RiboLace 360 Gel Free

A sample-to-data RiboSeq workflow that combines wet-lab reagents, Sequencing service, and Data Analysis service in one single and easy-to-use research solution.

Product	Catalog no	Rxns.
RiboLace 360 Gel Free	360SQ-12	12

Shipping: Blue Ice and Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: RiboLace 360 Gel Free is a complete RiboSeq solution combining the reagents to carry on the wet lab part and the associated sequencing* and bioinformatics services. The kit includes reagents and components for 12 RiboSeq reactions (from RPF pull down to NGS library prep) and a USB pen drive providing access to the bundled Sequencing and Data Analysis services.

Suitable for: Human, mouse, and Chinese hamster cell lines and tissues

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

**For US customers, next-generation sequencing following RiboLace360 sample prep will be performed in EU or UK. For customer outside Europe and USA, please contact us for information about the samples shipping for sequencing .*

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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
iUDIs plate	1 box	-20°C
USB pen drive	1 box	RT

Table 1. *Kit composition (in boxes and bag) and storage temperature.*

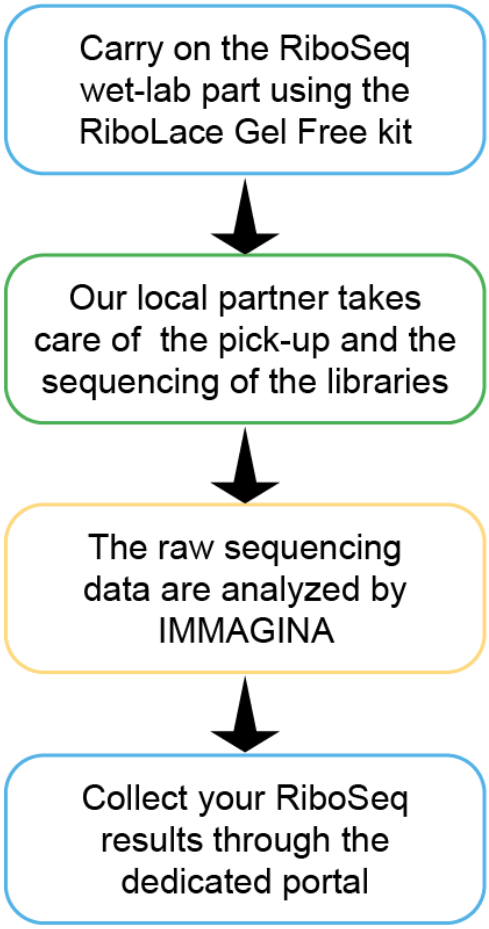
Additionally Required Materials

- 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
- RNA Clean & Concentrator™-5 (Zymo catalog. no. R1015 or R1016)
- AMPure XP for PCR Purification (Beckman Coulter catalog no. A63881)
- PCR Clean-up column kit (i.e., NucleoSpin® Macherey-Nagel catalog no 740609)
- Agilent 2100 Bioanalyzer
- Agilent High Sensitivity DNA Kit (Agilent Tech. catalog no. 5067-4626)

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)

INTRODUCTION



The RiboLace 360 Gel Free kit is a complete RiboSeq solution providing all the components and the reagents to perform 12 RiboSeq experiments and direct access to dedicated Sequencing and Data Analysis services.

The Gel Free technology is a fast, simple, and robust wet lab workflow that arises from the combination of IMMAGINA's RiboLace™ and LaceSeq™ technologies, for the isolation of active Ribosome Protected Fragments (RPF) and the preparation of efficient and precise NGS libraries. It is fast because RPF isolation takes 1 day and the entire workflow from 2 to 4 days depending on the organization of the working time. This method is straightforward, eliminating the need for gel purification, bulky equipment, or intricate manipulations such as ultracentrifugation. Furthermore, it demonstrates robustness by enhancing the recovery of RPF and their incorporation into the library. This not only amplifies method sensitivity but also diminishes operator variability.

The 360 approach provides straight forward access to dedicated Sequencing and Data Analysis services allowing the researcher to streamline the entire RiboSeq workflow from sample to result, reducing time investment, complexity, and costs.

How does it work: the user retains full experimental control performing the RPF's pull down and the library's preparation using the wet lab part of the kit. Once ready, the user gets in contact with IMMAGINA and sets up the pick-up date for the delivery to our local sequencing partner. IMMAGINA will then take care of the analysis of the sequencing data, and the user will be able to access the raw and the trimmed data, the results, and the report through a dedicated portal. Check the USB pen drive delivered with this kit for the complete set of guidelines. Please note that two pick-ups are included with the kit, it is possible to send multiple libraries at a time for sequencing, and the user can organize extra shipments at its cost.

The kit is suitable for human, mouse, and Chinese hamster primary, and immortalized cell lines (either freshly harvested or flash-frozen) and tissue (for tissues please purchase the dedicated Tissue Lysis Buffer Cat. no. #IBT0032),

The lowest starting material depends on the specimen. For specimens with very high translation levels (such as HeLa, HEK and CHO), it is possible to go as low as 300.000 cells. For lower input samples please contact us at techsupport@immaginabiotech.com.

IMMAGINA's mission is to develop unique and smart enabling technologies to break down the walls in complex Translational studies. Please visit our website https://immaginabiotech.com/ for a complete overview of our products and services and our proprietary technologies.

RiboLace™ Technology (Section B):

At the heart of the method lies a proprietary puromycin derivative, called 3P, that retains the ability to interact with active ribosomes while being covalently linked to a biotin molecule. The samples are first exposed to cycloheximide to clamp ribosomes on the mRNA fragments (recommended step), then lysate and nuclease digested to produce the 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. Eventually, the RPFs are purified by the ribosomal complex by simple differential elution. The original proof-of-concept of the technology was published on Cell Report in 2018.

LaceSeq™ Technology (Section C):

LaceSeq™, an Immagina proprietary technology, has been developed to elevate both the efficiency and simplicity of library preparation. The nucleases commonly used for the generation of the RPFs, leave a phosphate at the 3' extremity of the digested RNA fragments. Unlike the standard library preparation protocols that remove this molecular signature, LaceSeq™ exploits it to specifically target the RPFs and drive their preferential uptake into the NGS library.

After the initial selective ligation between the RPF 3'P end and our Linker, a second specific intra-molecular ligation is initiated. The kinetics of the second ligation ensures lower incorporation of RNA contaminants. The original proof-of-concept of the technology was published on NAR in 2021 with the circAID name.

The structure of the LaceSeq™ Linker has also been optimized to support downstream processes of NGS sequencing and Data analysis. Specific Unique Molecular Identifiers (UMI) are present in the linker, allowing the identification of PCR duplication products. Illumina adapters and Unique Dual Indexes (iUDIs) required for multiplexing are added after circularization and Reverse Transcription via a two-step PCR amplification.

It's important to note that the ALL-IN-ONE RiboLace Gel free includes the iUDIs plate and that we offer four different sets of iUDIs primers, 12 Forward and 12 Reverse per each set (Cat. no. #UDI0Z1-12, Z13-24, Z25-36 or Z37-48).

IMMAGINA's mission is to develop unique and smart enabling technologies to break down the walls in translational studies. Please visit our website https://immaginabiotech.com/ for a complete overview of our products & services and our proprietary technologies.

WORKFLOW OVERVIEW

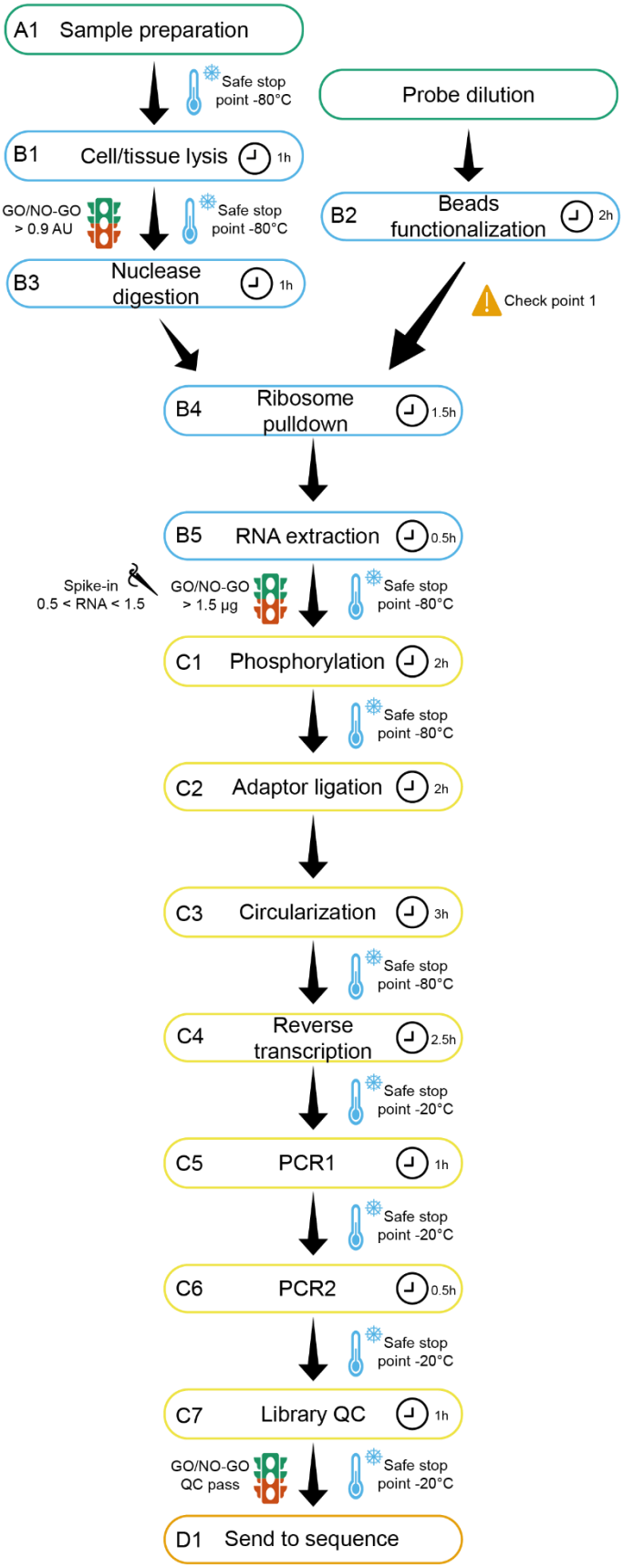


Fig.1 Overview of the RiboLace 360 Gel Free Kit workflow. In each box, steps are present in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures), GO/NO-GO conditions (traffic lights), and a checkpoint (caution signal) are placed right at the bottom of the corresponding steps.

Optimal Workflow Recommendations

- ⚠ Please avoid running more than 6 samples in parallel. Longer manipulation time may introduce an unwanted variability between the first and the last samples.
- Allocate at least 2 to 4 days for the completion of the entire workflow.
- If possible, please perform a preliminary lysis experiment to set the lysis volume following the suggested AU operational range (see section A3).
- The Beads Functionalization and the Nuclease Digestion (Steps B2 and B3) can be performed in parallel, to shorten the protocol length.
- This protocol has been optimized to perform the RPF pulldown and the library preparation starting with 0.9 AU (Abs260nm) of lysate, and 1.5 µg of RNA after RPF's pulldown. Crucially, if the sample amount does not allow it to reach 0.9 AU, it is still possible but not advisable, to lower the lysate input down to 0.6 AU without the need of modifying the kit stoichiometry.
- Starting with 0.9 AU of specimen lysate after RPF pulldown we expect to obtain at least 1.5 µg of RNA to start with the preparation of the library. To start with library preparation, the suggested quantity ranges from 0.8 to 1.5 µg of extracted RNA. If you have between 0.3 µg and 0.8 µg of extracted RNA in your sample, libraries can still be obtained but by changing the reagents and PCR cycles during library preparation. With amounts between 0.1 and 0.3 µg of RNA it is still possible to start with the preparation of the library by adding 0.2 ng of a "spike-in" or by increasing the starting material.
- 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

A.1 Sample Amounts Recommendations

The amount of Ribosome Protected Fragments (RPF) that can be isolated from a sample is strongly affected by its translational state and must be considered when programming experiments with the IMMAGINA RiboLace 360 Gel Free 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 A.U., and using it to fine-tune volumes and sample size during the real experiment (See Table 2 for lysis buffer volumes).

After ribosome pulldown (end of Step B) we expect to obtain between 0.3 and 1.5 µg of pulldown RNA to start with the preparation of the library. With amounts between 0.1 and 0.3 µg of RNA it is still possible to start with the preparation of the library by adding 0.2 ng of a “spike-in” or by increasing the starting material according to Appendix 10.

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 360 Gel Free 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 (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
B5	RNA Clean & Concentrator™-5	Additionally Required Material				
B5	G1F Buffer (G1F)	# IBT0081	200 µL	4°C	Vial	clear

Step B1. CELL LYSIS

⚠ **Before starting the experiment – Lysis Buffer Supplementing**

To ensure optimal reproducibility, for both cell and tissue lysis buffer, we recommend producing a fresh Supplemented Lysis Buffer (SLB) aliquot for each new experiment, right before proceeding with the Lysis Step. Working on ice, combine the SLB by following Table 3 instructions and multiply the volumes according to the number of samples being processed (N). please combine the different reagents following the left-to-right order.

	Lysis buffer (LB)	Sodium deoxycholate (SDC) 10% (W/V)	DNase I 1 U/µL	RiboLock RNase Inhibitor 40 U/µL	Final Volume
N=1	267 µL	30 µL	1.5 µL	1.5 µL	300 µL
N=___	____	____	____	____	____

Table 3. *Recipe for the supplementation of the provided lysis buffer or tissues lysis buffer.*

The SLB final concentration is Sodium deoxycholate (1%), DNase I (5U/mL), and RiboLock RNase Inhibitor (200 U/mL).

Please if the SLB appears as a whiteish and cloudy solution, do not proceed and check Appendix 3.

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 a 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. 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.

☐ **B1.2b** Collect the cells and centrifuge at 950g for 5min at 4°C, remove the media, and wash with **cold PBS** containing CHX (20 µg/mL).

☐ **B1.3b** Collect and centrifuge at 950g 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 20000 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 (20000 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 (20000 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 are concentrated and are NOT intended to be used without proper dilution. Before starting the experiment, as first time opening the kit, please thaw on ice the 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, 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. Proceed immediately with Step B5 without drying the beads for too long to avoid cracking them.

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

Step B5. RNA EXTRACTION

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

- ☐ **B5.1** Extract the RNA by directly adding 200 µL of the Zymo RNA Binding Buffer (**ZBB***) to the beads pipetting up and down.
- ☐ **B5.2** Transfer the bead suspension to a new nuclease-free 1.5 mL tube.
- ☐ **B5.3** Incubate the beads suspension at RT for 5 min with shaking at 600 rpm.
- ☐ **B5.4** 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.
- ☐ **B5.5** Add 200 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ **B5.6** Transfer the mixture to the **Zymo-Spin™ Column*** and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **B5.7** Add 400 µl **RNA Prep Buffer*** to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **B5.8** Add 700 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **B5.9** Add 400 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **B5.10** 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.
- ☐ **B5.11** Add 12 µL of **G1F Buffer** (**G1F**) directly to the column matrix directly to the column matrix and wait 1 min.
- ☐ **B5.12** 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.
- ☐ **B5.13** 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. To start with library preparation, the suggested quantity ranges from 0.8 to 1.5 µg of extracted RNA. If you have between 0.3 µg and 0.8 µg of extracted RNA in your sample, libraries can still be obtained but by changing the reagents and PCR cycles during library preparation. With amounts between 0.1 and 0.3 µg of RNA it is still possible to start with the preparation of the library by adding 0.2 ng of a “spike-in” or by increasing the starting material according to Appendix 10.

  - **If you have less than 0.1 µg of total RNA the chances of obtaining an informative library (or a library at all) are extremely small. Please contact us at techsupport@immaginabiotech.com.**

C. RPF's LIBRARY PREPARATION

RiboLace 360 Gel Free components and additional required materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
C1	Buffer BL1 (BL1)	#IBT0151	100 µL	-20°C	vial	Red
C1	L1 enzyme (L1)	#IBT0161	15 µL	-20°C	vial	Red
C1	ATP 10 mM	#IBT0171	100 µL	-20°C	vial	Red
C1/C2/C3	RNA Clean & Concentrator™-5	Additionally Required Material				
C1/C2/ C3/C5	Nuclease Free Water	Additionally Required Material				
C2	Buffer L2 (BL2)	#IBT0181	60 µL	-20°C	vial	Blue
C2	L2 enzyme (L2)	#IBT0191	15 µL	-20°C	vial	Blue
C2	MnCl2	#IBT0211	30 µL	-20°C	vial	Blue
C2	GTP	#IBT0201	20 µL	-20°C	vial	Blue
C2	Linker MC+ (0.1 µM) (MC+)	#IBT0222	30 µL	**-80°C**	vial	Blue
C3	Buffer L3 (BL3)	#IBT0231	50 µL	-20°C	vial	Yellow
C3	L3 Enzyme (L3)	#IBT0241	15 µL	-20°C	vial	Yellow
C3	PEG 8000 (PEG)	#IBT0251	300 µL	-20°C	vial	Yellow
C3	ATP 1 mM	#IBT0172	20 µL	-20°C	vial	Yellow
C4	Primer L4 (PL4)	#IBT0262	20 µL	-20°C	vial	Green
C4	Buffer L4 (BL4)	#IBT0271	75 µL	-20°C	vial	Green
C4	L4 enzyme (L4)	#IBT0281	15 µL	-20°C	vial	Green
C4	dNTPs	#IBT0301	20 µL	-20°C	vial	Green
C4	DTT	#IBT0291	20 µL	-20°C	vial	Green
C4	AR Enzyme (AR)	#IBT0311	10 µL	-20°C	vial	Green
C5	L5 enzyme (L5)	#IBT0321	1.5 mL	-20°C	vial	clear
C5	Fw PCR1 (F1)	#IBT0331	20 µL	-20°C	vial	clear
C5	Rev PCR1 (R1)	#IBT0341	20 µL	-20°C	vial	clear
C6	TR buffer (TR)	#IBT0351	0.5 mL	4°C	vial	clear
C1*	3P-RNA 1 µM (RNA)	#IBT0361	20 µL	-80°C	vial	clear

Step C1. 5' PHOSPHORYLATION

NOTE: We suggest starting the library preparation with 1.5 µg of extracted RNA, or, if the yield is lower, with all the amount of RNA that you extracted to maximize the output of each reaction and to obtain good libraries. Do not exceed 1.5 µg of extracted RNA to avoid overamplification of the final library.

☐ **C.1** Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

Buffer L1	5 µL
ATP (10 mM)	5 µL
L1	1 µL
RNA from Step B5.13	0.3-1.5 µg
H_2O	Up to 50 µL

☐ **C1.2** Incubate the reaction for 1h at 37 °C in a thermal cycler.

☐ **C1.3** Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 12,000 g for 30 seconds, unless otherwise specified.

☐ **C1.4** Prepare adjusted RNA Binding Buffer by mixing N x 50 µL of buffer and N x 50 µL of ethanol (95-100%).

☐ **C1.5** Add 100 µL adjusted RNA Binding Buffer (from step C1.4) to each sample and mix.

☐ **C1.6** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ **C1.7** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ **C1.8** Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

☐ **C1.9** Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

☐ **C1.10** Add 400 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through. To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 g at RT. Discard the flow-through. Carefully, transfer the column into a new RNase-free tube.

☐ **C1.11** Add 7 µL of **nuclease-free water** directly to the column matrix and wait 1 minute at RT.

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

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

Step C2. ADAPTOR LIGATION

☐ **C2.1** Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

	0.3-0.49 µg	0.5-0.79 µg	0.8-1.5 µg
RNA (from Step C1.12)	7 µL	7 µL	7 µL
Buffer L2	1 µL	1 µL	1 µL
GTP	0.5 µL	0.5 µL	0.5 µL
MnCl2	0.6 µL	0.6 µL	0.6 µL
L2 enzyme	1 µL	1 µL	1 µL
Linker MC+	0.25 µL	0.5 µL	1 µL

☐ **C2.2** Incubate the reaction for 1h at 37 °C in a thermal cycler.

☐ **C2.3** Add 40 µL **nuclease-free water.**

☐ **C2.4** Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 12,000 x g for 30 seconds, unless otherwise specified.

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

☐ **C2.6** Add the 100 µL adjusted RNA Binding Buffer (from step C2.5) to the sample and mix.

☐ **C2.7** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ **C2.8** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ **C2.9** Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

☐ **C2.10** Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

☐ **C2.11** Add 400 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through. To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 g at RT. Discard the flow-through. Carefully, transfer the column into a new RNase-free tube.

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

☐ **C2.13** Centrifuge and save the flow-through.

Step C3. CIRCULARIZATION

☐ C3.1 Mix the following reagents in a 0.2 mL nuclease-free PCR tube **(NOTE: use the 1 mM ATP vial (yellow cap color), not the 10 mM ATP vial (red cap color):**

RNA (from Step C2.13)	**9 µL**
Buffer L3	2 µL
ATP (1mM) yellow cup color	1 µL
PEG8000*	8 µL
Enzyme L3	1 µL

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

☐ C3.2 Incubate the reaction for 2h at 25 °C in a thermal cycler.

☐ C3.3 Add 30 µL **nuclease-free water.**

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

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

☐ C3.6 Add the 100 µL adjusted RNA Binding Buffer (from step C3.5) to the sample and mix.

☐ C3.7 Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ C3.8 Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

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

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

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

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

☐ C3.13 Centrifuge and save the flow-through.

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

Step C4. REVERSE TRANSCRIPTION

☐ **C4.1** For the generation of single-strand cDNA, combine the following reagents:

Circular RNA (from Step C3.13)	**12 µL**
dNTPs	1 µL
Primer L4	1 µL

☐ **C4.2** Incubate the circular RNA-primer mix at 70°C for 5 minutes and then transfer on ice for at least 1 minute.

☐ **C4.3** Add the following reagents to the annealed RNA-primer mix:

Buffer L4	4 µL
DTT	1 µL
L4 enzyme	1 µL

☐ **C4.4** Incubate for 40 min at 50 °C, then heat-inactivate for 10 min at 70 °C.

☐ **C4.5** Transfer on ice for at least 1 minute.

☐ **C4.6** Add 0.5 µL of AR Enzyme (AR).

☐ **C4.7** Incubate the solution at 37°C for 1h, then at 80°C for 20 minutes.

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

Step C5. PCR AMPLIFICATION – PCR 1

Note: the cycles of Step C5 – PCR 1 and Step C6 – PCR 2 depend on the starting RPFs that you used in Step C1. The number of cycles is important for avoiding overamplification, please refer to the following table for the correct number of cycles to utilize.

Table 4 *Number of cycles of PCR to use in Step C5 (PCR1) and C6 (PCR2)*

	RNA input amount		
	0.3-0.49 µg	0.5-0.79 µg	0.8-1.5 µg
PCR 1 cycles	9	8	8
PCR 2 cycles	6	6	6

☐ **C5.1** Combine the following reagents (for reaction) in a final volume of 100 µL:

cDNA (from Step C4.7)	20 µL
L5 enzyme (L5)	50 µL
F1	0.8 µL
R1	0.8 µL
H_2O	28.4 µL

☐ **C5.2** Place the tube(s) in a thermal cycler with a heated lid and run the following program:

Step	Temperature	Time
Initial denaturation	98°C	1 min
9-8 Cycles	98°C	30 secs
	61°C	30 secs
	72°C	10 secs
Hold	4°C	∞

☐ **C5.3** Transfer the reaction mix into a new 1.5 mL tube.

☐ **C5.4** Purify the PCR reaction by adding 160 µL of **Agencourt AMPure XP** beads **(Warm the beads at Room Temperature before use)** to each sample and mix well by pipetting the entire volume up and down at least 10 times.

☐ **C5.5** Incubate at room temperature for 5 minutes to let the library bind to the beads.

☐ **C5.6** Place the tubes on the magnetic rack until the solution is completely clear. While the tubes are still sitting on the magnetic separation device, discard the supernatant with a pipette.

☐ **C5.7** Keep the tubes on the magnetic rack. Wash the beads by adding 300 µL of 75% ethanol to each sample without disturbing the beads.

- ☐ **C5.8** Wait for 30 seconds and use a pipette to carefully remove the supernatant containing contaminants. The library will remain bound to the beads during the washing process.
- ☐ **C5.9** Repeat the washing step with 75% ethanol once, keeping the beads on the magnet.
- ☐ **C5.10** Let the beads pellet dry on the magnetic rack at room temperature for ~2–4 minutes. **Avoid to over dry the beads (pellet cracked) as this will significantly decrease elution efficiency.**
- ☐ **C5.11** Remove the tubes from the magnetic rack and add 50 µL of nuclease-free water to cover the pellet. Mix thoroughly by pipetting up and down to ensure complete bead dispersion. Incubate at room temperature for at least 3 minutes to rehydrate.
- ☐ **C5.12** Place the sample tubes on the magnetic rack for 2 minutes or longer until the solution is completely clear.

Step C6. PCR AMPLIFICATION – PCR 2

The LACEseq UDIs sequences can be found at this link. Please utilize one UDI for each reaction.

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

PCR1 (from Step C5.12)	49 µL
L5 enzyme (L5)	50 µL
LACEseq UDIs (10 µM)	1 µL

☐ **C6.2** Place the tube(s) in a thermal cycler with a heated lid and run the following program:

Step	Temperature	Time
Initial denaturation	98°C	1 min
6 Cycles	98°C	30 secs
	60°C	30 secs
	72°C	10 secs
Hold	4°C	∞

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

Step C7. LIBRARY QUALITY CHECK

☐ **C7.1** Evaluate each size selected library by **Agilent 2100 Bioanalyzer** using the **Agilent High Sensitivity DNA Kit**.

☐ **C7.2** Use the library profile results to determine whether each sample is suitable for sequencing. Successful library production should yield a major peak at ~200-220 bp (see Fig. 2). Additional peaks might be observed at about 170-190 bp that originate from adapter dimers. If the peak areas are higher than 50% of the principal 200 bp peak, you need to purify the libraries from gel before proceeding with sequencing.

☐ **C7.3** Perform a qPCR analysis using **P5 and P7 primers** on each library for highly accurate library quantification.

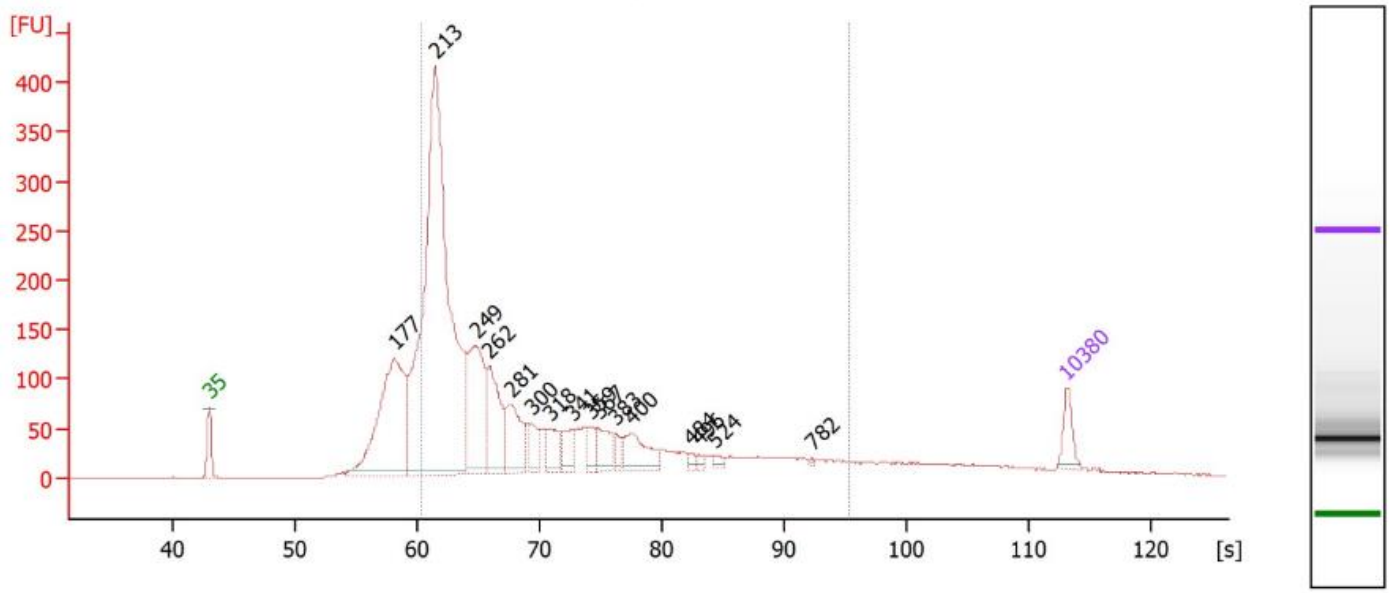


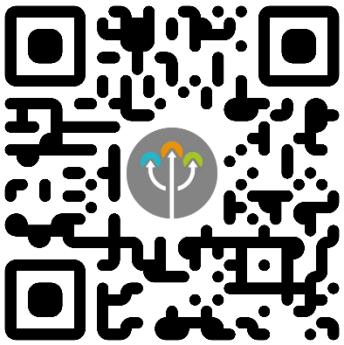
Figure 2. Example electropherogram libraries results. Typical electropherogram for a library prepared with an immortalized cell line. The library was analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The electropherogram need to present at least one major peak between 200 to 220. Tin this example, the peak at 213 bp corresponds to the size of RPFs, while the peaks at 170-190 bp correspond with the size of adaptor dimers**.**

☐ **C7.4** Before sequencing, for further QC of the library, please refer to the guidelines on the file present on the USB pen drive.

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 A.U	N=___ 0.9 A.U	Needed in Step	Step-by-Step Checklist
			B2.1	□ Place RmB v2-1 at RT for 30 min
			B2.2	□Vortex 30'
RiboLace magnetic beads (RmB)	144 µL	____	B2.3	□Add "___" Beads in 1.5 / 2 mL Tube □Place on magnet □REMOVE Supernatant
OH-buffer (OH)	270 µL	____	B2.4	□Remove from magnet □Wash OH Buffer: add "____" µL □Shake 5min 1400 rpm RT □Place on magnet □REMOVE Supernatant
Nuclease-free water	1000 µL	1000 µL	B2.5	□Remove from magnet □Wash N.F. Water: add 1000 µL □Shake 2min 1400 rpm RT □Place on magnet □REMOVE Supernatant
B-Buffer (BB)	270 µL	____	B2.6	□Remove from magnet □Wash B Buffer: add "____" µL □Shake 3min 1400 rpm RT □Place on magnet □REMOVE Supernatant □Repeat wash 2nd time
			B2.7	□Store 2 µL of diluted RsP for control
Diluted RiboLace Smart Probe (RsP)	81 µL	____	B2.8	□Remove from magnet □Resuspend in diluted RsP: "___" µL
			B2.9	□Incubate 1h shacking 1400 rpm RT
At this point, you can start the Nuclease digestion (step B3) in parallel				
			B2.10	□Place on magnet □Store 3 µl of Supernatant for control □Remove from magnet
mPEG	7.5 µL	____	B2.11	□Add mPEG "____" µL □Incubate 15min shacking 1400 rpm RT
Nuclease-free water	1000 µL	1000 µL	B2.12	□Place on magnet □REMOVE Supernatant □Remove from magnet □Wash with N.F. water: add 1000 µL □Shake 2 min 1400 rpm RT
W-buffer (WB)	1000 µL	1000 µL	B2.13	□Place on magnet □REMOVE Supernatant □Remove from magnet □Wash with W buffer 1000 µL □Shake 2 min 1400 rpm RT
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 A.U	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 0.2 AU as the starting material and W-buffer up to a final volume of 150 µL. Perform a titration assay, by adding to each reaction different quantities of Nux, below is an example:

Starting lysate	Quantity of diluted Nux (Step. 3.3)	Sample
0.2 AU	0	NT
0.2 AU	0.2 µL (AU x 1)	A
0.2 AU	1 µL (AU x 5)	B
0.2 AU	10 µL (AU x 50)	C

Table 10. *Components' volumes to use for the Nuclease Optimization of lysate. The table is intended as a guideline to follow when dealing with non-standard samples that require ad hoc digestion.*

- ☐ Digest the samples for 45 min at 25°C.
- ☐ Stop digestion with 1 µL of SUPERaseIn for 10 min on ice.
- ☐ Add 300 µL of the Zymo RNA Binding Buffer (**ZBB***).
- ☐ Incubate the solution at RT for 5 min with shaking at 600 rpm.
- ☐ Add 450 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ Transfer 700 µL of the mixture to the **Zymo-Spin™ Column*** and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ Transfer the remaining volume of the mixture to the **Zymo-Spin™ Column*** and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ Add 400 µl **RNA Prep Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ Add 700 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ Add 400 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
- ☐ To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 g at RT. Discard the flow-through. Carefully, transfer the column into a new RNase-free tube.
- ☐ Add 11 µL of **Nuclease Free Water** directly to the column matrix and wait 1 minute.

- ☐ Centrifuge for 30 seconds at 12,000 g at RT. The extracted RNA is present in the flow-through. Collect the flow through.
- ☐ With Nanodrop, measure the absorbance of each sample at 260 nm (set up the “nucleic acid” function of the Nanodrop), using 1 µL of Nuclease Free Water as blank.
- ☐ Extracted RNA needs to be run on a 15% TBE-urea gel.
- ☐ Pre-run the gel at 200 V for 30 min in TBE prepared with nuclease-free water. Clean well the gel wells with a syringe to remove UREA residuals before loading the samples.
- ☐ Prepare samples: add Gel Loading Buffer II to 1.5 µg of RNA (1:1 volume).
- ☐ Use an ultra-low range molecular weight marker as reference.
- ☐ Load the samples and the Marker on 15% TBE-urea polyacrylamide gel and run the gel for 1 h at 200V until the bromophenol blue band reaches the bottom of the gel.
- ☐ Stain the gel for 5 minutes with SYBR Gold in TBE and visualize the RNA using a UV-Transilluminator.

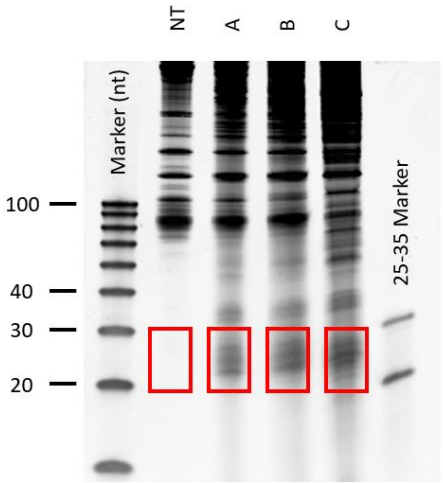


Fig.6 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel. In the red square the sizes between 25-35 nt.

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.6). 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.

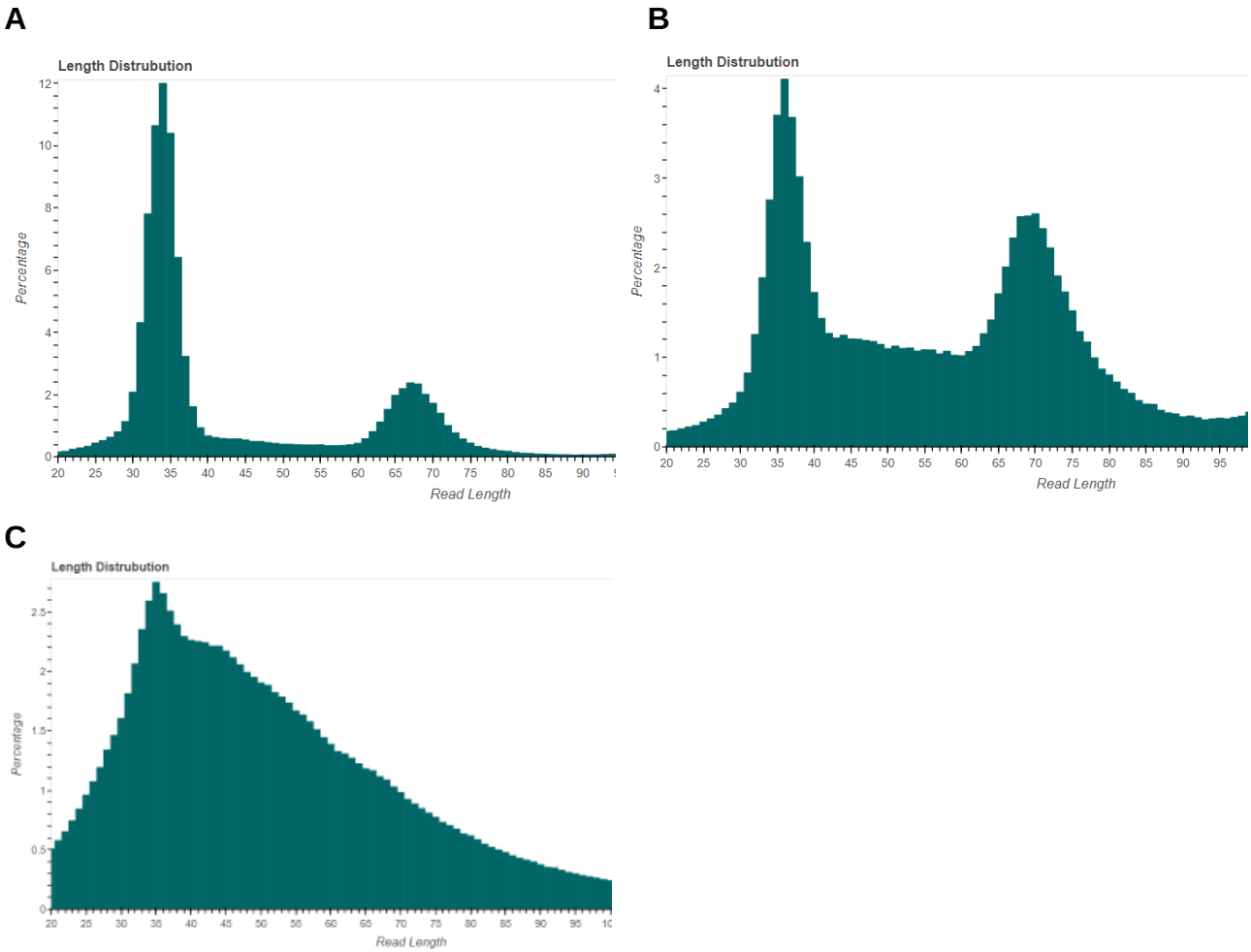


Fig.7 Example of read length distribution obtained by sequencing libraries originated from A) sample digested with the right amount of Nux B) underdigested sample or C) overdigested sample.

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

App.9 Optional intermediate checkpoint: PAGE visualization of the RPF after Ribosome pulldown (end of Step B5 – RNA extraction)

The RNA recovered at the end of Step B5 should be quantified by Nanodrop before proceeding with Step C1. This RNA contains the ribosome-protected fragments (RPFs) that are needed to prepare the library. If the quantity of RNA extracted is more than the 1.5 µg needed for the library preparation, we suggest running 1 µg of the extracted RNA on a 15% TBE-urea gel following the protocol below to check the presence of RPFs. If you do not have at least 2.5 µg of extracted RNA (1.5 µg for library and 1 µg for running the gel), prioritize the library preparation and avoid running this optional intermediate checkpoint, keeping 1.5 µg for library preparation.

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

- ☐ As a checkpoint, 1 µg of 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 Gel Loading Buffer II to 1 µg of RNA obtained from Step B5 (1:1 volume).
- ☐ Use 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.

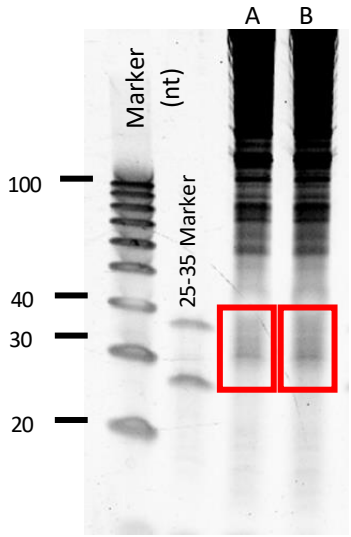


Fig.4 Example of RNA extracted after pulldown run on 15% TBE-Urea gel. The red boxes in lines A and B contain the bands relative to the RPF and corresponding to a length between 25 and 35. For proper RPF isolation via PAGE extraction please retrieve the gel portion within the red box.

- ☐ 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 with the kit.

App.10 Library Preparation: Low Amount of Starting Material – Troubleshooting Guidelines

Day one of this protocol ends with the pull-down or the RNA fraction containing the RPF fragments (end of section B) to be used for the production of the NGS libraries (beginning of section C). To properly enter the library preparation part of the protocol you should have at least 0.3 µg of pull-down RNA, this amount is required to compensate for the losses occurring during the multiple purification steps required to produce a library that can be sequenced. If this amount is not available, please refer to the following guidelines:

Increase Lysate AU:

In the case of low RNA extraction after pull-down, the suggested approach is to repeat the pull-down with an increased amount of AU by either loading a larger amount of lysate (if available with enough volume and concentration) or restarting the workflow from the beginning with a higher amount of starting material and rescale the volume of reagents for the pull-down accordingly. Please note that this will reduce the number of pull-down reactions that are possible to perform with the kit.

Example:

Reagent \ Lysate input	Standard Amounts	30% increase	Needed in Step
	0.9 AU	1.2AU	
RiboLace magnetic beads (RmB) v2-1	144 µL	187 µL	B2.3
OH-buffer (OH)	270 µL	351 µL	B2.4
Nuclease-free water	1000 µL	1000 µL	B2.5
B-Buffer (BB)	270 µL	351 µL	B2.6
Diluted RiboLace Smart Probe (RsP)	80 µL	104 µL	B2.8
mPEG	7.5 µL	10 µL	B2.11
Nuclease-free water	1000 µL	1000 µL	B2.12
W-buffer (WB)	1000 µL	1000 µL	B2.13
W-buffer (WB)	105 µL	105 µL	B2.14
Lysate	0.9 AU	1.2AU	B3.1

For further optimization, you can download the RiboLace Starter Protocol for a complete guide to the optimization of the reaction volumes (see Appendix 1).

Spike-in Strategy:

Should you not have the possibility to repeat the pull-down, but you have between 0.1 µg and 0.3 µg of pull-down RNA (the more the better), the protocol allows you to still move on with the production of the library by adding a so-called “spike-in”. **The “spike-in” approach comes at the cost of producing less informative libraries and should then be used only when strictly**

necessary. The “spike-in” is an exogenous RNA fragment of known sequence, with a length comparable to the RPF’s and a 3’P extremity (see fig. 9). Mimicking an RPF molecule allows to artificially boost the number of RPFs to a level sufficient to carry on with the multiple reaction and purification steps required for the production of the library. Thus, it should come as no surprise that, when using this strategy, after sequencing the library up to 60% of the counts will match the spike-in sequence.

- ☐ Calculate the total amount of RNA after pull-down (step B5.13)
- ☐ Add 0.5 µL of Spike-in (corresponding to 0.2 ng of spike-in Fig.9) to the total amount of RNA after pull-down.
- ☐ Start the Phosphorylation (Step C1) using the spiked-in RNA using the standard protocol for 0.3 µg.
- ☐ Follow up the normal protocol for 0.3 µg from Step C2.

Spike-in RNA exogenous sequence

5′-CTGAGAAAGTAGAGCAAGAAGAAATAGAGC-3′

(20 µL, 0.4 ng/uL)

Fig.9 Spike-in RNA exogenous sequence, with amount and concentration

Less Than 0.1 µg of pull-down RNA:

Having less than 0.1 µg of pull-down RNA the chances of producing a library that can be sequenced are close to none. The best strategy is to restart the pull-down with an increased amount of material to be able to get at least within the operational range. If you cannot perform again the pulldown, please write to techsupport@immaginabiotech.com,

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Notes: