Ribosome Profiling All-In-One Set

from your sample to sequencing

Product	Catalog no	Rxns.
Ribosome Profiling All-In-One Set	#RS-001s	12

Shipping: Blue Ice and Dry ice

Storage Conditions: store components according to storage conditions reported on labels and on this manual

Shelf Life: 12 months

Description: Ribosome Profiling All-In-One Set contains all reagents to perform ribosome profiling from cell/tissues lysis to final Next Generation Sequencing library. This set includes RiboLace Mod.1, LaceSeq, PAGE Extraction Gel and UDIs for 12 reactions. This product is suggested for the experimental designs which include challenging samples, like ones with low translational rates. The kit is suitable for Illumina platforms (MiSeq, NovaSeq 6000, NextSeq550/1000/2000).

Suitable for: Eukaryotic cell lines and tissues

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

Kit contents	Qty.	Storage
Ribosome Profiling All-In-One 4°C components	1 box	4°C
Ribosome Profiling All-In-One -20°C components	1 box	-20°C
Ribosome Profiling All-In-One -80°C components	1 bag	-80°C
Filters and Tubes	1 package	RT
iUDIs plate	1 plate	-20°C

Additionally Required Materials

- o Sodium deoxycholate 10% solution in DNase/RNase free water
- o Cycloheximide (Sigma-Aldrich, catalog no. C4859-1ML)
- o DNase I (Thermo Scientific catalog no. 89836)
- o RiboLock RNase Inhibitor (Thermo Scientific catalog no. EO0381)
- o SUPERase●In (Invitrogen, catalog no. AM2696)
- o RNAse free water and DEPC water
- o Acid-phenol:chloroform (Ambion catalog no. AM9720)
- o Nanodrop ND-1000 UV-VIS Spectrophotometer
- o GlycoBlue (Ambion catalog no. AM9515)
- o Isopropanol (Sigma catalog no. 278475)
- o Microcentrifuge and nonstick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- o Automatic wheel (rotator)
- o Magnetic stand for 1.5mL tube
- o Qubit Fluorometer
- o Qubit™ microRNA Assay Kit
- o 15% TBE-Urea polyacrylamide gel (e.g., BioRad catalog no. 450-6053 or Thermo Scientific catalog no. EC6885BOX)
- o Gel Loading Buffer II (Denaturing PAGE) (Thermo Scientific catalog no. AM8546G)
- o SYBR Gold (Thermo Scientific, catalog no. S11494)
- o RNA Clean & Concentrator™-5 (Zymo catalog. no. R1015 & R1016)
- o AMPure XP for PCR Purification (Beckman Coulter catalog no. A63881)
- o NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel catalog no 740609.10/.50/.250)
- o Agilent 2100 Bioanalyzer
- o High-Sensitivity DNA chip (Agilent Tech. catalog no. 5067-4626)
- o 10% TBE polyacrylamide gel (e.g. Thermo Scientific catalog no. EC6275BOX)
- o DNA Gel Loading Dye (e.g., Thermo Scientific catalog no. R0611)

Recommendations

Sample Recommendations

Please note that the success of the experiment is strongly affected by the translational state of your biological samples. Two lysates similarly concentrated (i.e., similar Abs260nm) could have different amounts of translating ribosomes due to a different efficiency in protein synthesis. For example, immortalized cells are known to have higher rates of translation than primary cells. In addition, treatments such as drugs and transfection reagents could affect ribosome activity and ribosomes distribution along translated RNAs. Thus, the rate of protein synthesis of each sample must be considered when programming experiments with the IMMAGINA - Ribosome Profiling All-In-One Set. If possible, always use the maximum available AU input in the suggested range.

Please start the LACEseq NGS library only if you have at least 5 ng of Ribosomes protected fragments (RPFs) after PAGExt extraction (step 6.11). If you do not have at least 5 ng of RPFs, please contact out specialists at techsupport@immaginabiotech.com before starting the library preparation.

Input lysate preparation and quantification

Cells and tissues should be lysed following Step 1 of this manual using the provided IMMAGINA lysis buffer (Cat nr. #RL001-1) or IMMAGINA Tissue lysis buffer (Cat. nr. #RL001-2, not included please purchase separately). Both lysis buffers have to be supplemented as indicated in Table 1 page 9 immediately before use. The use of different lysis buffers is strongly discouraged because it may interfere with the efficiency of ribosomes pull-down.

The kit has been optimized for a cell lysate input between 0.1 - 0.3 total AU (Abs260 nm). The Abs260nm should be measured using Nanodrop (selecting Nucleic Acid function) with the supplemented lysis buffer (Table 1, page 9) as blank. Briefly, use 1.5 µL of supplemented lysis buffer for blank subtraction and then quantify 1.5 µL of lysed specimen. If the instrument does not allow to utilize the supplemented lysis buffer as blank, please use water instead. After blank subtraction, quantify both the supplemented lysis buffer and the lysed specimen, then subtract the absorption calculated at 260 nm for both for the following quantifications steps.

Example:

- ☐ Lysis buffer Abs260nm = 7 AU
- ☐ Specimen Abs260nm = 17 AU
- ☐ Absorbance value of lysate = 17 – 7 = 10 AU

In general, starting with 5 million immortalized cells lysed in 300 µL of lysis buffer, an absorbance after blank subtraction between 7 to 15 AU is expected.

After proper quantification of the lysate, you should consider it as concentration in AU/mL. Thus, for calculating the volume of lysate to utilize, divide the concentration for 1000 to obtain AU/µL. The volume calculated as in the example below, has to be used for the pulldown experiment in Step 3.

Examples:

- Nanodrop absorbance value of lysate at 260 nm: 10 AU. This means that the absorbance of the lysate is 10 AU/ml (= 0.01AU/µL).

  - ☐ To start with 0.1 AU use: 0.1AU/0.01 AU/µL = 10 µL of lysate
  - ☐ To start with 0.2 AU use: 0.2AU/0.01 AU/µL = 20 µL of lysate
  - ☐ To start with 0.3 AU use: 0.3AU/0.01 AU/µL = 30 µL of lysate

- Nanodrop absorbance value of lysate at 260 nm: 4 AU. This means that the absorbance of the lysate is 4 AU/ml (=0.004 AU/µl).

  - ☐ To start with 0.1 AU use: 0.1AU/0.004 AU/µL = 25 µL of lysate
  - ☐ To start with 0.2 AU use: 0.2AU/0.004 AU/µL = 50 µL of lysate
  - ☐ To start with 0.3 AU use: 0.3AU/0.004 AU/µL = 75 µL of lysate

Please consider starting with the maximum amount of material (0.3 AU) to maximise the possibility of pulling down sufficient material for library preparation. This is particularly important, especially if specimen present a low translational rate.

Using the Positive Controls

If you are using this kit for the first time, we strongly recommend performing the positive control reaction for library preparation. The positive control (CTRL, Cat. Nr. #LS001-22) is an RNA fragment with a 5'OH and 3'P (1 µM). For library preparation of the positive control, use 1 µl of the RNA control (10 ng) starting from Step 6 – phosphorylation until Step 13.4 gel run and staining. Two bands as depicted in the image should be visible of about the same quantity (Fig.3 page 29).

Optional Nuclease optimization (for Step 3)

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 (#RL001-7) that is intended to be diluted before using, and that need to be added to the lysate sample in a fix quantity, depending on the amount of AU as starting material. This quantity is suitable for the majority of cell lines; however, it can be modulated depending on the needs and the type of specimen. To optimize this quantity, after lysing the sample in Step 1, start with 0.3 AU as starting material and W buffer up to a final volume of 200 µL. Perform a titration assay, by adding to each reaction different quantities of Nux, below and example:

Starting lysate	Quantity of diluted Nux (Step. 3.3)	Sample
0.3 AU	0	NT
0.3 AU	0.3 µL (AU x 1)	A
0.3 AU	1.5 µL (AU x 5)	B
0.3 AU	15 µL (AU x 50)	C

- Digest the samples for 45 min at 25°C.
- Stop digestion with 0.5 µL of SUPERaseIn for 10 min on ice.
- Add 20 µL SDS 10% and 5 µL of Proteinase K and incubate at 37°C for 75 min.
- Add 225 µL of Acid Phenol:Chloroform:Isoamyl Alcohol.
- Vortex and centrifugate at 14,000 x g for 5 min.
- Keep the aqueous phase and transfer it into a new vial.
- Add 500 µL Isopropanol and 2 µL GlycoBlue.
- Mix and incubate a RT for 3 min, then store at -80°C for 2 hours.
- Pellet the RNA by centrifugation (20000g) for 30 min at 4°C.
- Remove the supernant and wash the pellet once with 70% cold ethanol. Centrifuge for 5 min at 20000g, 4°C.
- Resuspend the pellet in 10 µL of Nuclease Free Water.
- Extracted RNA need 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 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.

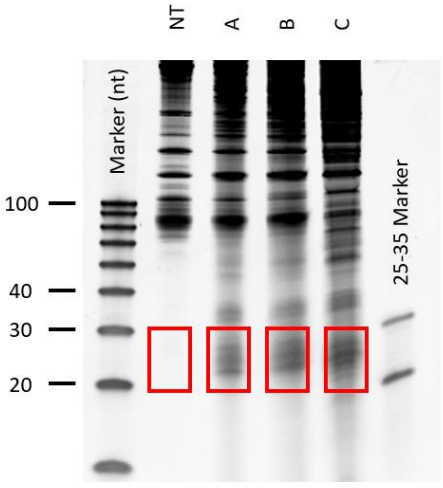

Fig.1 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel. In the red square the sizes between 25-35 nt.

As depicted in the figure above, not digested sample do not present the typical enrichment of fragments at 25-35 nt (red square in Fig.1). Under-digested sample (A) do not display a high enrichment of RPFs, while over-digested sample (C) shows a smear of signal on the gel. In this example, 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.

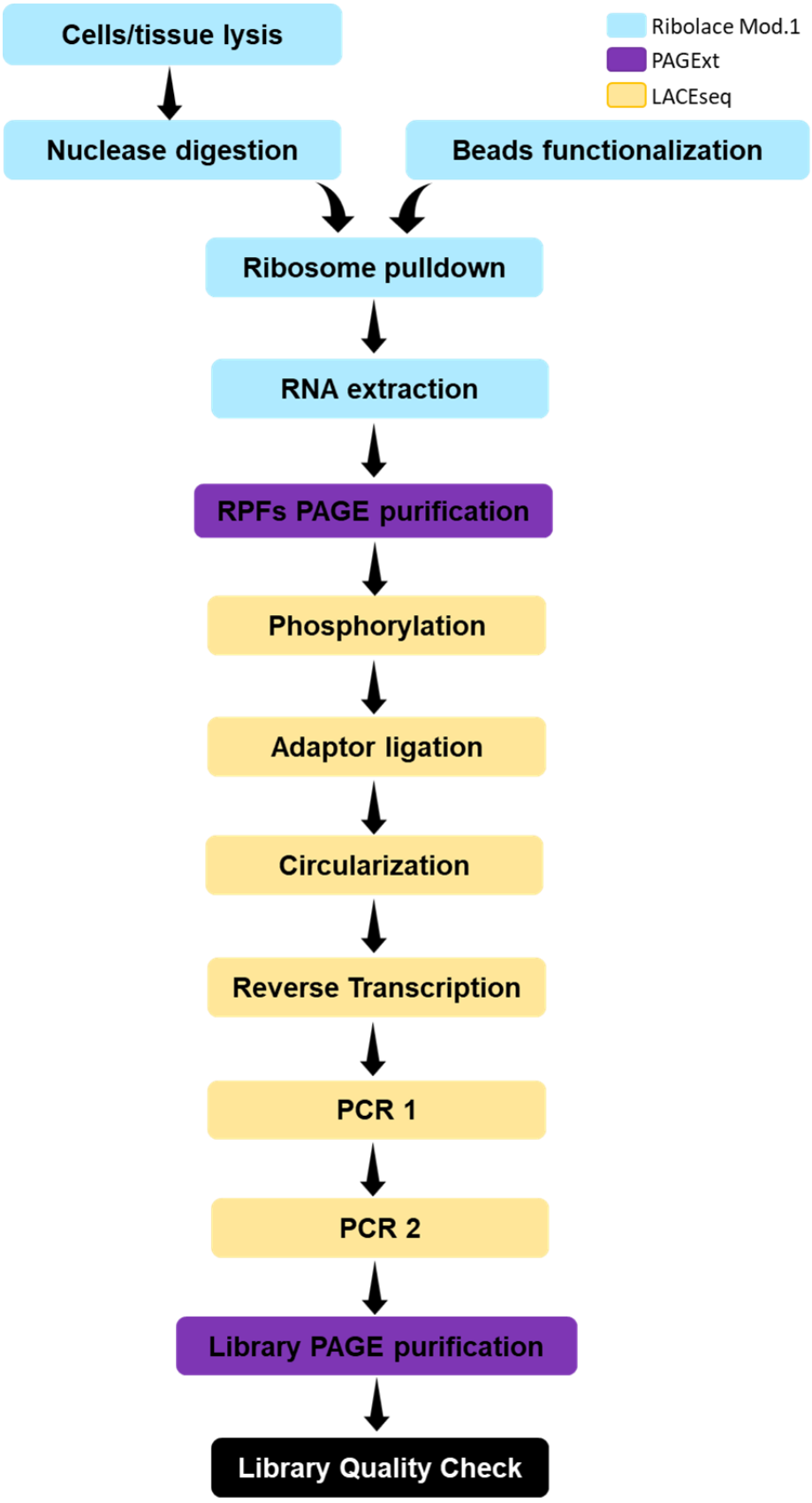

Figure 2. ***Overview of the Ribosome Profiling All-In-One Set workflow.***

Pull-down of active Ribosomes

Ribosome Profiling All-In-One Set components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial
SDS 10% (SDS)	#RL001-9	0.5 mL	RT	Vial	clear
B-Buffer (BB)	#RL001-3	5 mL	4°C	Bottle	--
W-buffer (WB)	#RL001-4	25 mL	4°C	Bottle	--
RiboLace magnetic beads (RmB) v2-0	#RL001-25	1.2 mL	4°C	Vial	clear
OH-buffer (OH)	#RL001-14	5 mL	4°C	Bottle	--
Proteinase K (K)	#RL001-17	65 µL	4°C	Vial	clear
Lysis buffer (LB)	#RL001-1	2x 1.9 mL	-20°C	Vial	clear
RiboLace smart probe (RsP)	#RL001-5	76 µL	-20°C	Vial	clear
Nuclease (Nux)	#RL001-7	19.5 µL	-20°C	Vial	clear
mPEG	#RL001-22	39 µL	-20°C	Vial	clear
Stabilizing Nux Solution (SS)	#RL001-24	5 µL	-20°C	Vial	clear

Note:

Step 2 (page 13) and Step 3 (page 15) can be performed in parallel.

Before starting the experiment

RiboLace smart probe dilution: add 324 µL of B-buffer to the RiboLace smart probe vial previously thaw on ice. After use, it is suggested to aliquot the mix, and store the aliquots at -80°C to avoid more than two freeze-thaw cycles. The aliquots need to take into account the amount to utilize in Step 2.8. As a suggestion, creating aliquots of 100 µL will allow to perform 3 experiments with each vial when following the protocol using 0.1- 0.3 AU lysate input. By implementing this aliquoting strategy, you can maximize the efficient utilization of the vials and ensure that the number of experiments aligns seamlessly with the established protocol.

Preparation of the lysis buffer: keep the required optimal volume of lysis buffer on ice and add the following components: Sodium deoxycholate (1% final concentration), DNase I (5U/mL final concentration), and RiboLock RNase Inhibitor (200 U/mL final concentration) (Table 1).

Table 1. *Recipe for the supplementation of the provided lysis buffer or tissues lysis buffer.*

Final volume	Lysis buffer (LB)	Sodium deoxycholate	DNase I	RiboLock RNase Inhibitor
Stock concentration		10%	1 U/µL	40 U/µL
200 µL	178 µL	20 µL	1 µL	1 µL

We recommend creating a fresh supplemented lysis buffer right before proceeding with the Lysis Step to ensure optimal reproducibility. If you have three samples to lyse, each with a volume of 200 µL, you can prepare a total of 600 µL of supplemented lysis buffer by following these steps:

- Start with 534 µL of lysis buffer (LB).
- Add 60 µL of SDC 10% (sodium deoxycholate) to the LB.
- Include 3 µL of DNAse I to the mixture.
- Lastly, add 3 µL of RiboLock.

By supplementing the LB with these specific quantities, you will have freshly prepared 600 µL of supplemented lysis buffer that can be used for lysis in three separate samples of 200 µL each. This approach ensures consistency and reproducibility in your experimental workflow.

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 contact our tech support (techsupport@immaginabiotech.com).

Please consider lysing your specimen according to the following table:

Table 2. *Quantity of lysis buffer depending on specimen amount.*

Specimen	Quantity	Lysis buffer	Volume of supplemented LB (µL)
Cell	0.3 – 1 million	#RL001-1	50 µL
Cell	1 – 5 million cells	#RL001-1	150 µL
Cell	> 5 million cells	#RL001-1	300 µL
Tissue	< 10 mg	#RL001-2	500 µL
Tissue	> 10 mg	#RL001-2	800 µL

STEP 1. CELL LYSIS

NOTE: 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 cell pellet in ice and proceed with treating the sample from step 1.4b (lysing the pellet cells in supplemented lysis buffer).

Adherent Cells lysis

☐ **1.1a** 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 treatments is suggested – but it is not mandatory - to increase the efficiency of the ribosomes' affinity purification. CHX treatment could induce accumulation of ribosomes within the first 10 codons. If you choose to avoid CHX treatment, it is crucial to ensure the prompt and proper flash freezing of the sample according to procedure described at the beginning of this session.

☐ **1.2a** After incubation, place the cells on ice and wash them quickly with **cold PBS** containing CHX (20 µg/mL).

☐ **1.3a** Remove all residual PBS with a pipette. It is crucial that all the PBS is removed before proceeding with the lysis to avoid diluting the lysis buffer.

☐ **1.4a** Perform the lysis directly adding the complete **supplemented lysis buffer** (Table 1) to each cell dish and scrape vigorously. Mechanical scraping helps disrupt the cell membrane and release the cellular contents, including ribosomes, for downstream processing. To ensure a good lysis, follow these guidelines for mechanical scraping:

- Prior to 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 cause damage to the cells or 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.

☐ **1.5a** 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.

☐ **1.6a** Transfer the supernatant to a new tube and keep it on ice for 20 min.

☐ **1.7a** With Nanodrop, check the absorbance of the cell lysate at 260 nm (setup the “nucleic acid” function of the Nanodrop), using 1.5 µL of the supplemented lysis buffer previously prepared (Table 1) as blank (for troubleshooting check guidelines at page 3 Input lysate preparation and 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

☐ **1.1b** 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 treatments is suggested – but it is not mandatory - to increase the efficiency of the ribosomes’ affinity purification. CHX treatment could induce accumulation of ribosomes within the first 10 codons. If you choose to avoid CHX treatment, it is crucial to ensure the prompt and proper flash freezing of the sample according to procedure described at the beginning of this session.

☐ **1.2b** Collect the cells and centrifuge at 950g for 5min at 4°C, remove the media and quickly wash the cells with **cold PBS** containing CHX (20 µg/mL).

☐ **1.3b** Centrifuge at 950g for 5 min at 4°C. Remove the supernatant completely.

☐ **1.4b** Resuspend cell pellet in complete **lysis buffer** (Table 1).

☐ **1.5b** 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).

☐ **1.6b** Pellet the nuclei by centrifugation at 20,000 g for 5 min at 4°C.

☐ **1.7b** Transfer the supernatant to a new tube and leave it on ice for 20 min.

☐ **1.8b** With Nanodrop, check the absorbance of the cell lysate at 260 nm (setup the “nucleic acid” function of the Nanodrop), using 1.5 µL of the supplemented lysis buffer previously prepared (Table 1) as blank (for troubleshooting check guidelines at page 3 Input lysate preparation and 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

☐ **1.1c** Pulverize the tissue under liquid nitrogen with mortar and pestle. Recover the powder in a 1.5 mL tube.

☐ **1.2c** Resuspend with 800 µL of **Tissues Lysis Buffer** (not included - IMMAGINA catalog no. #RL001-2) supplemented as in Table 1. Pease note that both Tissues Lysis buffer and W-buffer contain CHX (20 µg/mL).

☐ **1.3c** Centrifuge at max speed (20000 g) for 2 min at 4°C to remove tissue and membrane debris and collect the supernatant.

☐ **1.4c** Centrifuge again the supernatant for 5 min at max speed (20000 g) at 4°C and collect the supernatant. Keep on ice for 20 min**.**

☐ **1.5c** With Nanodrop, check the absorbance of the cell lysate at 260 nm (setup the “nucleic acid” function of the Nanodrop), using 1.5 µL of the supplemented tissues lysis buffer previously prepared (Table 1) as blank (for troubleshooting check guidelines at page 3 Input lysate preparation and 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 2. BEADS FUNCTIONALIZATION

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

☐ **2.1** Remove the **RiboLace magnetic beads (RmB) v2-0** from 4°C and place the tube at RT for at least 30 min.

☐ **2.2** Vortex the RiboLace magnetic beads (**RmB**) v2-0 tube for > 30 sec.

☐ **2.3** Put 90 µL of **RiboLace magnetic beads (RmB) v2-0** in a new 1.5 mL tube. You can functionalize more beads depending on the number of samples that you want to process in parallel. In this case, final volume = 90 µL x N (N = number of sample). Place the tube on the magnet to separate the RiboLace magnetic beads (RmB) v2-0. Remove supernatant.

☐ **2.4** Remove the tube from the magnet and wash the RiboLace magnetic beads (RmB) v2-0 with 90 µL x N of **OH-buffer (OH)** for 5 min shaking at 1,400 rpm at RT. Remove the supernatant.

☐ **2.5** Wash with 900 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 RiboLace magnetic beads (RmB) v2-0 are binding to the plastic tube, you can add Triton X-100 to a final concentration of 0.1%.

☐ **2.6** Wash the beads in 90 µL x N of **B-buffer (BB)** shaking for 3 min at 1,400 rpm at RT. Place the tube on the magnet for at least 1 min and remove the supernatant. If RiboLace magnetic beads v2-0 (RmB) are binding to the plastic tube, you can add 0.1% final Tx100. Repeat the wash once again with the same volume.

☐ **2.7** Keep at least 2 µL of diluted RiboLace smart probe (**RsP, see "Before starting the experiment",** page 8) for security check point (see grey box below).

☐ **2.8** Resuspend the RiboLace magnetic beads (RmB) v2-0 with 30 µL x N of diluted RiboLace smart probe (**RsP**).

☐ **2.9** 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 treatment (STEP. 3).

☐ **2.10** After the incubation, place the tube on a magnet and take out 3 µL of the supernatant (unbound probe) for security point (see below). Leave the rest in the vial.

☐ **2.11** Add 3 µL x N of **mPEG** to the tube, mix in a shaker at 1,400 rpm at RT for 15 min. Do not allow the beads to precipitate.

☐ **2.12** Place the tube on a magnet for 2–3 min, discard the supernatant and wash with 500 µL **Nuclease-free water** for 2 min with shaking at 1,400 rpm at RT.

☐ **2.13** Wash the RiboLace magnetic beads v2-0 (RmB) two times with 500 µL **W-buffer** (**WB**) for 2 min with shaking at 1,400 rpm at RT. Remove the supernatant.

☐ **2.14** Resuspend the RiboLace magnetic beads v2-0 (**RmB**) in a 100 µL x N of **W-buffer (WB)**, and equally divide the functionalized beads in individual tubes according to the (N) number of samples.

Remove the W-buffer (WB) only before adding the cell lysate (**Step 4**) to avoid drying the beads.

⚠ Security Check Point

CHECK PROPER BEADS FUNCTIONALIZATION

Comparing the difference in the absorbance measured at A 270 nm (Nanodrop ND-1000) for the unbound probe (collected in Step 2.10) and the staring solution of the diluted RiboLace smart probe (**RsP**) (collected in Step 2.7) allows an estimation of the binding efficiency. In particular, ~ 10-50 % 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.

STEP 3. NUCLEASE TREATMENT

☐ **3.1** Start with a total volume of lysate corresponding to 0.1 - 0.3 A.U (260 nm) (see Pag 3 for calculation) and add **W-buffer** (**WB**) to 150 µL final volume.

☐ **3.2** Add 0.3 µL **Stabilizing Nux Solution (SS)** and pipet.

☐ **3.3** In a vial, 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. If you are dealing with not conventional samples (e.g., not immortalized cell lines) or if your specimen presents some changes that might hamper to correct protein translation, you could perform a titration curve with the Nux prior performing your experiment. This step is not mandatory but could assess the proper quantity of Nux to add in your specific sample (see. Optional Nuclease optimization Note at page 5).

☐ **3.4** Digest the sample in a 1.5 mL tube for 45 min at 25 °C with the **diluted Nuclease (Nux)** prepared before using a volume (µL) according to this formula.

Diluted Nux µL = A.U x 5.

Trash the remaining diluted Nux solution.

☐ **3.5** Stop digestion with 0,5 µL µL **SUPERase•In** for 10 min on ice.

STEP 4. RIBOLACE PULL-DOWN

Remove the W-buffer (WB) from Step 2.14 only immediately before adding the cell lysate!

⚠**4.1** Add the **digested cell lysate** to the **functionalized beads** from Step 2.14 (to avoid dilution, discard the supernatant of the beads before adding the cell lysate) and mix well.

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

⚠**4.3** 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 pull down the beads by gently hand shaking. Place the tubes on ice and put them on a magnet at 4°C.

⚠**4.4** 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.**

⚠**4.5** Remove the supernatant. Carefully wash the beads twice with 500 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.

⚠**4.6** Remove the beads from the magnet and resuspend them with 200 µL **W-buffer** (**WB)**

⚠**4.7** Transfer the beads suspension to a new nuclease-free 1.5 mL tube.

⚠***Your ribosomes are attached to the beads, don't discard them!***

STEP 5. ACTIVE RPFs EXTRATION

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

☐ **5.1** Add 20 µL **SDS 10%** (**SDS**) and 5 µL **Proteinase K** (**K**) to the bead's suspension, and incubate at 37 °C in a water bath for 75 min.

☐ **5.2** Add 225 µL **Acid Phenol:Chloroform:Isoamyl Alcohol**.

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

☐ **5.4** If there is no phase separation, add 20 µL **NaCl 2M in DEPC water** and repeat the centrifugation.

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

☐ **5.6** Add 500 µL **Isopropanol** and 2 µL **GlycoBlue**

☐ **5.7** Mix and incubate a RT for 3 min, then store at -80°C for:

- at least 2 hours (fast procedure)
- overnight (safe procedure, recommended with total lysate input is < 0.3 A.U)

☐ **5.8** Pellet the RNA by centrifugation (20000g) for 30 min at 4°C.

☐ **5.9** Remove the supernatant and wash the pellet once with 500 µL 70% cold ethanol. Centrifuge for 5 min at 20000g, 4°C.

☐ **5.10** Remove the supernatant and resuspend the pellet in 5 µL of **Nuclease Free Water**.

☐ **5.11** Proceed with RPFs PAGE Purification.

STEP 6. PAGE PURIFICATION OF THE RIBOSOME PROTECTED FRAGMENTS

RiboSeq All In One Set components needed in this part:

Kit component	Cat. nr.	Quantity	Storage	Type	Vial
Filters tubes	#KGE002-6	24 pcs	RT	Bag	--
Pierced tubes	#KGE002-7	24 pcs	RT	Bag	--
TR buffer (TR)	#KGE002-1	0.5 mL	4°C	Vial	yellow
RNA Extraction Buffer (REB)	#KGE002-2	5.2 mL	4°C	Bottle	--
Marker 1 (M1)	#KGE002-4	13 µL	-20°C	Vial	yellow
25-35 Marker (25-35 M)	#RL001XL-26	13 µL	**-80°C**	Vial	clear

Note:

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

⚠ ***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.***

☐ **6.1** Prepare samples: add 5 µL of Gel Loading Buffer II to 5 µL of RPFs obtained from Step 5.

☐ **6.2** Prepare M1 marker: mix 1 µL M1, 4 µL nuclease-free water and 5 µL of Gel Loading Buffer II.

☐ **6.3** Prepare 25-35 Marker: mix 2 µL of 25-35 Marker, 3 µL nuclease-free water and 5 µL Gel Loading Buffer II.

☐ **6.4** Denature the samples, 25-35 Marker and marker M1 for 90 sec at 80 °C. Place the tubes immediately on ice.

☐ **6.5** Load the samples, 25-35 Marker, and Marker 1 on 15% TBE-urea polyacrylamide gel and run the gel at 200V until the bromophenol blue band reaches the bottom of the gel (about 50 min to 1 hour).

☐ **6.6** Stain the gel for 5 minutes with a solution made of 10 mL of TBE and 1.5 µL of SYBR Gold and visualize the RNA using a UV-Transilluminator.

☐ **6.7** Size select the ribosome protected fragments (RPF) between 25-nt and 35-nt according to the marker M1 and 25-35 Marker (see Figure below).

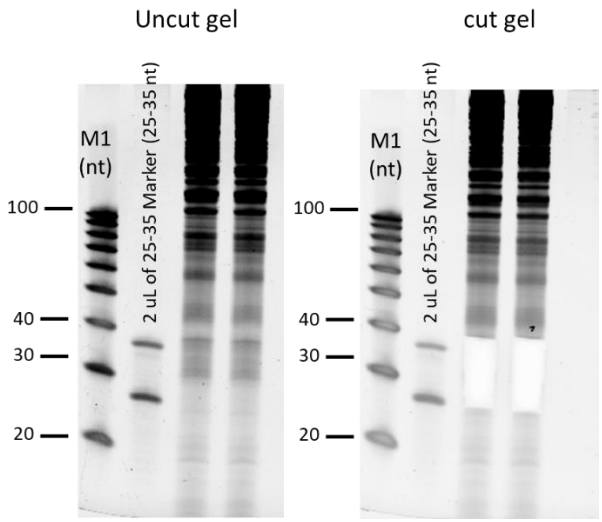

Fig.2 The RPFs are ~25-35 nt in length. The 25-35 Marker is a mix of two oligos 25 nt and 35 nt in length. It can be used as a size marker. Please note that the part that needs to be cut is between 25-35 depicted in the white rectangle on right gel.

- ☐ **6.8** Place each gel slice in a pierced tube (provided) and nest the pierced small tube inside a 1.5-ml RNase-free Microcentrifuge tube (provided). Spin at maximum speed for 3 min at RT. If part of the gel pieces is still in the pierced tube, repeat the spinning. In any case, transfer carefully any remaining gel debris from the pierced tube, discard the pierced tube and keep the 1.5 ml microcentrifuge tube.
- ☐ **6.9** Add 400 µL of **RNA Extraction Buffer** (**REB**), close the vial with the provided cap and incubate the tubes for 1 hour at - 80°C. Thaw the tubes at RT and then place the samples on a wheel in slow motion (3-10 rpm), at RT overnight.
- ☐ **6.10** With a 1 mL cut-tip, add the gel slurry to the provided filter tube and spin at 1,000g for 3 min at RT to remove the gel debris. Transfer the eluted solution to a new tube.
- ☐ **6.11** Add 700 µL of isopropanol and 1.5 µL GlycoBlue to the eluted sample.
- ☐ **6.12** Store at - 80°C for 2h (fast procedure) or overnight (safe procedure).
- ☐ **6.13** Thaw the samples on ice and pellet the RNA by centrifugation at 20,000g for 30 min at 4°C.
- ☐ **6.14** Remove the supernatant and wash the pellet once with 500 µL of 70% cold ethanol. Centrifuge for 5 min at 20,000g, 4°C.
- ☐ **6.15** Remove the supernatant being careful to get rid of all ethanol residues. Resuspend the pellet in 11 µL TR buffer.
- ☐ **6.16** Quantify the RPFs (1 µL) using a Qubit™ microRNA Assay Kit.

LIBRARY PREPARATION OF RIBOSOME PROTECTED FRAGMENTS

RiboSeq All In One Set components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial
Buffer BPK	#LS001-1	80 µL	-20°C	Vial	Red
PK enzyme (PK)	#LS001-2	13 µL	-20°C	Vial	Red
ATP	#LS001-3	80 µL	-20°C	Vial	Red
Buffer BA	#LS001-4	20 µL	-20°C	Vial	Blue
Enzyme Mix A (Mix A)	#LS001-5	13 µL	-20°C	Vial	Blue
MnCl2	#LS001-6	10 µL	-20°C	Vial	Blue
GTP	#LS001-7	10 µL	-20°C	Vial	Blue
Linker MC (1 µM)	#LS001-8	26 µL	**-80°C**	Vial	Blue
Buffer BLB	#LS001-9	30 µL	-20°C	Vial	Yellow
Enzyme Mix B (Mix B)	#LS001-10	13 µL	-20°C	Vial	Yellow
PEG 8000	#LS001-11	200 µL	-20°C	Vial	Yellow
RT_T Primer (RT_T)	#LS001-12	13 µL	-20°C	Vial	Green
Buffer BRT	#LS001-13	60 µL	-20°C	Vial	Green
RT enzyme (RT)	#LS001-14	13 µL	-20°C	Vial	Green
dNTPs	#LS001-15	13 µL	-20°C	Vial	Green
DTT	#LS001-16	15 µL	-20°C	Vial	Green
Amplification mix (AM)	#LS001-17	1.25 mL	-20°C	Vial	Clear
Fw PCR1 (F1)	#LS001-18	12 µL	-20°C	Vial	Clear
Rev PCR1 (R1)	#LS001-19	12 µL	-20°C	Vial	Clear
Control (CTRL)	#LS001-22	10 µL	**-80°C**	Vial	Clear

Note:

Input RPFs amount: ≥ 5 ng (quantified by Qubit™ microRNA Assay Kit after gel extraction). In case of less RPFs amount, combine multiple ribosomes' pulldowns for the same sample. The RPFs amount you utilize for library preparation is important to tailor specifically the amount of linker to add in Step 8 and the number of PCR cycles to run for Step 11 and Step 12 respectively. We strongly suggest starting the library preparation with all the amount of RPFs that you extracted (from 5 to 40 ng) to maximize the output of each reaction and to obtain good libraries.

If you are using this kit for the first time, we recommend performing the positive control reaction. The positive control (CTRL, clear cap) is an RNA fragment with a 5'OH and 3'P (1 µM). For library preparation of the positive control, use 1 µL of the RNA control (10 ng).

STEP 7. 5' PHOSPHORYLATION

☐ **7.1** Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

Buffer BPK	5µL
ATP (10 mM)	5 µL
PK	1 µL
RNA from step **6.16**	10 µL
H_2O	29 µL

☐ **7.2** Incubate the reaction for 1h at 37 °C in a thermal cycler.

☐ **7.3** Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 10,000-16,000 g for 30 seconds, unless otherwise specified.

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

☐ **7.5** Add 100 µL adjusted RNA Binding Buffer (from step 6.4) to the sample and mix.

☐ **7.6** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Keep the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ **7.7** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

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

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

☐ **7.10** Add 400 µL RNA Wash Buffer to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer. Carefully, transfer the column into a new RNase-free tube.

☐ **7.11** Add 7 µL of **nuclease-free water** directly to the column matrix and centrifuge.

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

STEP 8. LIGATION

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

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

☐ **8.1** Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

	RPFs amount (25 – 35 nt)			
	5-7 ng	**8-14 ng**	**15-24 ng**	**25-40 ng**
RNA (from Step 7)	6 µL	6 µL	6 µL	6 µL
Buffer BA	1 µL	1 µL	1 µL	1 µL
GTP	0.5 µL	0.5 µL	0.5 µL	0.5 µL
$MnCl_2$	0.6 µL	0.6 µL	0.6 µL	0.6 µL
Enzyme Mix A	1 µL	1 µL	1 µL	1 µL
Linker MC 1µM	0.25 µL	0.5 µL	1 µL	2 µL
H_2O	0.75 µL	0.5 µL	-	-

☐ **8.2** Incubate the reaction for 1h at 37 °C in a thermal cycler.

☐ **8.3** Add 40 µL **nuclease-free water.**

☐ **8.4** Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 10,000-16,000 x g for 30 seconds, unless otherwise specified.

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

☐ **8.6** Add the 100 µL adjusted RNA Binding Buffer (from step 7.5) to the sample and mix.

☐ **8.7** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ **8.8** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

- ☐ **8.9** Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.
- ☐ **8.10** Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
- ☐ **8.11** Add 400 µL RNA Wash Buffer to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer. Carefully, transfer the column into a RNase-free tube.
- ☐ **8.12** Add 9 µL of **nuclease-free water** directly to the column matrix and centrifuge.

STEP 9. CIRCULARIZATION

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

RNA (from Step 8)	**8 µL**
Buffer BLB	2 µL
ATP (1mM)	1 µL
PEG8000	8 µL
Enzyme Mix B	1 µL

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

☐ 9.2 Incubate the reaction for 2h at 25 °C in a thermal cycler.

☐ 9.3 Add 30 µL **nuclease-free water.**

☐ 9.4 Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 10,000-16,000 g for 30 seconds, unless otherwise specified.

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

☐ 9.6 Add the 100 µL adjusted RNA Binding Buffer (from step 8.5) to the sample and mix.

☐ 9.7 Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ 9.8 Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

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

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

☐ 9.11 Add 400 µL RNA Wash Buffer to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer. Carefully, transfer the column into a RNase-free tube.

☐ 9.12 Add 12 µL of **nuclease-free water** directly to the column matrix and centrifuge.

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

STEP 10. REVERSE TRASCRIPTION

☐ **10.1** For the generation of single strand cDNA, combine the following reagents:

Circular RNA (from step 9)	**12 µL**
dNTPs	1 µL
RT_T Primer	1 µL

☐ **10.2** Incubate the circular RNA-primer mix at 70°C for 5 minutes and then transfer to ice for at least 1 minute.

☐ **10.3** Add the following reagents to the annealed RNA:

Buffer BRT	4 µL
DTT	1 µL
RT enzyme	1 µL

☐ **10.4** Incubate 40 min at 50°C, then heat-inactivate for 5 min at 80 °C.

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

STEP 11. PCR AMPLIFICATION – PCR 1

☐ **Note:** the cycles of Step 11 – PCR 1 and Step 12 – PCR 2 depend on the starting RPFs that you used in Step 7. The number of cycles is important for avoiding overamplification, please refer to the following table for the correct number of cycles to utilize.

Table 6 *Number of cycles of PCR to use in Step 11 (PCR1) and 12 (PCR2)*

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

☐ **11.1** Combine the following reagents (for reaction) in final volume of 100 µL:

cDNA (from Step 10)	**20 µL**
Amplification Mix	50 µL
F1	0.8 µL
R1	0.8 µL
H_2O	28.4 µL

☐ **11.2** Place the tube(s) in a thermal cycler with a heated lid and run the following program:

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

*Please refers to Table 6 for correct number of cycles.

☐ **11.3** Purify the PCR reaction by adding 160 µL of **Agencourt AMPure XP** beads to each sample and mix well by pipetting the entire volume up and down at least 10 times.

☐ **11.4** Incubate at room temperature for 5 minutes to let the library bind to the beads.

☐ **11.5** Place the tubes on the magnetic rack until the solution is completely clear. While the tubes are still sitting on the magnetic separation device, discard the supernatant with a pipette.

☐ **11.6** Keep the tubes on the magnetic rack. Wash the beads by adding 300 µL of 75% ethanol to each sample without disturbing the beads.

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

STEP 12. PCR AMPLIFICATION – PCR 2

☐ **12.1** Combine the following reagents for reaction (final volume 100 µL):

PCR1 **(from Step 11)**	49 µL
Amplification Mix	50 µL
LACEseq UDIs (10 µM)	1 µL

☐ **12.2** Place the tube(s) in a thermal cycler with a heated lid and run the following program:

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

*Please refers to Table 6 for correct number of cycles.

☐ **12.3** Purify the PCR reaction by using NucleoSpin Gel and PCR CleanUp kit (or equivalent) follow the PCR clean-up protocol of the manufacture manual. Elute each sample in 20 µl of nuclease-free water.

STEP 13. PAGE PURIFICATION OF LIBRARIES

RiboSeq All In One Set components needed in this part:

Kit component	Cat. nr.	Quantity	Storage	Type	Vial Cap color
Filters tubes	#KGE002-6	24 pcs	RT	Bag	--
Pierced tubes	#KGE002-7	24 pcs	RT	Bag	--
TR buffer (TR)	#KGE002-1	0.5 mL	4°C	Vial	yellow
DNA Extraction Buffer (DEB)	#KGE002-3	5.2 mL	4°C	Bottle	--
Marker 2 (M2)	#KGE002-5	13 µL	-20°C	Vial	yellow

☐ **13.1** Prepare samples: add 4 µL of 6x DNA loading dye to 20 µL of cleaned-up PCR (from Step 12.3).

☐ **13.2** Prepare M2 marker: mix 1 µL M2, 9 µL nuclease-free water and 2 µL of 6xDNA loading dye.

☐ **13.3** Load the samples in two separate lanes, while Marker 2 in one lane on 10% TBE polyacrylamide gel. Run the gel for 50 min at 200V. If the loading dye contains xylene cyanol, run the gel till the xylene cyanol reaches the bottom of the gel.

☐ **13.4** Stain the gel for 5 minutes with a solution made of 10 mL of TBE and 1.5 µL of SYBR Gold and visualize the RNA using a UV-Transilluminator.

☐ **13.5** Excise the library band at ~ 200- nt according to M2 (see Figure 3); take care not to excise the ~170 nt adapter dimers band!

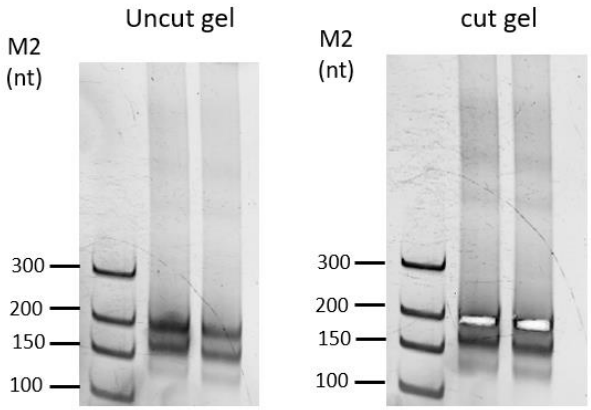

Fig.3 The Library is 200 nt length, while the adaptor dimer is 170 nt. Please note that the part that needs to be cut is exactly the band at 200 nt, leaving the whiskies out as depicted in the white rectangle on right gel. The sharper the cut, the lower the quantity of adaptor dimer that remains in the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

☐ **13.6** Place each gel slice in a pierced tube (provided) and nest the pierced small tube inside a 1.5-ml RNase-free Microcentrifuge tube (provided). Spin at maximum speed for 3 min at RT. If part of the gel pieces is still in the pierced tube, repeat the spinning. In any case, transfer carefully

any remaining gel debris from the pierced tube, discard the pierced tube and keep the 1.5 ml microcentrifuge tube.

☐ **13.7** Add 400 µL of **DEB** (**DNA Extraction Buffer**), close the vial with the provided cap, incubate the tubes for 1 hour at -80°C. Thaw the tubes at RT and then place the samples on a wheel in slow motion (3-10 rpm), at RT overnight.

☐ **13.8** With a 1 mL cut-tip, transfer the liquid and gel slurry into a spin filter (provided) and spin at 1,000g for 3 min at RT to remove the gel debris. Transfer the eluted solution to a new 1.5 ml tube.

☐ **13.9** Add 700 µL of **Isopropanol** and 1.5 µL **GlycoBlue** to the eluted sample.

☐ **13.10** Store at - 80°C for 2h (fast procedure) or overnight (safe procedure).

☐ **13.11** Thaw the samples on ice and pellet the DNA by centrifugation (20,000g) for 30 min at 4°C.

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

☐ **13.13** Remove the supernatant and resuspend the pellet in 11 µL TR buffer. Proceed with Library Quality Check

STEP 14. LIBRARY QUALITY CHECK

☐ **14.1** Evaluate each size selected library by **Agilent 2100 Bioanalyzer** using the **Agilent High Sensitivity DNA Kit**.

☐ **14.2** Use the library profile results to determine whether each sample is suitable for sequencing. Successful library production should yield a major peak at ~200 bp (see Fig. 4). Additional peaks might be observed at about 170-190 bp that are originated from adapter dimers. If the peaks areas are higher than 40% of the principal 200 bp peak, you need to purify again the libraries from gel before proceeding with sequencing.

☐ **14.5** Perform a qPCR analysis using **P5 and P7 primers** on each library for high accurate library quantification.

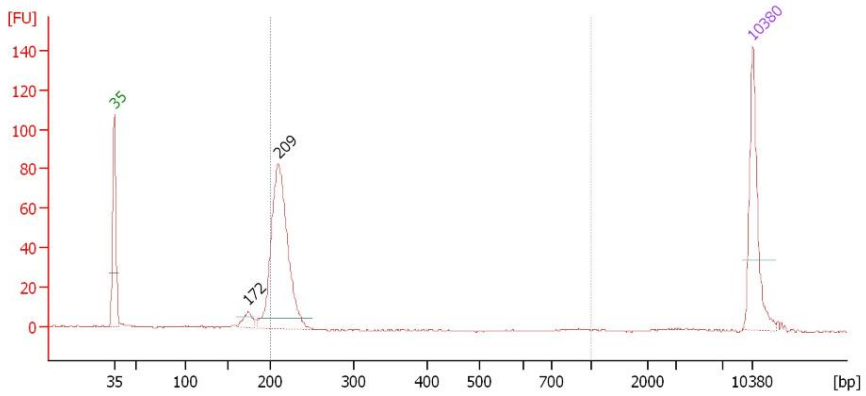

Fig. 4 Example electropherogram results for RiboSeq XL All In One Set libraries. Library was analysed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The peak at 209 bp corresponds to the size of RPFs, while the peaks at 172 bp correspond with the size of adaptor dimers.

STEP 15. HOW TO SEQUENCE

The libraries produced are suitable for Illumina platforms MiSeq, NovaSeq 6000, and NextSeq 550/1000/2000.

We suggest sequencing 100 bp SE with deepness between 100 and 120 M reads/sample. If you would like to observe rare translational events, such as uORF, and ribosome readthrough, we suggest you sequence 200 M reads/sample. Please note that, if you are willing to visualize disomes and trisomes, longer reads are required, thus in this case we suggest sequencing 150-200 bp SE between 100 and 120 M reads.

If it is possible, please utilize a sequencer with pattern flow cells such as NovaSeq 6000.

For Novaseq 6000 we suggest entering for XP protocol with the pool concentrated 470 pM, while for standard protocol 700 pM. In general, we prefer adding 3.5% quantity of PhiX.

For MiSeq you should load the libraries pool concentrated 12 pM.

For NextSeq 1000/2000 we suggest loading the library at 500 pM, with a 10% spike in of PhiX.

Finally, it is possible to sequence our libraries PE 150 and using only the FW reads as input for data analysis. For this reason, we suggest sequencing deep to retrieve 100 to 120 M forward reads/sample.

Contacts

Info

info@immaginabiotech.com

Sale support (quoting, ordering and order status update)

orders@immaginabiotech.com

Technical service (technical enquiries and quality complaints)

techsupport@immaginabiotech.com

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

www.immaginabiotech.com

+39 04611787270

Notes: