RiboLace Module 1

Product	Catalog no	Rxns.
RiboLace Mod. 1	#RL001_Mod.1_12	12

Shipping: Blue Ice and Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: : RiboLace Mod.1 contains all reagents to isolate active ribosomes by affinity purification and magnetic separation.

The kit is compatible with the PAGE Gel Extraction Kit (Cat. no. #KGE-002_12) and with the LACEseq library preparation kit (LACEseq Cat. no. #LS-001_12).

Suitable for: Eukaryotic cell lines and tissues

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

Kit contents	Qty.	Storage
RiboLace Mod.1 4°C components	1 box	4°C
RiboLace Mod.1 -20°C components	1 box	-20°C
RiboLace Mod.1 -80°C components	1 bag	-80°C

Additionally Required Materials

- 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)
- SUPERase●In (Invitrogen, catalog no. AM2696)
- RNAse free water and DEPC water
- Acid-phenol:chloroform (Ambion catalog no. AM9720)
- Nanodrop ND-1000 UV-VIS Spectrophotometer
- GlycoBlue (Ambion catalog no. AM9515)
- Isopropanol (Sigma catalog no. 278475)
- Microcentrifuge and nonstick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- Automatic wheel (rotator)
- Magnetic stand for 1.5mL tube

Optional material:

- 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)

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. Thus, the rate of protein synthesis of each sample has to be taken into account when programming experiments with the IMMAGINA - RiboLace Mod.1. In any case, always use the maximum available AU input in the suggested range.

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 7 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 7) 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.

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.

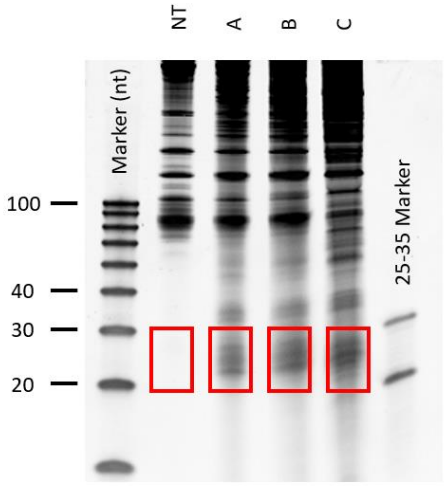

Fig.1 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel. In the red square the sizes between 25-35 nt.

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.

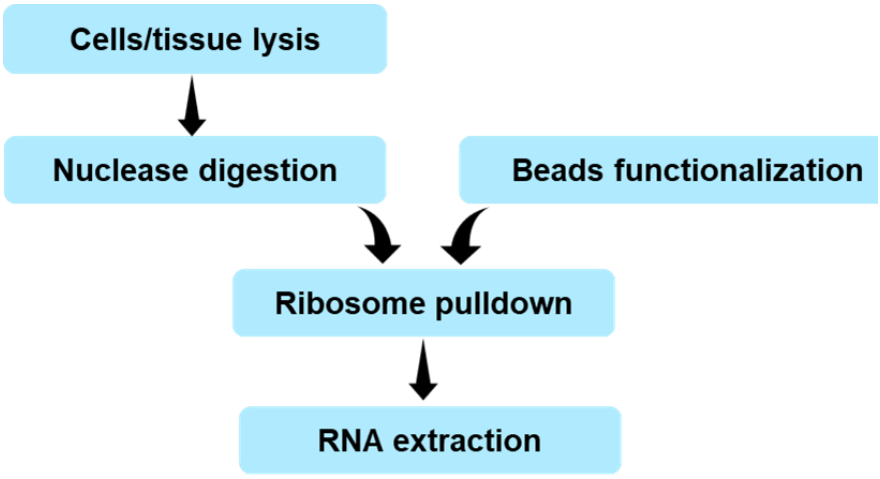

Figure 2. ***Overview of the RiboLace kit workflow***

Pull-down of active Ribosomes

RiboLace Mod.1 components list

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 11) and Step 3 (page 13) 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 9) 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 (20,000 g) 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 20,000 g, 4°C.

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

☐ **5.11** At this stage, the extracted RNA can be run on a gel and RPFs can be visualize and eventually extract the RPFs from gel via PAGE Purification using PAGExt kit (Cat. no #KGE-002_12).

OPTIONAL CHECKPOINT: PAGE VISUALIZATION OF THE RPF AFTER RIBOSOME PULLDOWN (END OF STEP 5)

RiboLace Mod.1 components needed in this part:

Kit component	Cat. nr.	Quantity	Storage	Type	Vial
25-35 Marker (25-35 M)	#RL001-26	13 µL	**-80°C**	Vial	clear

Optional material:

- 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)

The RNA recovered at the end of Step 5 contains the ribosomes 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).

Protocol for optional checkpoint PAGE visualization of the RPF after Ribosome pulldown

- ☐ The extracted RNA could 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 5 µL of Gel Loading Buffer II to the RNA obtained from Step 5.10.
- ☐ Prepare 25-35 Marker: mix 2 µL of 25-35 Marker, 3 µL nuclease-free water and 5 µL Gel Loading Buffer II.
- ☐ Use also an ultra-low range molecular weight marker as reference.
- ☐ 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).
- ☐ Stain the gel for 5 minutes with SYBR Gold in TBE and visualize the RNA using a UV-Transilluminator.

- ☐ Please note that a signal between 25 nt and 35 nt should be visible. Bands present in the red square belong to the 80S ribosomes protected fragments (RPFs) and their presence in the gel indicates a proper ribosome pulldown with the RiboLace Mod.1 kit.
- ☐ RPFs can be extracted from gel by using the PAGE Gel Extraction Kit (Cat. no #KGE-002_12).

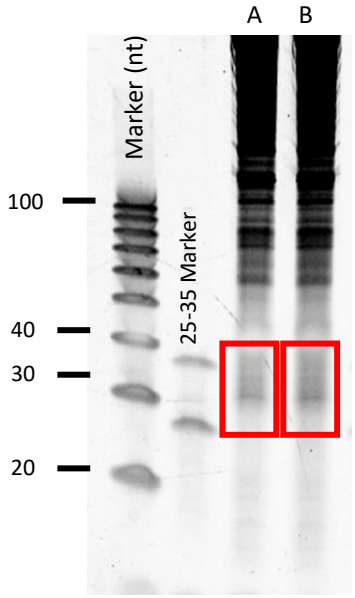

Fig.3 Example of RNA extracted after pulldown run on 15% TBE-Urea gel.

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: