RiboLace Pro kit

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

Product	Catalog no	Rxns.
RiboLace Pro kit	# RL00P-12	12

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 Pro kit contains all reagents to isolate active ribosomes by affinity purification and magnetic separation.

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
- 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) 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 Pro 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 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.

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RiboLace™ Technology (Section B):

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

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WORKFLOW OVERVIEW

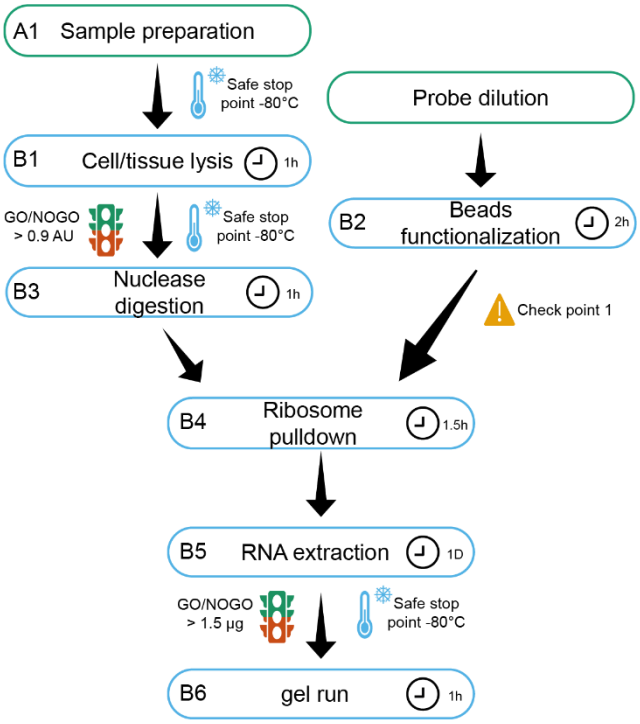


Fig.1 Overview of the RiboLace Pro Kit workflow. *For each box, the step name and number are shown in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures), GO/NO/GO conditions (traffic lights), and a checkpoint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

Optimal Workflow Recommendations

-  Please run up to 6 samples in parallel. Longer manipulation time may introduce an unwanted variability between the first and the last sample.
- 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 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 starting with 0.9 AU (Abs260nm) of lysate. 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 sample lysate, we expect to obtain between 0.5 to 1.5 µg of RNA after RPF pulldown.
- 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. SAMPLE PREPARATION

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 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 RPF pulldown.
- The second, is the amount of RNA retrieved after Ribosome & RPF pulldown (Step B5).

As a general indicator 5 million non-treated cells, coming from an immortalized line (such as HeLa, HEK, CHO, and K562) at 70 to 80% confluence represent a comfortable starting point. 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 AU, 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 AU of your sample is measured using a spectrophotometer, most commonly a Nanodrop. Set the instrument so to measure the Abs at 260 nm (usually Nucleic Acid function) and measure the absorbance of your lysate using the Supplemented Lysis Buffer (SLB) as blank (see Before starting the experiment – Lysis Buffer Supplementing & Table 3). The use of different lysis buffers is strongly discouraged because it may interfere with the efficiency of ribosome pull-down and with the AU calculation (some components may absorb at 260 nm).

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

Example:

- ☐ *Supplemented Lysis buffer SLB 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	# IBT0031	50 µL
Cell	1 – 5 million cells	# IBT0031	150 µL
Cell	> 5 million cells	# IBT0031	300 µL
Tissue	< 10 mg	# IBT0032	500 µL
Tissue	> 10 mg	# IBT0032	800 µL

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

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

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

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

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

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

- ☐ To start with 0.9 AU use: 0.9AU/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 Pro 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)	# IBT0031	4 x 1.3 mL	-20°C	Vial	clear
B1	SDC 10%	Additionally Required Material				
B1	DNAse I	Additionally Required Material				
B1	RiboLock RNase Inhibitor	Additionally Required Material				
B1	Cycloheximide (CHX)	Additionally Required Material				
B1	PBS	Additionally Required Material				
B2	B-Buffer (BB)	# IBT0021	10 mL	4°C	Bottle	--
B2	RiboLace magnetic Beads v2.1 (RmB v2.1)	# IBT0042	1.8 mL	4°C	Vial	clear
B2	OH-buffer (OH)	# IBT0051	5 mL	4°C	Bottle	--
B2	mPEG	# IBT0061	120 µL	-20°C	Vial	clear
B2/B5	Nuclease free water	Additionally Required Material				
B2*	RiboLace smart probe (RsP)	# IBT0011	200 µL	-20°C	Vial	clear
B2	diluted RiboLace smart probe (dRsP)	Dilute Aliquot from RsP		-80°C		
B2/B3/B4	W-buffer (WB)	# IBT0071	2 x 25 mL	4°C	Bottle	--
B3	Nuclease (Nux)	# IBT0091	21 µL	-20°C	Vial	clear
B3	Diluted Nuclease (dNux)	Dilute Aliquot from Nux				
B3	Nux Enhancer (NE)	# IBT0081	13 µL	-20°C	Vial	clear
B3	SuperRNAse In	Additionally Required Material				
B5	Proteinase K	# IBT0111	130 µL	-20°C	Vial	clear
B5	SDS 10%	# IBT0121	600 µL	4°C	Vial	clear
B5	Acid Phenol: Chloroform:Isoamyl Alcohol	Additionally Required Material				
B5	Isopropanol	Additionally Required Material				
B5	GlycoBlue	Additionally Required Material				
B5	70% cold ethanol	Additionally Required Material				

Step B1. CELL LYSIS

⚠ **Before starting the experiment – Supplemented Lysis Buffer (SLB)**

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.

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 it is not mandatory - to increase the efficiency of the ribosomes' affinity purification. CHX treatment could induce the accumulation of ribosomes within the first 10 codons. Should you not wish to add CHX check Appendix 2 for the alternative protocol.

☐ **B1.2a** After incubation, place the cells on ice and wash them quickly with **cold PBS** containing CHX (20 µg/mL).

☐ **B1.3a** Remove all residual PBS with P200 pipette. **All the PBS must be removed before proceeding with the lysis to avoid diluting the lysis buffer.**

☐ **B1.4a** Perform the lysis directly adding the complete **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.3 - Input lysate preparation and quantification - & Table 3) to each cell dish and scrape vigorously. Mechanical scraping helps the downstream processing by disrupting the cell membrane and releasing the cellular contents, including ribosomes.

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

- Before scraping, make sure you are working in a sterile environment using appropriate aseptic techniques.
- Prepare your sample by adding the necessary lysis buffer or solution as per the protocol.
- Using a suitable tool such as a cell scraper, spatula, or pipette tip, gently scrape the surface of the cell culture dish or tissue to dislodge the cells.
- Apply consistent but gentle pressure to ensure thorough scraping while avoiding excessive force that may introduce debris.
- Scrape in a systematic manner, covering the entire surface area to ensure an even distribution of lysed cells.
- Continue scraping until you observe the desired level of cell detachment and release of cellular material.
- Transfer the lysate to a suitable collection vessel, such as a microcentrifuge tube, for further processing or analysis.

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

☐ **B1.6a** Transfer the supernatant to a new tube and keep it on ice for 20 min.

☐ **B1.7a** Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 AU calculation - Input lysate quantification). If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing.

Suspension Cells lysis

☐ **B1.1b** Treat the cells with 10 µg/mL of **cycloheximide (CHX)** for 5 min at 37°C before lysis, should you not wish to add CHX check Appendix 2 for the alternative protocol. CHX treatment is suggested – but it is not mandatory - to increase the efficiency of the ribosomes’ affinity purification. CHX treatment could induce the accumulation of ribosomes within the first 10 codons. Should you not wish to add CHX check Appendix 2 for the alternative protocol.

☐ **B1.2b** Collect the cells and centrifuge at 950 g 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 g for 5 min at 4°C. Remove the supernatant completely.

☐ **B1.4b** Resuspend cell pellet in **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.3 - Input lysate preparation and quantification - & Table 3).

☐ **B1.5b** Lysate cells by passing them through a G26 needle ~20 times (please note that if the volume is below 50 µL, using the syringe will lead to the loss of specimen, as a possibility you could pipette up and down ~20 times avoiding creating bubbles).

☐ **B1.6b** Pellet the nuclei and cell debris by centrifugation at 20,000 g for 5 min at 4°C.

☐ **B1.7b** Transfer the supernatant to a new tube. Leave on ice for 20 min.

☐ **B1.8b** Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 AU calculation - Input lysate quantification). If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing.

Tissues lysis

☐ **B1.1c** Pulverize the tissue under liquid nitrogen with mortar and pestle. Recover the powder in a 1.5 mL tube.

☐ **B1.2c** Resuspend with 800 µL of **Tissues Lysis Buffer** (not included - Immagina catalog no. #IBT0032) supplemented as per instruction in the section “Before starting the experiment – Lysis Buffer Supplementing” & Table 3. Please note that both Tissues Lysis buffer and W-buffer contain CHX (20 µg/mL).

☐ **B1.3c** Centrifuge at max speed (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 at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 AU calculation - Input lysate quantification). If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing.

Step B2. BEADS FUNCTIONALIZATION

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

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

The RiboLace smart probe (RsP) present in the kit 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 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 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 **B-buffer** (**BB**), shaking for 3 min at 1,400 rpm at RT. Place the tube on the magnet for at least 1 minute and remove the supernatant. If RmB are binding to the plastic tube, you can add Triton X-100 to a final concentration of 0.1%. Repeat the wash once again with the same 270 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 the 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 **W-buffer** (**WB**) for 2 min with shaking at 1,400 rpm at RT. After the first wash, put the tube on the magnet to remove the supernatant before adding the solution. After the second wash, place the tube on the magnet and remove completely the supernatant.

☐ **B2.14** Resuspend the functionalized RmB beads with 100 µL of W-buffer (**WB**).

☐ **B2.15** If the beads were functionalized for more than one reaction, equally divide the functionalized beads in individual tubes according to the (N) number of samples you are processing.

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

⚠Security Check Point

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

Step B3. NUCLEASE DIGESTION

The Ribosome Protected Fragments are generated during the Nuclease Digestion step (ribosome footprinting). The suggested Nuclease amount and digestion timing are well-suited for most organisms and tissues. Nonetheless, 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 8 - Optional Nuclease Optimization.**

- ☐ **B3.1** Start with a total volume of lysate corresponding to 0.9 A.U. (260 nm) (see Section A2.3 for calculation) diluted in W-buffer (**WB**) to the final volume of 450 µL.
- ☐ **B3.2** Add 0.9 µL of **Nux Enhancer (NE)**.
- ☐ **B3.3** Dilute 1.5 µL of **Nuclease** (**Nux**) by adding 98.5 µL W-buffer (**WB**). Pipet up and down 5 times to mix well the diluted Nux solution (dNux).
- ☐ **B3.4** Digest the sample in a 1.5 mL tube for 45 min at 25 °C with 4.5 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 W-buffer (WB) from Step B2.14 only immediately before adding the cell lysate!

☐ **B4.1** Add the **digested cell lysate** to the functionalized beads (to avoid dilution, discard the supernatant of the beads before adding the cell lysate) and mix well.

☐ **B4.2** Incubate for 70 min, on a wheel in slow motion (3-10 rpm) at 4°C.

☐ **B4.3** Remove the tubes from the wheel. **DO NOT CENTRIFUGATE** but allow the entire solution with the beads to settle at the bottom of the tube. If residual solution is present on the lid, pull down the beads by gently flicking down the tube by hand 2 or 3 times. Place the tubes on ice. Place the magnet in an ice bucket before putting the tubes on it.

☐ **B4.4** Keep working on ice and separate the beads with a magnet.

☐ **DO NOT REMOVE THE BEADS FROM THE MAGNET and NEVER TOUCH THE BEADS IN THE NEXT WASHING STEPS.**

☐ **B4.5** Remove the supernatant. Carefully wash the beads twice with 1000 µL W-buffer (**WB**). Do not remove the samples from the magnet. Carefully add the WB on the opposite side of the Eppendorf to where the beads are present. Carefully remove the supernatant without disturbing the beads.

☐ **B4.6** Remove completely the W-buffer (WB) before removing the beads from the magnet and resuspend them in 400 µL of W-buffer (WB).

☐ **B4.6** Transfer the bead suspension to a new nuclease-free 1.5 mL tube.

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

Step B5. RNA EXTRACTION

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

☐ **B5.1** Add 40 µL **SDS 10%** (**SDS**) and 10 µL **Proteinase K** (**K**) to the bead's suspension and incubate at 37 °C for 75 min.

☐ **B5.2** Add 450 µL **Acid Phenol:Chloroform:Isoamyl Alcohol**.

☐ **B5.3** Vortex and centrifugate at 14,000 x g for 5 min at 4°C.

☐ **B5.4** If there is no phase separation, add 20 µL **NaCl 2M** and repeat the centrifugation.

☐ **B5.5** Keep the aqueous phase and transfer it into a new vial.

☐ **B5.6** Add 1000 µL **Isopropanol** and 2 µL **GlycoBlue**

☐ **B5.7** Mix and incubate at RT for 3 min, then store at -80°C for:

- at least 2 hours (fast procedure)
- overnight (safe procedure, recommended for better recovery yields)

☐ **B5.8** Pellet the RNA by centrifugation (20,000 g) for 30 min at 4°C.

☐ **B5.9** Remove the supernatant and wash the pellet once with 1000 µL 70% cold ethanol. Centrifuge for 5 min at 20,000 g, 4°C.

☐ **B5.10** Remove the supernatant and resuspend the pellet in 12 µL of **Nuclease Free Water**.

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 RiboLace Pro kit (Cat. No # RS0XL-12).

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

RiboLace Pro kit components and additional required materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
B6.1	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).

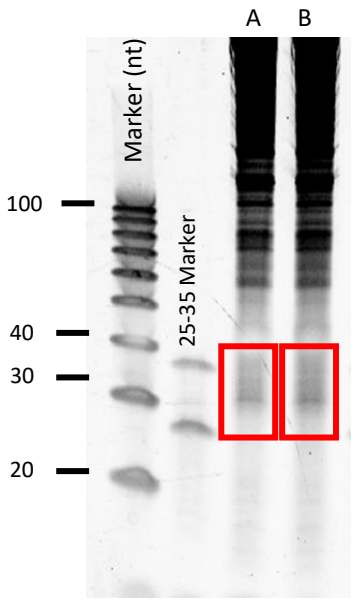
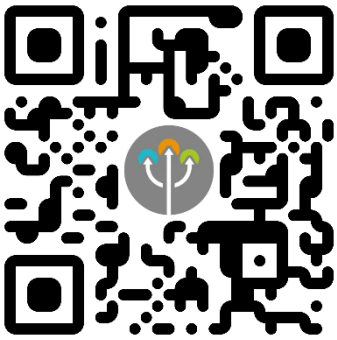


Fig.2 Example of RNA extracted after pulldown run on 15% TBE-Urea gel. *The red boxes in lines A and B contain the bands relative to the RPF and corresponding to a length between 25 and 35. For proper RPF isolation via PAGE extraction please retrieve the gel portion within the red box.*

APPENDIX

App.1 Guidelines for Sample Input Amount Optimization

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



Click or scan me to access the RiboLace Starter protocol.

App.2 Alternative CHX removal protocol

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

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

App.3 Lysis buffer supplementation issues

Please check if, after adding Sodium deoxycholate a whiteish and cloudy solution appears. If so, please do not proceed with the lysis of the sample and toss the supplemented LB. Subsequently, warm up the SDC at RT and add it to a new aliquot of the not-supplemented LB. If the whiteish and cloudy solution persists, please contact our tech support (techsupport@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 4. *Sample Lysis Output Summary*

App.5 Beads functionalization checklist

Reagent	N=1 0.9 AU	N=___ 0.9 AU	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 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 1400 rpm RT □Place on magnet □REMOVE Supernatant
B-Buffer (BB)	270 µL	____	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)	81 µL	____	B2.8	□Remove from magnet □Resuspend in diluted RsP: "___" µL
			B2.9	□Incubate 1h shacking 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	7.5 µL	____	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 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 1,400 rpm RT
W-buffer (WB)	100 µL	____	B2.14	□Place on magnet □REMOVE Supernatant □Resuspend in "____"µL w-Buffer
			B2.15	□Aliquot in 105 µL of equal volumes in N tubes

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

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

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

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

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				
5				
6				

Table 6*. Sample beads functionalization summary*

App.7 Nuclease digestion checklist

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

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

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

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

Reagent	0.9 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)	0.9 µL	B3.2	□ Add 0.9 µL of NE to the lysate
Nux (Nux) + W-Buffer		B3.3	□ Dilute 1.5 µL of Nux in 98.5 µL W-buffer to create diluted Nux (dNux)
Diluted Nux (dNux)	4.5 µL	B3.4	□ Add 4.5 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 8. *Components' volumes to use for the Digestion of lysate Step B3. The table is intended as a guideline to follow when dealing with non-standard nuclease amounts.*

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

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

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

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

- ☐ Digest the samples for 45 min at 25°C.
- ☐ Stop digestion with 1 µL of SUPERaseIn for 10 min on ice.
- ☐ Add 30 µL SDS 10% and 5 µL of Proteinase K and incubate at 37°C for 75 min.
- ☐ Add 310 µL of Acid Phenol:Chloroform:Isoamyl Alcohol.
- ☐ Vortex and centrifugate at 14,000 g for 5 min.
- ☐ Keep the aqueous phase and transfer it into a new vial.
- ☐ Add 500 µL Isopropanol and 2 µL GlycoBlue.
- ☐ Mix and incubate an RT for 3 min, then store at -80°C for 2 hours.
- ☐ Pellet the RNA by centrifugation (20,000 g) for 30 min at 4°C.
- ☐ Remove the supernatant and wash the pellet once with 70% cold ethanol. Centrifuge for 5 min at 20,000 g, 4°C.
- ☐ Resuspend the pellet in 10 µL of Nuclease Free Water.
- ☐ Extracted RNA needs to be run on a 15% TBE-urea gel.
- ☐ Pre-run the gel at 200 V for 30 min in TBE prepared with nuclease-free water. Clean well the gel wells with a syringe to remove UREA residuals before loading the samples.
- ☐ Prepare samples: add Gel Loading Buffer II to 1.5 µg of RNA (1:1 volume).
- ☐ Use an ultra-low range molecular weight marker as reference.
- ☐ Load the samples and the Marker on 15% TBE-urea polyacrylamide gel and run the gel for 1 h at 200V until the bromophenol blue band reaches the bottom of the gel.

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

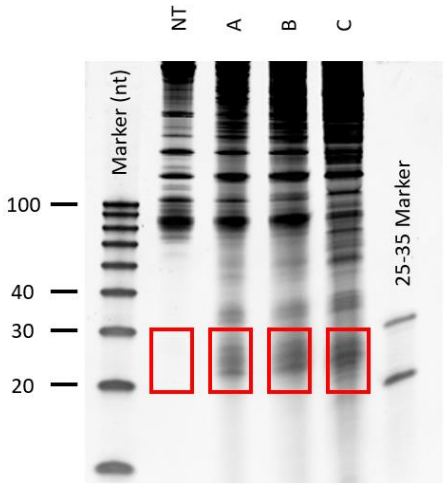


Fig.3 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel. In the red square the sizes between 25-35 nt.

As depicted in the figure above, not digested sample does not present the typical enrichment of fragments at 25-35 nt (red square in Fig.3). Under-digested sample (A) does not display a high enrichment of RPFs, while the over-digested sample (C) shows a smear of signal on the gel. In this example, the quantity of Nux used in sample B (AU x 5) needs to be utilized for all the reactions.

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

App.9 RNA extraction checklist

Table 10 is intended as a guideline to follow for RNA extraction.

Reagent	0.9 A.U.	Needed in Step	Step-by-Step Checklist
W-buffer (WB)	400 µL	B4.6	□ Remove completely the W-buffer □ Remove the beads from the magnet □ Resuspend the RmB v2-1 in 400 µL of W-buffer
		B4.7	□Transfer the bead suspension to a new tube
SDS 10% (SDS)	40 µL	B5.1	□Add 40 µL of SDS 10% to the Tube
Proteinase K (K)	10 µL	B5.1	□Add 10 µL of Proteinase K to the Tube □ Digest for 75 min at 37°C
Acid Phenol: Chloroform: Isoamyl Alcohol	450 µL	B5.2	□Add 450 µL of Acid P:C:IA to the tube
		B5.3	□Vortex the tube □centrifugate at 14,000 g for 5 min at 4°C
		B5.5	□Transfer the aqueous phase to a new tube
Isopropanol	1000 µL	B5.6	□Add 1000 µL 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	1000 µL	B5.9	□REMOVE Supernatant □Wash with 1000 µL 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 12 µL of Nuclease Free Water

Table 10. *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.10 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 **G1F Buffer** (**G1F**) 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 G1F Buffer (G1F) as blank.

Contacts

Info

info@immaginabiotech.com

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

orders@immaginabiotech.com

Technical service (technical inquiries and quality complaints)

techsupport@immaginabiotech.com

Viale Industria, 47, 38057, Pergine Valsugana (TN), ITALY

https://www.immaginabiotech.com/

+39 04611787270

Notes: