPEG	3 µL	5.2 µL	7.5 µL	10 µL	B2.11
Nuclease-free water	1000 µL	1000 µL	1000 µL	1000 µL	B2.12
W-buffer (WB)	1000 µL	1000 µL	1000 µL	1000 µL	B2.13
W-buffer (WB)	105 µL	105 µL	105 µL	105 µL	B2.14

Table 5. *Components' volumes to use in Step B2.*

App.6 Beads functionalization checklist

Reagent	N=1 X AU	N=___ X AU	Needed in Step	Step-by-Step Checklist
			B2.1	□ Place RmB v2-0 at RT for 30 min
			B2.2	□Vortex 30'
RiboLace magnetic beads (RmB)	____	____	B2.3	□Add "___" Beads in 1.5 / 2 mL Tube □Place on magnet □REMOVE Supernatant
OH-buffer (OH)	____	____	B2.4	□Remove from magnet □Wash OH Buffer: add "____" µL □Shake 5min 1,400 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 1,400 rpm RT □Place on magnet □REMOVE Supernatant
B-Buffer (BB)	____	____	B2.6	□Remove from magnet □Wash B Buffer: add "____" µL □Shake 3min 1,400 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)	____	____	B2.8	□Remove from magnet □Resuspend in diluted RsP: "___" µL
			B2.9	□Incubate 1h shaking 1,400 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	____	____	B2.11	□Add mPEG "____" µL □Incubate 15min shaking 1,400 rpm RT
Nuclease-free water	1000 µL	1000 µL	B2.12	□Place on magnet □REMOVE Super Natant □Remove from magnet □Wash with N.F. water: add 1000 µL □Shake 2 min 1,400 rpm RT
W-buffer (WB)	1000 µL	1000 µL	B2.13	□Place on magnet □REMOVE Super Natant □Remove from magnet □Wash with W buffer 1000 µL □Shake 2 min 1,400 rpm RT
W-buffer (WB)	105 µL	____	B2.14	□Place on magnet □REMOVE Super Natant □Resuspend in "____"µL w-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.7 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.8 Alternative digestion requirement (for Step B3 – Nuclease treatment)

The protocol is intended for the digestion of 0.9 AU of input material per reaction. If needed the user can decide to modify the input material to satisfy specific experimental requirements, thus the Nuclease and the accessory reagents need to be adjusted accordingly. The amount of AU dictates the amount of diluted nuclease (dNux). For dNux, NE and SUPERaseIn amounts please check the table below.

Please refer to the following table for quantities to utilize in Step B3.

Reagent	0.1 < AU < 0.3	0.31 < AU < 0.6	0.61 < AU < 0.9	0.91 < AU < 1.2	Needed in Step
W-buffer (WB)	Final volume 150 µL	Final volume 300 µL	Final Volume 450 µL	Final Volume 600 µL	B3.1
Nux Enhancer (NE)	0.3 µL	0.6 µL	0.9 µL	1.2 µL	B3.2
Diluted Nux (dNux)	3 µL	6 µL	9 µL	12 µL	B3.4
SUPERaseIn	0.5 µL	1.0 µL	1.5 µL	2 µL	B3.5

Table 4. *Components' volumes to use in Step B3.*

App.9 Alternative digestion checklist

Table 9 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/µL	V for __ AU (µL)	V of W-Buffer up to __ (µL)
1				
2				
3				
4				

Table 9. *Summary of the volumes to utilize for the dilution of lysate Step B3.*

Table 10 is intended as a guideline to follow for digesting the lysate when dealing with different amounts.

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

Table 10. *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.10 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 W-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 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 g at RT. Discard the flow-through.
- ☐ Add 400 µl RNA Prep Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ Add 700 µl RNA Wash Buffer to the column and centrifuge for 30 seconds at 12,000 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 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 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.

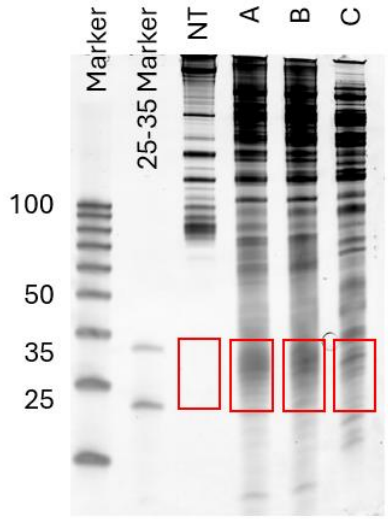


Fig.6 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel. In the red square the sizes are between 25-35 nt.

As depicted in the figure above, not 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.

App.11 Alternative RNA extraction (Step B5)

The protocol is intended for extraction of the RNA obtained after pulldown starting with 0.9 AU of input material. If needed the user can decide to modify the input materials to satisfy the specific experiment requirements, thus the reagents need to be adjusted accordingly. The amount of AU dictates the amount of W-buffer used to resuspend the beads in Step B4.6. Together, SDS 10%, Proteinase K, Acid Phenol:Chloroform:Isoamyl Alcohol, Isopropanol, and 70% cold ethanol need to be adjusted accordingly. Please refer to the following table for quantities to utilize in Step B4.7 and Step B5.

Reagent	0.1 < AU < 0.3	0.31 < AU < 0.6	0.61 < AU < 0.9	0.91 < AU < 1.2	Needed in Step
W-buffer (WB)	200 µL	300 µL	400 µL	400 µL	B4.6
SDS 10% (SDS)	20 µL	30 µL	40 µL	40 µL	B5.1
Proteinase K (K)	5 µL	7.5 µL	10 µL	10 µL	B5.1
Acid Phenol:Chloroform:Isoamyl Alcohol	225 µL	338 µL	450 µL	450 µL	B5.2
Isopropanol	500 µL	750 µL	1000 µL	1000 µL	B5.6
GlycoBlue	2 µL	2 µL	2 µL	2 µL	B5.6
70% cold ethanol	500 µL	750 µL	1000 µL	1000 µL	B5.9
Nuclease Free Water	12 µL	12 µL	12 µL	12 µL	B5.10

Table 12. *Components' volumes to use in Step B5.*

App.12 Alternative RNA extraction checklist

Table 13 is intended as a guideline to follow for RNA extraction when dealing with different amounts.

Reagent	X AU	Needed in Step	Step-by-Step Checklist
W-buffer (WB)	____	B4.6	□ Remove completely the W-buffer □ Remove the beads from the magnet □ Resuspend the RmB v2-0 in "___" of W-buffer
		B4.7	□Transfer the bead suspension to a new tube
SDS 10% (SDS)	____	B5.1	□Add "___" of SDS 10% to the Tube
Proteinase K (K)	____	B5.1	□Add "___" of Proteinase K to the Tube □ Digest for 75 min at 37°C
Acid Phenol: Chloroform: Isoamyl Alcohol	____	B5.2	□Add "___" of Acid P:C:IA to the Tube
		B5.3	□Vortex the tube □centrifugate at 14,000 x g for 5 min at 4°C
		B5.5	□Transfer the aqueous phase to a new tube
Isopropanol	____	B5.6	□Add "___" of Isopropanol to the Tube
GlycoBlue	2 µL	B5.6	□Add 2 µL of GlycoBlue to the Tube
		B5.7	□Mix by inversion of the tube □Incubate at RT for 3 min □Store at -80°C overnight
		B5.8	□centrifugate at 20,000 g for 30 min at 4°C
70% cold ethanol	____	B5.9	□REMOVE Supernatant □Wash with "___" of 70% cold ethanol □centrifugate at 20,000 g for 5 min at 4°C
Nuclease Free Water	12 µL	B5.10	□REMOVE Supernatant □Let ethanol evaporate □Resuspend in "____"µL of Nuclease Free Water

Table 13. *Components' volumes to use for the RNA extractions Step B5. The table is intended as a guideline to follow when dealing with non-standard samples.*

App.13 RNA extraction column based.

Should you not wish to use Acid Phenol:Chloroform:Isoamyl Alcohol to extract the RNA after pulldown, it is possible to utilize the column instead.

⚠ ***The reagents are part of the RNA Clean & Concentrator™-5 kit (Zymo catalog. no. R1015 or R1016)**

- ☐ **Ba5.1** Place the 1.5 mL tube from step B4.6 on the magnet and remove completely the W-buffer (**WB**).
- ☐ **Ba5.2** Remove the tube from the magnet and extract the RNA by directly adding 200 µL of the Zymo RNA Binding Buffer (**ZBB***) to the beads pipetting up and down.
- ☐ **Ba5.3** Transfer the bead suspension to a new nuclease-free 1.5 mL tube.
- ☐ **Ba5.4** Incubate the beads suspension at RT for 5 min with shaking at 600 rpm.
- ☐ **Ba5.5** After the incubation, place the tube on a magnet and **collect the supernatant**, transferring it to a new nuclease-free 1.5 mL tube. Discard the beads.
- ☐ **Ba5.6** Add 200 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ **Ba5.7** Transfer the mixture to the **Zymo-Spin™ Column*** and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **Ba5.8** Add 400 µl **RNA Prep Buffer*** to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **Ba5.9** Add 700 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **Ba5.10** Add 400 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **Ba5.11** To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 g at RT. Discard the flow-through. Carefully, transfer the column into a new RNase-free tube.
- ☐ **Ba5.12** Add 12 µL of **Nuclease Free Water** (NFW) directly to the column matrix directly to the column matrix and wait 1 min.
- ☐ **Bs5.13** Centrifuge for 30 seconds at 12,000 g at RT. The extracted RNA is present in the flow-through. Keep the Eppendorf with the flow through.
- ☐ **Ba5.14** With Nanodrop, measure the absorbance of each sample at 260 nm (set up the “nucleic acid” function of the Nanodrop), using 1 µL of NFW as blank.

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