# ALL-IN-ONE RiboLace Gel Free

A fast, easy, and robust RiboSeq workflow (sample to NGS library) that provides deeper insights without the need for any gel extraction steps.

| Product | Catalog no | Rxns. |
|---|---|---|
| ALL-IN-ONE RiboLace Gel Free | GF001-12 | 12 |

Shipping: Blue Ice and Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: ALL-IN-ONE RiboLace Gel Free contains all reagents to perform ribosome profiling from cells/tissues to the final Next Generation Sequencing library without the need for RNA size selection and PAGE-gel extraction steps. The kit includes components for 12 reactions, and it is suitable for Illumina platforms (MiSeq, NovaSeq 6000, NextSeq1000/2000).

Suitable for: Eukaryotic cell lines and tissues

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

# Table of Contents

## 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 |

**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)
- If not present in the kit, DNAse I (Zymo catalog. No. E1010)
- Etanol 95-100%
- Agilent Eukaryote Total RNA Nano Kit (Agilent Tech. catalog no. 5067-1512)

# INTRODUCTION

The ALL-IN-ONE RiboLace Gel free kit is a complete RiboSeq solution combining IMMAGINA's RiboLace technology for the isolation of active Ribosome Protected Fragments (RPFs) with the LaceSeq technology for the preparation of efficient and precise NGS libraries of small RNA fragments. The synergy between those two technologies results in a fast, simple, and robust workflow. 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 kit is suitable for eukaryotic primary, and immortalized cell lines (either freshly harvested or flash-frozen) and tissue (for tissues please purchase the dedicated Tissue Lysis Buffer Cat. no. #IBT0032),

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

IMMAGINA's mission is to develop unique and smart enabling technologies to break down the walls in complex Translational studies. Please visit our website https://www.immaginabiotech.com/ for a complete overview of our products 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. Ribosomes are thus purified by affinity purification and magnetic separation and the ribosomes are extracted from the ribosomal complex. 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 of nuclease cleavage, 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 under the "circAID" name since the technology was originally developed for nanopore sequencing.

The structure of the LaceSeq™ Linker has also been optimized to support downstream processes of NGS sequencing and Data analysis by a sharp trimming of the 5'-end of the RPF. 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://www.immaginabiotech.com/ for a complete overview of our products & services and our proprietary technologies.

# WORKFLOW OVERVIEW

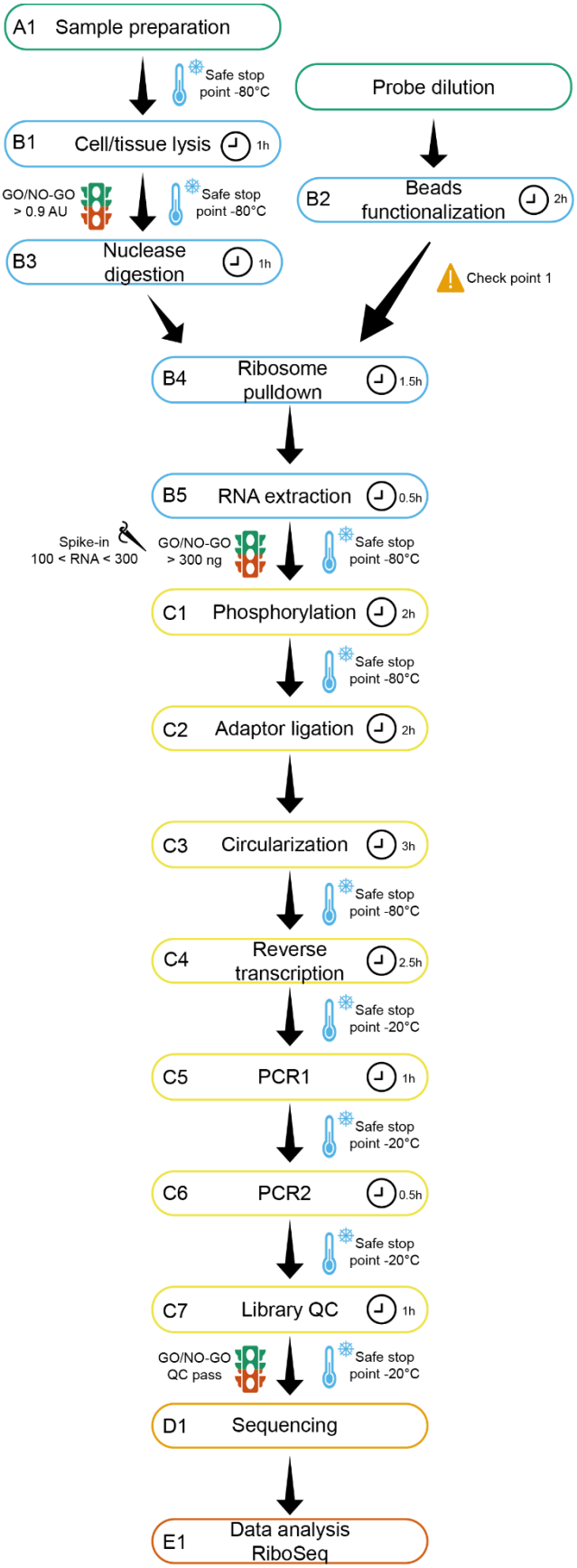


**Fig.1 Overview of the ALL-IN-ONE RiboLace Gel Free Kit workflow.** In each box, steps are present in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures), GO/NO-GO conditions (traffic lights), and a checkpoint (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 ALL-IN-ONE RiboLace 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. With amounts lower than 0.3 µg it is worth considering a multiplexing solution available at https://www.immaginabiotech.com/products/riboseq-sample-to-library.

## 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

ALL-IN-ONE RiboLace 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 | Binding Buffer (BB) | # IBT0021 | 10 mL | 4°C | Bottle | -- |
| B2 | RiboLace magnetic Beads v2.1 (RmB v2.1) | # IBT0042 | 1.8 mL | 4°C | Vial | clear |
| B2 | OH Buffer (OH) | # IBT0051 | 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) | # IBT0012 | 200 µL | -20°C | Vial | clear |
| B2 | diluted RiboLace smart Probe (dRsP) | Dilute Aliquot from RsP | | -80°C | | |
| B2/B3/B4 | Wash Buffer (WB) | # IBT0071 | 2 x 25 mL | 4°C | Bottle | -- |
| B3 | Nuclease (Nux) | # IBT0091 | 21 µL | -20°C | Vial | clear |
| B3 | Diluted Nuclease (dNux) | Dilute Aliquot from Nux | | | | |
| B3 | Nux Enhancer (NE) | # IBT0081 | 13 µL | -20°C | Vial | clear |
| B5 | RNA Clean & Concentrator™-5 | Additionally Required Material | | | | |
| B5 | G1F Buffer (G1F) | # IBT0101 | 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 P200 pipette. **All the PBS must be removed before proceeding with the lysis to avoid diluting the lysis buffer.**

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

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

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

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

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

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

## Suspension Cells lysis

☐ **B1.1b** Treat the cells with 10 µg/mL of **cycloheximide (CHX)** for 5 min at 37°C before lysis, should you not wish to add CHX check Appendix 2 for the alternative protocol. 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, please note that the concentration of the nuclease is critical for the outcome as, using the incorrect quantity, might lead to varying effects on the read length distribution. ⚠ **Should you need to perform a titration curve to assess the proper quantity of Nux to add to your (non-conventional) sample, you can check the guidelines in Appendix 8 - Optional Nuclease Optimization.**

- ☐ **B3.1** Start with a total volume of lysate corresponding to 0.9 A.U. (260 nm) (see Section A2.3 for calculation) diluted in W-buffer (**WB**) to the final volume of 450 µL.
- ☐ **B3.2** Add 0.9 µL of **Nux Enhancer (NE)**.
- ☐ **B3.3** Dilute 1.5 µL of **Nuclease** (**Nux**) by adding 98.5 µL W-buffer (**WB**). Pipet up and down 5 times to mix well the diluted Nux solution (dNux).
- ☐ **B3.4** Digest the sample in a 1.5 mL tube for 45 min at 25 °C with 4.5 µL of the diluted Nuclease **(dNux)** prepared before. Trash the remaining diluted Nux solution, for experiments performed on other days, prepare fresh diluted Nux.
- ☐ **B3.5** Stop digestion with 1.5 µL of **SUPERaseIn** for 10 min on ice.

# Step B4. RIBOSOMES PULLDOWN

**Remove the W-buffer (WB) from Step B2.14 only immediately before adding the cell lysate!**

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

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

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

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

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

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

☐ **B4.6** Remove completely the W-buffer (WB) before removing the beads from the magnet. 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

ALL-In-ONE RiboLace 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 L1 (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 | 100 µL | -20°C | vial | Blue |
| C2 | L2 enzyme (L2) | #IBT0191 | 15 µL | -20°C | vial | Blue |
| C2 | GTP | #IBT0201 | 20 µL | -20°C | vial | Blue |
| C2 | MnCl2 | #IBT0211 | 30 µL | -20°C | vial | Blue |
| C2 | Linker MC+ (MC+) | #IBT0222 | 30 µL | -80°C | vial | Blue |
| C3 | Buffer L3 (BL3) | #IBT0231 | 100 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 | 100 µL | -20°C | vial | Green |
| C4 | L4 enzyme (L4) | #IBT0281 | 15 µL | -20°C | vial | Green |
| C4 | DTT | #IBT0291 | 50 µL | -20°C | vial | Green |
| C4 | dNTPs | #IBT0301 | 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 | FWD 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.

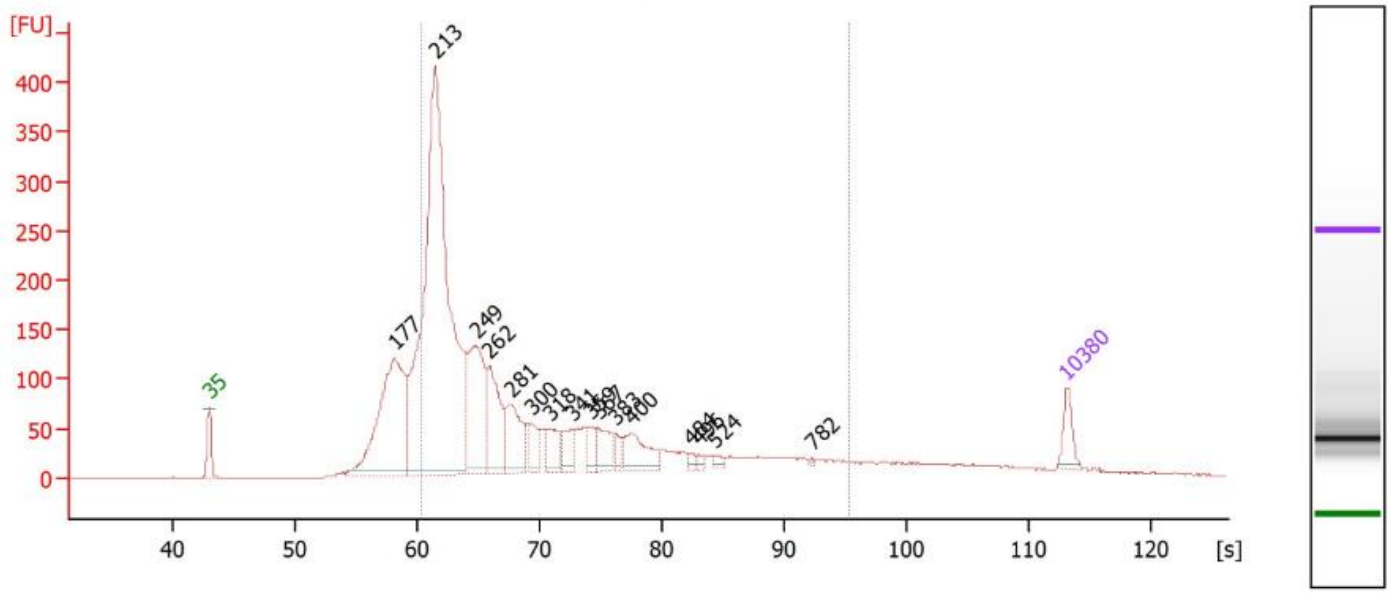


**Figure 2. Example electropherogram libraries results.** Typical electropherogram for a library prepared with an immortalized cell line. The library was analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The electropherogram need to present at least one major peak between 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**.**

# D. SEQUENCING

### Step D1. HOW TO SEQUENCE – DO IT YOURSELF

The libraries produced are suitable for Illumina platforms, we strongly suggest the use of sequencing platforms based on pattern flow cells such as MiSeq, NovaSeq 6000, and NextSeq 1000/2000. Although compatible, we do not advise the use of open-flow cells such as NextSeq 550, which in our experience yield less satisfactory sequencing runs.

Sequencing lengths shorter than 100 bp should be avoided, although theoretically sufficient, shorter read length may lead to loss of reads during computational analysis. We suggest Single-End sequencing run with depth between 100 and 120 Million reads/sample, and if you would like to observe rare translational events, such as uORF, and ribosome readthrough, to increase the depth to 200 M reads/sample.

To visualize RPFs deriving from the footprint of ribosome disome and trisome the sequencing length must be increased to at least 150 bp or 200bp.

In common practice, Single-End sequencing is less frequently used than Pair-End sequencing, and some facilities are more prone to performing PE sequencing. It is possible to sequence our library in PE (with a length of 150bp) but with a depth of 200 - 240 Million reads, this is because only the Forward Reads are retained for the Data analysis.

For Novaseq 6000 using the XP protocol, we suggest an entry concentration of the pool of 470 pM, while for standard protocol of 700 pM. In general, we prefer adding a 3.5% quantity of PhiX.

For MiSeq the loading concentration of the library pool should be 12 pM, while for NextSeq 1000/2000 we suggest loading the library at 500 pM, with a 10% spike-in of PhiX.

### Step D2. HOW TO SEQUENCE – OUTSOURCE TO IMMAGINA

Supporting the whole RiboSeq workflow requirements, IMMAGINA provides sequencing services for RiboSeq NGS libraries, RNAseq libraries, and the required downstream data analysis:

- a basic package for the trimming of the sequencing data, the alignment of the reads and the counts,
- a package for the calculation of the Translation Efficiency,
- a Premium package for the development of ad-hoc analysis.

You can contact us at info@immaginabiotech.com to discuss your needs in detail.

# E. BIOIT GUIDELINES – do it yourself

## Expected Illumina sequencing output.

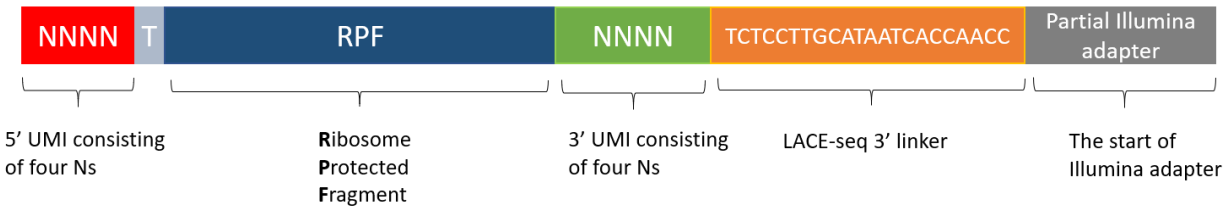


**Figure 1. Expected Illumina sequencing output:** example of a read generated.

Unique molecular identifiers (UMIs) are strings of random nucleotides that are attached to RPFs prior to PCR amplification and can be used to accurately detect PCR duplicates.

The T at position fifth precedes the start of the RPF. The sequence content of a high-quality library has a T peak in position fifth in 90-100% of the reads (Figure 2).

To check T peak use fastqc command:

- fastqc --outdir outputdir input.fastq

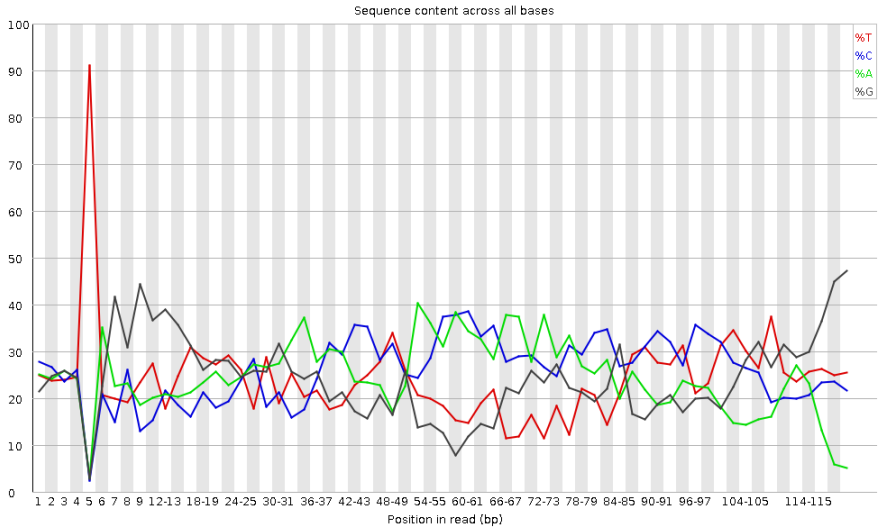


**Figure 2. Sequence content across all bases graph.** The sequence content of a high-quality library has a T peak in position five in 90-100% of the reads.

## Workflow overview

There are 5 main steps in the analysis pipeline:

E1. Software installation

E2. Trimming/UMI extraction

E3. Filtering rRNA, tRNA and ncRNA

E4. STAR alignment

E5. RiboWaltz pipeline

**Step E1. Software installation:**

Information and guides to install the required tools. Though more recent versions of the programs will also be compatible with this pipeline, the workflow is intended to function with the versions listed:

- Dependencies
  - Trimming:
    - Cutadapt (https://cutadapt.readthedocs.io/en/stable/installation.html)
  - Quality Control:
    - Fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
  - Alignment:
    - bowtie2 (https://bowtie-bio.sourceforge.net/bowtie2/index.shtml)
    - STAR (https://github.com/alexdobin/STAR)
  - Utilities:
    - umi_tools (https://github.com/CGATOxford/UMI-tools)
    - samtools (https://www.htslib.org/ )
  - Ribosomal Footprint Analysis:
    - RiboWaltz (R) (https://github.com/LabTranslationalArchitectomics/riboWaltz)
- Build Aligner Indexes
  - To build bowtie2 indexes fasta files of tRNAs, rRNAs and snRNAs or ncRNAs are necessary. You can find those files https://rnacentral.org/.
  - To build STAR index also gtf file is needed. And those files can be found at https://www.gencodegenes.org/ and https://www.ensembl.org

Once the tools have been installed, you will need to make sure that the UNIX environment variables are appropriately set. You can either add the location of the executables installed to your PATH variable or create a new directory called bin in your home directory, copy the executables to this location, and add the location of the bin directory to your PATH variable.

To change your PATH variable, enter (assuming bash shell):

```
> export PATH = <list of paths>:$PATH
```

| Parameter | Definition |
|---|---|
| `PATH = <list of paths>:$PATH` | specify number of threads in computer for this job (Depends on the computer) |

## Step E2: UMI extraction/Trimming

Proper trimming of the reads is important for efficient mapping. Here we provide some guidance on the use of (E2.1) cutadapt (Martin M. 2011) to remove Linker MC+ (MC+), (E2.2) UMI-tools extract (Smith T. 2017) to move the UMI sequence from the read to the read name so that PCR duplicates can be removed after the alignment, (E2.3) cutadapt to remove the T preceding the RPF.

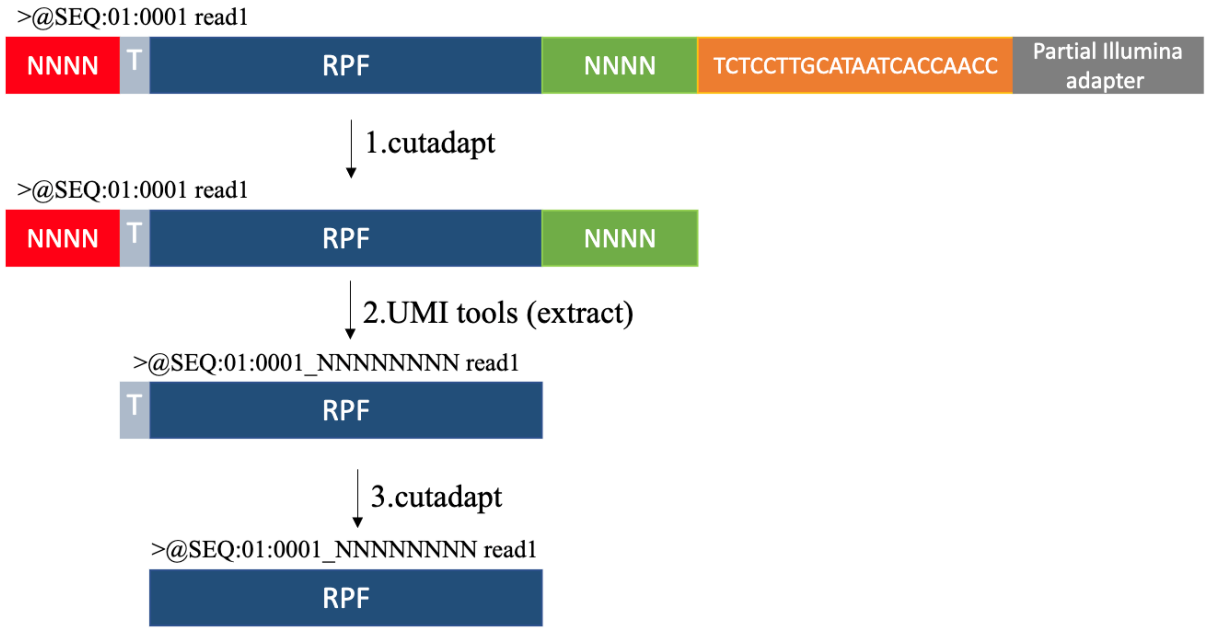


**Figure 3. UMI extraction and trimming step schematic.** The RPF extraction is done in 3 steps: linker removal, UMI extraction, T removal.

## E2.1: cutadapt

First the Linker MC+ (MC+) is trimmed from the 3' end of each read and only reads longer than X+9 nt are retained, while shorter reads are discarded:

```
cutadapt --cores N --minimum-length X+9 -a TCTCCTTGCATAATCACCAACC --discard-untrimmed -
o trim.fastq input.fastq
```

| Parameter | Definition |
|---|---|
| `--cores N` | specify number of threads in computer for this job (Depends on the computer) |
| `--minimum-length X+9` | Reads are retained if they are longer than X+9 nt, where X is the length of the RPF (usually X=20 for ribosome profiling analysis), and 9 is the sum of the lengths of the 5' and 3' UMIs |
| `-a TCTCCTTGCATAATCACCAACC` | Removal of the LACE-seq 3' linker and any sequence that may follow |
| `--discard-untrimmed` | Reads in which *no* adapter is found are discarded |
| `-o trim.fastq` | The output file name |
| `input.fastq` | The input file name |

### E2.2: UMI-tools (extract)

The sequence of the 5' and 3' UMIs are moved from the read sequence to the read name:

```
umi_tools extract -I trim.fastq --bc-pattern='^(?P<umi_1>.{4}).+(?P<umi_2>.{4})$' --
extract-method=regex -S extract.fastq --log=<umi_extract.log>
```

| Parameter | Definition |
|---|---|
| `-I trim.fastq` | The input file name must be the same as the output file name in step1 |
| `--bc-pattern='^(?P<umi_1>.{4}).+(?P<umi_2>.{4})$'` | extract the first 4 (5'UMI) and the last 4 bases (3'UMI) of each read |
| `--extract-method` | defines method for UMI extraction |
| `-S extract.fastq` | The output file name |

NOTE: UMI-tools dedup can be used after alignment to remove duplicates based on the mapping coordinate and the UMI attached to the read name.

### E2.3: cutadapt

The T preceding the RPF is then removed:

```
cutadapt --cores N -g ^T --discard-untrimmed -o trim2.fastq extract.fastq
```

| Parameter | Definition |
|---|---|
| `--cores N` | specify number of threads in computer for this job (Depends on the computer) |
| `-g ^T` | Removal of the first T at the start of each read |
| `-o trim2.fastq` | The output file name |
| `extract.fastq` | The input file name must be the same as the output file name in step E2.2 |

## Step E3: Filtering rRNA, tRNA and ncRNA

In order to remove and quantify ribosomal RNA (rRNA) content or other contaminants (tRNAs and snRNAs etc) in your sample prior to alignment to the genome, you can align the trimmed reads against specific contaminant sequences. The first step in removing contaminants is to create a FASTA formatted file containing contaminating sequences from your sample to align against, using the Bowtie aligner (Bowtie2-build https://bowtie-bio.sourceforge.net/bowtie2/index.shtml).

To build bowtie2 indexes fasta files of tRNAs, rRNAs and snRNAs or ncRNAs are necessary. You can find those files https://rnacentral.org/.

```
bowtie2-build --threads N - f <reference.fasta.file> <given_index_name>
```

| Parameter | Definition |
|---|---|
| `--threads N` | specify number of threads in computer for this job (Depends on the computer) |
| `- f <reference.fasta.file> <given_index_name>` | f: specify fasta file location and name (Eg: /go/to/reference.fa) and given_index_name refers to the location and name of the indexes (Eg: /go/to/index/rRNA) |

## E3.1: removing rRNA contaminant

```
bowtie2 --threads N -N 1 --no-1mm-upfront -q <trimmed.fastq.gz> --
un=<norRNA.fastq.gz> -x <rRNA_bowtie_index>
```

| Parameter | Definition |
|---|---|
| --threads N | specify number of threads in computer for this job (Depends on the computer) |
| -N 1 | Number of allowed mismatches |
| --no-1mm-upfront | This option prevents Bowtie 2 from searching for 1-mismatch end-to-end alignments |
| -q <trimmed.fastq.gz> | Input filename |
| --un=<norRNA.fastq.gz> | output not aligned reads |
| -x <rRNA_bowtie_index> | Index file for alignment |

## E3.2: removing tRNA contaminant

```
bowtie2 --threads N -N 1 --no-1mm-upfront -q <norRNA.fastq.gz> --
un=<norRNA_notRNA.fastq.gz> -x <tRNA_bowtie_index>
```

| Parameter | Definition |
|---|---|
| --threads N | specify number of threads in computer for this job (Depends on the computer) |
| -N 1 | Number of allowed mismatches |
| --no-1mm-upfront | This option prevents Bowtie 2 from searching for 1-mismatch end-to-end alignments |
| -q <trimmed.fastq.gz> | Input filename |
| --un=<norRNA_notRNA.fastq.gz> | output not aligned reads |
| -x <tRNA_bowtie_index> | Index file for alignment |

## E3.3: removing ncRNA contaminant

```
bowtie2 --threads N -N 1 --no-1mm-upfront -q <norRNA_notRNA.fastq.gz> --
un=<norRNA_notRNA_noncRNA.fastq.gz> -x <ncRNA_bowtie_index>
```

| Parameter | Definition |
|---|---|
| --threads N | specify number of threads in computer for this job (Depends on the computer) |
| -N 1 | Number of allowed mismatches |
| --no-1mm-upfront | This option prevents Bowtie 2 from searching for 1-mismatch end-to-end alignments |
| -q <trimmed.fastq.gz> | Input filename |
| --un=<norRNA_notRNA_noncRNA.fastq.gz> | output not aligned reads |
| -x <ncRNA_bowtie_index> | Index file for alignment |

### Step E4: Sequence Alignment

The next step for analysis is to align the remaining reads to the genome using the STAR (https://github.com/alexdobin/STAR).

To build STAR index also gtf file is needed. And those files can be found at https://www.gencodegenes.org/ and https://www.ensembl.org

```
STAR --runMode genomeGenerate --runThreadN N --genomeDir <location_for_index> --
genomeFastaFiles <location_of_fasta_file> --genomeSAindexNbases <calculated size>
--sjdbGTFfile <location_of_gtf_file>
```

| Parameter | Definition |
|---|---|
| `--runMode genomeGenerate` | option directs STAR to run genome indices generation job |
| `--runThreadN N` | specify number of threads in computer for this job (Depends on the computer) |
| `--genomeDir <location_for_index>` | location_for_index: refers to the location and name of the indexes |
| `--genomeFastaFiles <location_of_fasta_file>` | location_of_fasta_file: specifies one or more FASTA files with the genome reference sequences. The tabs are not allowed in chromosomes' names, and spaces are not recommended. |
| `--genomeSAindexNbases <calculated size>` | genomeSAindexNbases: can be find with; min (14, log2(GenomeLength)/2-1) for hg38 genome its min (14, log2(3272116950)/2-1) = 14 |
| `--sjdbGTFfile <location_of_gtf_file>` | location_of_gtf_file: specifies the path to the file with annotated transcripts in the standard GTF format. |

### Step E5: RiboWaltz pipeline

For the RiboSeq Quality Metrics analysis you can use RiboWaltz, an R package that integrates quality controls of the ribosome profiling data, P-site identification for improved interpretation of positional information and a variety of graphical representations.

Use transcriptome BAM file and GTF annotation file to run riboWaltz (https://github.com/LabTranslationalArchitectomics/riboWaltz).

# 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 5**. *Sample Lysis Output Summary*

## App.5 Beads functionalization checklist

| Reagent | N=1<br>0.9 A.U | N=___<br>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<br>□Place on magnet<br>□REMOVE Supernatant |
| OH-buffer (OH) | 270 µL | ____ | B2.4 | □Remove from magnet<br>□Wash OH Buffer: add "____" µL<br>□Shake 5min 1400 rpm RT<br>□Place on magnet<br>□REMOVE Supernatant |
| Nuclease-free water | 1000 µL | 1000 µL | B2.5 | □Remove from magnet<br>□Wash N.F. Water: add 1000 µL<br>□Shake 2min 1400 rpm RT<br>□Place on magnet<br>□REMOVE Supernatant |
| B-Buffer (BB) | 270 µL | ____ | B2.6 | □Remove from magnet<br>□Wash B Buffer: add "____" µL<br>□Shake 3min 1400 rpm RT<br>□Place on magnet<br>□REMOVE Supernatant<br>□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<br>□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<br>□Store 3 µl of Supernatant for control<br>□Remove from magnet |
| mPEG | 7.5 µL | ____ | B2.11 | □Add mPEG "____" µL<br>□Incubate 15min shacking 1400 rpm RT |
| Nuclease-free water | 1000 µL | 1000 µL | B2.12 | □Place on magnet<br>□REMOVE Supernatant<br>□Remove from magnet<br>□Wash with N.F. water: add 1000 µL<br>□Shake 2 min 1400 rpm RT |
| W-buffer (WB) | 1000 µL | 1000 µL | B2.13 | □Place on magnet<br>□REMOVE Supernatant<br>□Remove from magnet<br>□Wash with W buffer 1000 µL<br>□Shake 2 min 1400 rpm RT<br>□Repeat wash 2nd time |
| W-buffer (WB) | 100 µL | ____ | B2.14 | □Place on magnet<br>□REMOVE Supernatant<br>□Resuspend in "____"µL w-Buffer |
| | | | B2.15 | □Aliquot in 105 µL of equal volumes in N tubes |

**Table 6.** *Components' volumes to use for the Bead Functionalization Step B2. N = number of reactions. The table is intended as a guideline to follow when dealing with non-standard bead amounts and multiple samples.*

## App.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 | | | | |

**Table 7**. *Sample beads functionalization summary*

## App.7 Nuclease digestion checklist

Table 8 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 8.** *Summary of the volumes to utilize for the dilution of lysate Step B3.*

Table 9 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<br>□ 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<br>□ Incubate for 10 min on ice |

**Table 9.** *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.

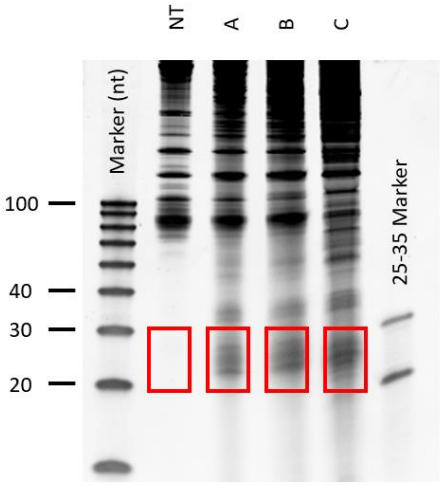


**Fig.6 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel. In the red square the sizes between 25-35 nt.**

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.

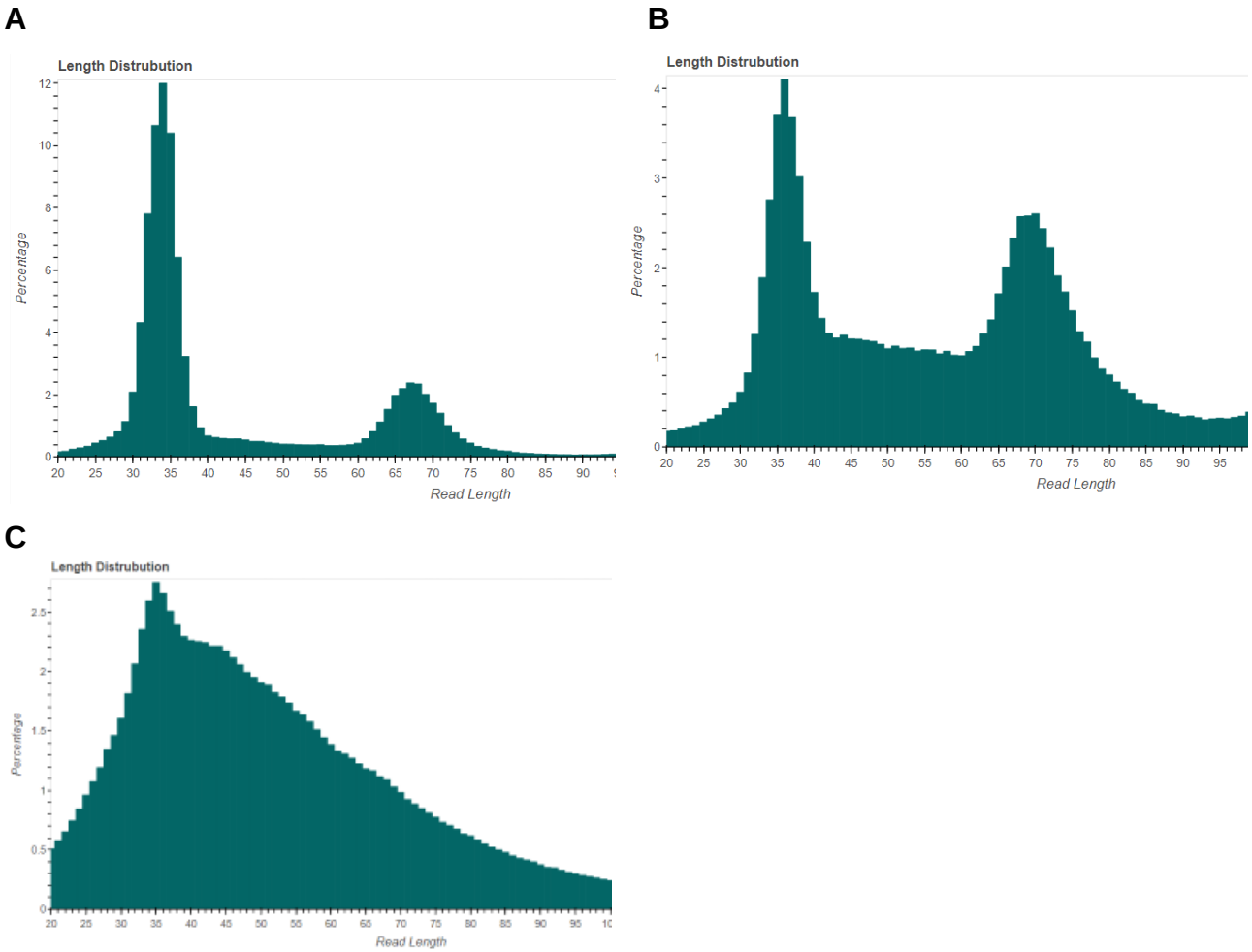


**Fig.7 Example of read length distribution obtained by sequencing libraries originated from A) sample digested with the right amount of Nux B) underdigested sample or C) overdigested sample.**

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.

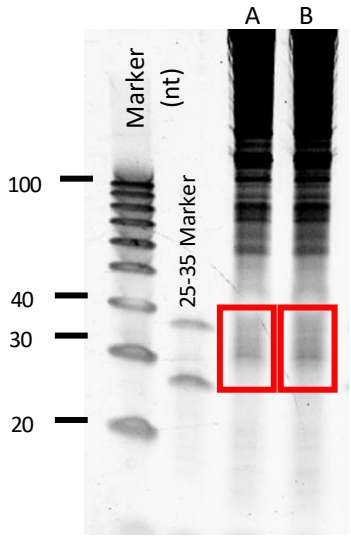


**Fig.8 Example of RNA extracted after pulldown run on 15% TBE-Urea gel.** The red boxes in lines A and B contain the bands relative to the RPF and corresponding to a length between 25 and 35. For proper RPF isolation via PAGE extraction please retrieve the gel portion within the red box.

- ☐ 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,

## App.11 Translation Efficiency.

Amongst the many regulatory mechanisms occurring within the cell, two steps are mainly responsible for governing protein production, the transcription of the information stored in the genome into (messenger) RNA and its translation into protein. High levels of transcription do not necessarily correlate with high levels of translation or vice versa, explaining why RNAseq data have generally poor correlation with proteomics data. RiboSeq data and particularly active RiboSeq data representing the fraction of messenger that is being translated, show a much better correlation but tell only half of the story. To get a full picture of the regulatory mechanism it is thus important to combine the two pieces of information, normalizing the rate at which RNA molecules are translated (RiboSeq data - and particularly active RiboSeq data), to the amount of RNA available (RNAseq data). This type of analysis is called Translation Efficiency (TE for short) and is an extremely powerful tool that allows the researcher to directly correlate changes in experimental conditions with changes in cellular behavior. On one hand, TE provides a clear overview of each gene expression pathway and correlation with other patterns, on the other, it constitutes a cost-effective and informational-richer proxy to proteomics, with one TE analysis hitting ~30'000 targets against the ~10'000 of a proteomics experiment.

This appendix provides the guidelines we suggest you follow to perform a TE experiment when using our RiboLace technology-based products, that -coincidentally- allow you to select for the actively translating part of the translatome.

**Important note**

If you wish to perform Translation Efficiency analysis, it is important to **originate RiboSeq and RNAseq data from the same sample** to avoid sample-to-sample variability. In our experience, starting both protocols with the same lysate obtained in Step B1. CELL LYSIS provides the highest correlation between the resulting data sets.

### TE1 Total RNA extraction for RNAseq library preparation

- ☐ **TE1.1** Start with a total volume of lysate corresponding to 0.4 A.U. (260 nm see Section A2.3 for calculation) and dilute to a final volume of 50 µL in W-buffer. If the required volume to reach 0.4 AU is more than 50 µL, use 50 µL instead.
- ☐ **TE1.2** Add 150 µL of the Zymo RNA Binding Buffer (**ZBB***).
- ☐ **TE1.3** Incubate the solution at RT for 5 min with shaking at 600 rpm.
- ☐ **TE1.4** Add 200 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ **TE1.5** Transfer the mixture to the **Zymo-Spin™ Column*** and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ **TE1.6** Add 400 µl **RNA Prep Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ **TE1.7** Add 700 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ **TE1.8** Add 400 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT Discard the flow-through.

- ☐ **TE1.9** 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.
- ☐ **TE1.10** Add 21 µL of **Nuclease Free Water** directly to the column matrix and wait 1 minute.
- ☐ **TE1.11** Centrifuge for 30 seconds at 12,000 g at RT. The extracted RNA is present in the flow-through. Collect the flow through.
- ☐ **TE1.12** With Nanodrop, measure the absorbance of each sample at 260 nm (set up the "nucleic acid" function of the Nanodrop), using 1.5 µL of Nuclease Free Water as blank.

At this point, we suggest performing a DNAse 1 treatment to allow complete removal of DNA contamination and obtain a better sequencing outcome. Depending on the DNAse I provider, the following digesting protocol (from Step TE1.13 to TE1.15) might be changed according to manufacturer instructions. The following protocol is based on DNAse I from Zymo.

- ☐ **TE1.13** Add to 20 µL of extracted RNA, 20 µL of Nuclease Free Water and 5 µL of DNAse I buffer.
- ☐ **TE1.14** Add 1 U of DNAse I every 10 µg of extracted RNA (5 µL of the reconstituted 1 U/ µL).
- ☐ **TE1.15** Incubate the 50 µL of solution 15 min at 25°C.
- ☐ **TE1.16** Extract the RNA by directly adding 100 µL of the Zymo RNA Binding Buffer (**ZBB***) pipetting up and down.
- ☐ **TE1.17** Add 150 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ **TE1.18** Transfer the mixture to the **Zymo-Spin™ Column*** and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ **TE1.19** Add 400 µl **RNA Prep Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ **TE1.20** Add 700 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ **TE1.21** Add 400 µl **RNA Wash Buffer*** to the column and centrifuge for 1 minute at 12000 g at RT to ensure complete removal of the wash buffer. Discard the flow-through.
- ☐ **TE1.22** 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.
- ☐ **TE1.23** Add 15 µL of **Nuclease Free Water** directly to the column matrix and wait for 1 minute.
- ☐ **TE1.24** Centrifuge for 30 seconds at 12,000 g at RT. The extracted RNA is present in the flow-through. Collect the flow through.
- ☐ **TE1.25** With Nanodrop, measure the absorbance of each sample at 260 nm (set up the "nucleic acid" function of the Nanodrop), using 1.5 µL of Nuclease Free Water as blank.

It is important to check for proper RNA integrity before processing the library preparation. The best approach is evaluating each RNA by **Agilent 2100 Bioanalyzer** using the **Eukaryote Total RNA Nano Kit** (for eukaryotic samples, for other species please use equivalent). An expected RNA Integrity Number (RIN) between 7 and 10 is expected and needed for producing informative libraries (Fig.10).

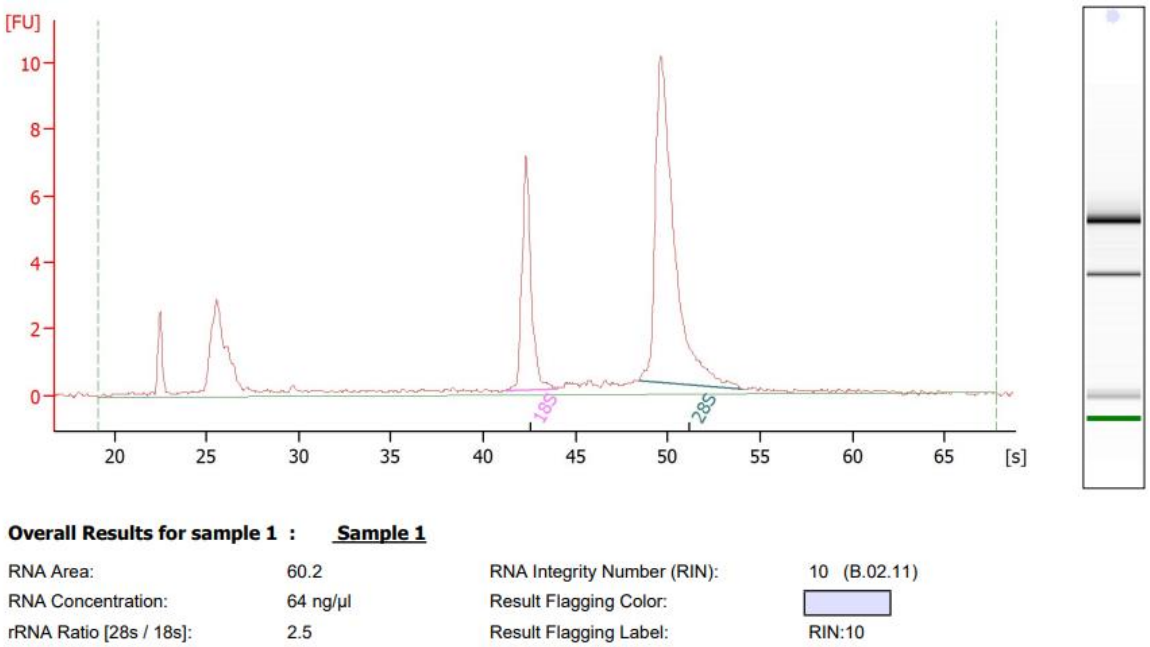


**Figure 10. Example electropherogram RNA integrity.** Typical electropherogram for a total RNA prepared with an immortalized cell line. The RNA was analyzed on an Agilent 2100 Bioanalyzer using the Eukaryote Total RNA Nano Kit. The electropherogram needs to present an RNA Integrity Number (RIN value) between 7-10**.**

## TE2 RNAseq library preparation guidelines

The extracted RNA is now ready to be processed for library preparation. There are two main methods with which libraries can be obtained:

- polyA selection
- ribodepletion and total-RNA library preparation

The choice between the two libraries depends on which information you want to retrieve but both can be utilized. Keep in mind that if you are enriching your sample for polyA transcripts, you might lose information on transcripts that do not present this feature (e.g. noncoding transcripts that might be translated in your samples but do not present polyA). Usually, we prefer to utilize a kit that includes all the transcripts, thus a library preparation kit with a ribodepletion step to remove ribosomal RNA contamination is needed.

## TE3 RNAseq library sequencing guidelines

RNAseq libraries will require sequencing specifications that depend on the type of library that you are creating. Depending on the total RNA library preparation that you want to utilize, please follow the other vendor guidelines for sequencing. In general, we sequence deeper the Riboseq (100-120M reads SE100 or 200-240M reads PE150) than the RNAseq (30-40M reads PE150).

## TE4 TE BioIT guidelines

For the analysis, we usually analyze active RiboSeq data to identify the Differentially Translated Genes (DTG), and in parallel, we analyze RNAseq data to calculate the Differentially Expressed Genes (DEG). Once we have both DTG and DEG data we can compare them and calculate the Translational Efficiency.

Supporting the whole RiboSeq workflow requirements, IMMAGINA provides sequencing services for RiboSeq NGS libraries, RNAseq libraries, and the required downstream data analysis with a package for the calculation of the Translation Efficiency or a Premium package for the development of ad-hoc analysis.

You can contact us at info@immaginabiotech.com to discuss your needs in detail.

## Contacts

**Info**

info@immaginabiotech.com

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

orders@immaginabiotech.com

**Technical service (technical inquiries and quality complaints)**

techsupport@immaginabiotech.com

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

https://www.immaginabiotech.com/

+39 04611787270

Notes: