ALL-IN-ONE RiboLace Multiplexing

A fast, simple, and reliable RiboSeq pooling workflow that processes 36 samples, multiplexed into 6 barcodes across 6 libraries, without requiring gel extraction from sample to sequencing.

Product	Catalog no	Rxns.
ALL-IN-ONE RiboLace Multiplexing	MX001-36	36

Shipping: Blue Ice and Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: ALL-IN-ONE RiboLace Multiplexing 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 36 pulldown reactions and 6 barcodes for 36 multiplexed (6x6) library preparations. It is suitable for Illumina platforms (MiSeq, NovaSeq 6000, NovaSeqX, NextSeq1000/2000).

Suitable for: Eukaryotic cell lines and tissues

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

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Kit storage info

	Qty.	Storage
4°C components	1 box	4°C
-20°C components	1 box	-20°C
-80°C components	1 bag	-80°C
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)
- Etanol 95-100%

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)
- Agilent Eukaryote Total RNA Nano Kit (Agilent Tech. catalog no. 5067-1512)

INTRODUCTION

The ALL-IN-ONE RiboLace Multiplexing kit is a complete RiboSeq solution combining Immagina's RiboLace™ technology for the isolation of active Ribosome Protected Fragments (RPFs) with the LaceSeq™ Multiplexed technology for the preparation of efficient and precise NGS libraries of small RNA fragments.

This comprehensive multiplexing solution enables processing of up to 36 samples, grouped into 6 libraries, with 6 samples per library (1 sample = 1 barcode; 6 barcodes = 1 library). The protocol is intended for multiplexing 6 samples per library, as shown in Figure 1. The kit allows for various pooling configurations (e.g., 3x6, 4x6), with a minimum of 3 samples per pool but all reagents are optimized for a 6x6 scheme. Please note that using configurations different from 6x6 will reduce the number of reactions that can be performed with the kit. **Thus, if you are not multiplexing in the 6x6 configurations, you will not be able to process the total of 36 samples (e.g.,** 3 samples x 6 LaceSeq multiplexed reactions = 18 samples).

The protocol here described is for a pooling configuration 6x6.

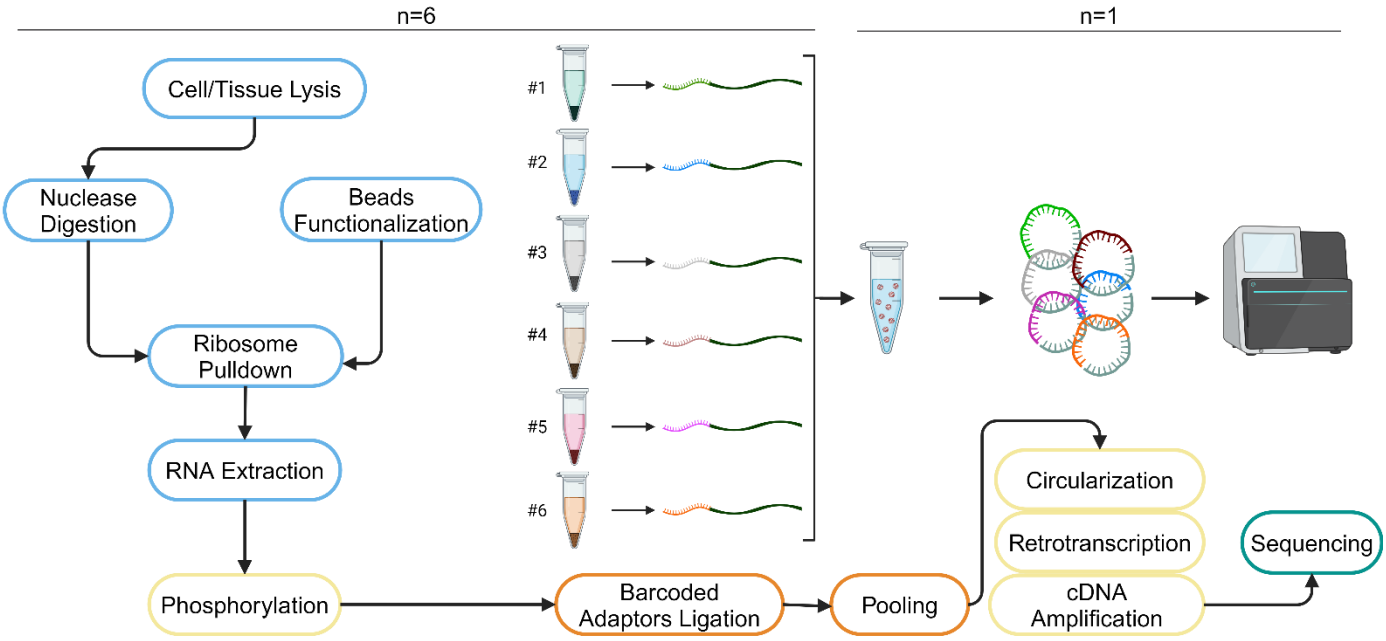

Figure 1. *Simple overview of the multiplexing-pooling protocol.*

This multiplex kit offers a fast, simple, and robust workflow with 6x greater sensitivity compared to standard single-reaction kits. It is ideal for users needing a flexible solution that works with both immortalized cells and complex samples with low translation activity (e.g., primary neuron cultures). Additionally, the kit allows pooling samples from different experiments into defined libraries for streamlined analysis.

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 ALL-IN-ONE RiboLace Multiplexing kit is validated for use with eukaryotic immortalized cell lines (e.g., K562, MCF7, HEK293), primary cell lines (e.g., primary fibroblasts, cortical neurons), whether freshly harvested or flash-frozen, and various tissues (e.g., mouse brain, lung, liver). For tissue processing, please purchase the dedicated Tissue Lysis Buffer (Cat. no. #IBT0032). The lowest starting material depends on the specimen type and its translational state**.**

For immortalized, highly translated cell lines the minimum number of cells is 35,000. For primary cells we suggest starting from 300,000 cells. However, consider that the amount of RPF per cell is highly dependent on the cell type and translational state. This is especially true for primary cell lines. For this reason, feel free to adjust the number of cells to a comfortable starting point compatible with your experimental need, as long as you can retrieve at least 0.05 total A.U. after sample lysis. For lower input samples please contact us at techsupport@immaginabiotech.com.

RiboLace™ Ribosome Isolation Technology (page 12-23, of this protocol, 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 monosomes. 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™ Multiplexed Technology (page 24-33, of this protocol, 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 5'-OH linkers, 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 use of barcoded linkers allows for the multiplexing of up to 6 samples (minimum 3) that can be processed simultaneously as a single library prep reaction. The barcodes are unique 8-nt sequences inserted at the 3' of the linker that allow the user to distinguish between samples without risk of cross-contamination. The possibility of pooling up to 6 samples together after the first ligation of LaceSeq library prep not only makes the protocol faster, but also increases the sensitivity of the reactions.

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 Multiplexing 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).

Sequencing and Data Analysis (page 33-42, of this protocol, Section D-E):

All necessary information for protein sequencing and data analysis is provided, with basic bioinformatics skills required for the analysis. If needed, Immagina offers support through its proprietary interactive software, Martian™. This tool enables non-experts to easily explore and interact with their data, addressing the majority of translation-related questions that may arise.

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.

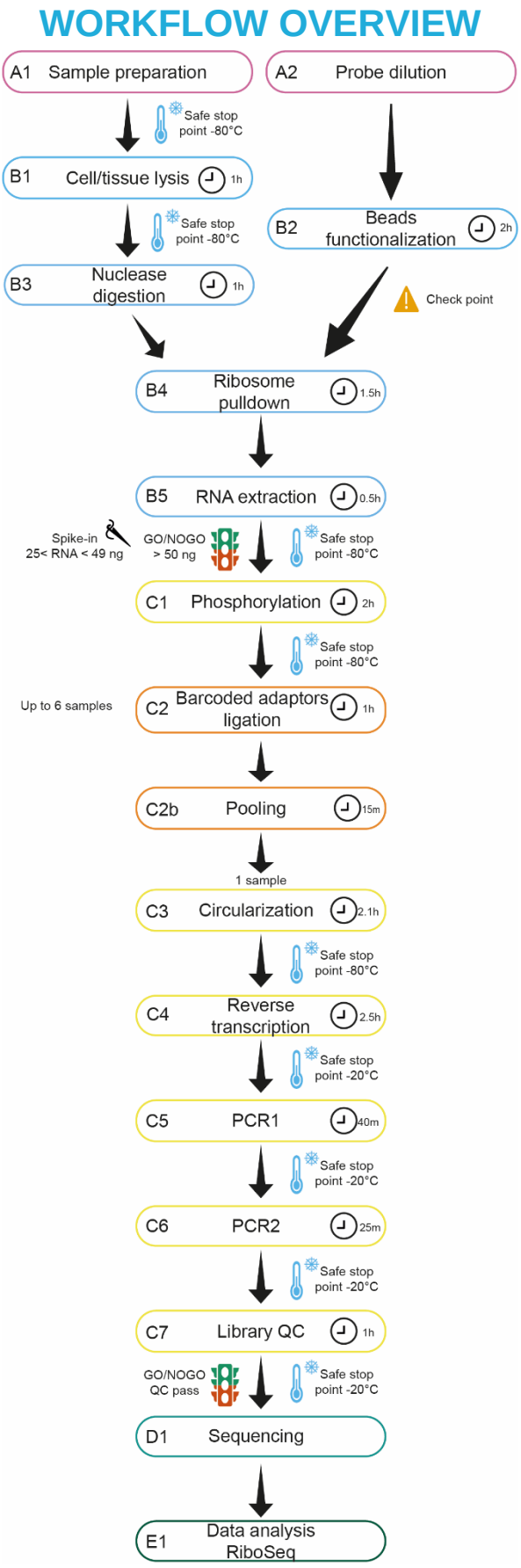

Figure 2. Overview of the ALL-IN-ONE RiboLace Kit workflow. *In each box, steps are present in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures), GO/NO-GO conditions (traffic lights), and a checkpoint (caution signal) are placed right at the bottom of the corresponding steps.*

Optimal Workflow Recommendations

- ⚠ For RiboLace section, please avoid running more than 6 samples in parallel. Longer manipulation time may introduce an unwanted variability between the first and the last samples.
- The multiplexing protocol allows you to process a total of 36 samples by generating **6 libraries composed of 6 samples** each in the LaceSeq section (36 pulldown reactions, 6 LaceSeq multiplexed reactions). If you wish, you can multiplex less sample per reaction (n ≥ 3), but this will reduce the total number of samples that can be processed (3 samples x 6 LaceSeq multiplexed reactions = 18 samples).
- We suggest you to multiplex in one library only samples with similar translational activity (i.e., primary cells in one pool, immortalized cells in a different pool), to ensure an equal distribution of the reads between different samples after sequencing. Moreover, if possible, please multiplex samples originated from the same model organism to allow an easier data analysis.
- Allocate at least 2 days for the completion of the entire workflow.
- If possible, please perform a preliminary lysis experiment to set the lysis volume following the suggested A.U. 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. Beads functionalization and nuclease digestion steps should be performed on **at least 3 samples in parallel**.
- This protocol is optimized for ribosome pulldown and RPF library preparation from low-translating cells or low-concentration lysates, starting with as little as 50 ng of total RNA after ribosome pulldown (representing all RNA on the beads after separation of ribosomes). If after pulldown you obtain less than 50 ng of RNA you can perform other pulldown reactions of the same sample and pool together the RNA after the extraction.
- After Ribosome pulldown we expect to obtain at least 50 ng of RNA to start with the preparation of the library. With recovered amounts between 25 and 49 ng of RNA it is still possible - but not advisable - to continue with the library preparation by adding a “spike-in” (see Appendix 9).
- Do not proceed with library prep if the total RNA amount after ribosome pull-down is lower than 25 ng of RNA
- 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.
- If you are interested in Translation Efficiency analysis, please check Appendix 11 before starting the experiment.

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 Multiplexing 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, one indicator that can be used as a checkpoint and go/no-go during the protocol is the amount of RNA retrieved after Ribosome & RPF pulldown (Step B5).

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 at least 50 ng of pulldown RNA to start with the preparation of the library. With amounts between 25 ng and 49 ng of RNA it is still possible to start with the preparation of the library by adding 0.02 ng of a “spike-in” or by increasing the starting material. With amounts lower than 25 ng, the chances to obtain a library are extremely low and we suggest you contact our tech support at techsupport@immaginabiotech.com.

A.2 AU Calculation - Input lysate Quantification

A.2.1 Measure Lysate A.U.

Cells and tissues should be lysed following Step B1 a, b, or c instructions depending on your specimen type. The A.U. of your sample is measured using a spectrophotometer, most commonly a Nanodrop. Set the instrument 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 A.U. 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 A.U.*
- ☐ *Specimen Abs260nm = 9 A.U.*
- ☐ *Absorbance value of lysate = 9 – 7 = 2 A.U.*

A.2.2 Lysis Volume Selection

It is important to lysate the specimen in an appropriate volume to obtain a lysate with a range of Abs260 ≈ 1 at least (after SLB subtraction).

The resuspension values suggested in Table 2 should set you within a readable A.U. range. For instance, starting with 500,000 neurons lysed in 40 µL of lysis buffer an absorbance between 1 to 2 A.U. is expected after SLB subtraction. Feel free to modify those resuspension values according to your sample behavior.

IMMORTALIZED CELL LINES	Quantity	Lysis buffer	Volume of supplemented LB (µL)
	35,000-50,000 cells	# IBT0031	20 µL
	50,000-200,000	# IBT0031	40 µL
	200,000 - 1million	# IBT0031	100 µL
	> 1million	# IBT0031	200 µL
PRIMARY CELL LINES	Quantity	Lysis buffer	Volume of supplemented LB (µL)
	300,000 – 800,000	# IBT0031	30 µL
	800,000 – 1 million	# IBT0031	40 µL
	1 million – 5 million	# IBT0031	80 µL
	> 5 million cells	# IBT0031	200 µL
TISSUES	Quantity	Lysis buffer	Volume of supplemented LB (µL)
Tissue	< 10 mg	# IBT0032	300 µL
Tissue	> 10 mg	# IBT0032	800 µL

Table 2. *The quantity of lysis buffer depends on the specimen's 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 A.U. read out as a concentration, and we can decide arbitrarily to set it as A.U./mL. IMPORTANT: Please, remove all the PBS carefully before proceeding with the lysis of your sample, as the presence of PBS leftover influences greatly the A260 reading.

If the total A.U. in your sample is less than 0.05 (see examples below for calculation), try to increase the number of cells. If the A260 read at the Nanodrop is < 1, prepare again your samples increasing the number of cells and/or reducing the volume of lysis buffer.

To calculate the volume of lysate to utilize for 0.6 A.U., follow the examples below.

Example 1:

- Started with 1 million immortalized cells. Lysis in 100 µL of Supplemented Lysis Buffer.
- Nanodrop absorbance value of lysate at 260 nm after SLB subtraction = 9 A.U.
- This means that, arbitrarily, we set the absorbance of the lysate at 9 A.U./mL, which is divided by 1000 µL/mL to get the concentration per µL = 0.009 A.U./µL.
- **The total A.U. in my sample is = 0.009 A.U./µL * 100 µL = 0.9 A.U**.
- To start with 0.6 A.U. use: 0.6 A.U./0.009 A.U./µL = 66 µL of lysate.

Example 2:

- Started with 800,000 primary cells. Lysis in 40 µL of Supplemented Lysis Buffer.
- Nanodrop absorbance value of lysate at 260 nm after SLB subtraction = 2 A.U.
- This means that, arbitrarily, we set the absorbance of the lysate at 2 A.U./mL, which is divided by 1000 µL/mL to get the concentration per µL = 0.002 A.U./µL.
- **The total A.U. in my sample is = 0.002 A.U./µL * 40 µL = 0.08 A.U**.
- Perform the pulldown using all the lysate.

Example 3:

- Started with 800,000 primary cells. Lysis in 40 µL of Supplemented Lysis Buffer.
- Nanodrop absorbance value of lysate at 260 nm after SLB subtraction = 1 A.U.
- This means that, arbitrarily, we set the absorbance of the lysate at 2 A.U./mL, which is divided by 1000 µL/mL to get the concentration per µL = 0.001 A.U./µL.
- **The total A.U. in my sample is = 0.001 A.U./µL * 40 µL = 0.04 A.U**.
- You should stop and prepare a new sample increasing the number of cells and/or adjusting the lysis buffer volume (e.g., 1.6 million cells in 40 µ L of Supplemented Lysis Buffer).

B. ACTIVE RIBOSOME PULLDOWN

ALL-IN-ONE RiboLace Multiplexing 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	26 mL	-20°C	Bottle	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	26 mL	4°C	Bottle	--
B2	RiboLace magnetic Beads v2 (RmB v2)	# IBT0042	2 x 1.8 mL	4°C	Vial	clear
B2	OH Buffer (OH)	# IBT0052	10 mL	4°C	Bottle	--
B2	mPEG	# IBT0062	240 µL	-20°C	Vial	clear
B2/B5	Nuclease free water	Additionally Required Material				
B2*	RiboLace smart Probe (RsP)	# IBT0012	2x200 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)	# IBT0082	25 µL	-20°C	Vial	clear
B3	SUPERaseIn	Additionally Required Material				
B5	RNA Clean & Concentrator™-5	Additionally Required Material	**1column/ sample**			
B5	G1F Buffer (G1F)	# IBT0102	1.5 mL	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
For 100 µL suppl. LB	89 µL	10 µL	0.5 µL	0.5 µL	100 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 1.

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 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** Either trypsinize the cells according to standard protocols for your cell lines of interest or directly scrape them in PBS/detaching solution. Please avoid using any detaching reagent containing EDTA which could damage the ribosomal integrity.

1) **MECHANICAL SCRAPING.** 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 PBS/detaching solution needed.
   - 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.

2) **TRYPSINIZATION**. To ensure good lysis, follow these guidelines for trypsinization:

- Wash cells quickly with cold PBS containing CHX (20 µg/mL).
- Remove PBS and cover the cells in the desired amount of trypsin.
- Place at 37°C and wait until the cells start detaching, checking under the microscope.
- Stop trypsin action by adding desired amount of complete medium.
- Wash the plate thoroughly to remove all attached cells, then transfer everything into a tube for further processing.

☐ **B1.3a** Centrifuge cells at 950 g (*this value can be adjusted according to the cell line used*) for 5 minutes at 4°C. Remove the media and wash the pellet with **cold PBS** containing CHX (20 µg/mL).

☐ **B1.4a** Centrifuge cell pellet at 950 g (*this value can be adjusted according to the cell line used*) for 5 minutes at 4°C. Remove supernatant completely. **IMPORTANT: It is crucial to remove all the leftover PBS, to avoid sample dilution during lysis.**

☐ **B1.5a** Perform the lysis directly adding the **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.2.2 - Input lysate preparation and quantification - & Table 2) to the cell pellet and resuspending with P200 pipette. Avoid creating bubbles.

☐ **B1.6a** 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.

☐ **B1.7a** Transfer the supernatant to a new tube and keep it on ice for 20 min.

☐ **B1.8a** 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. Appendix 3 can be used to take notes of the volumes used for sample lysis and relative A260 absorption.

Suspension Cells lysis

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

- ☐ **B1.2b** Collect the cells and centrifuge at 950 g (*this value can be adjusted according to the cell line used*) for 5min at 4°C, remove the media, and wash with cold PBS containing CHX (20 µg/mL).
- ☐ **B1.3b** Collect and centrifuge at 950 g (*this value can be adjusted according to the cell line used*) for 5 min at 4°C. Remove the supernatant completely. **IMPORTANT: It is crucial to remove all the leftover PBS, to avoid sample dilution during lysis.**
- ☐ **B1.4b** Resuspend cell pellet in **Supplemented Lysis Buffer** (for resuspension volumes check the guidelines in section A.2.2 - Input lysate preparation and quantification - Table 2).
- ☐ **B1.5b** 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 the cell pellet and resuspending with P200 pipette. Avoid creating bubbles.
- ☐ **B1.6b** Pellet the nuclei and cell debris by centrifugation at 20,000 g for 5 min at 4°C.
- ☐ **B1.7b** Transfer the supernatant to a new tube. Leave on ice for 20 min.
- ☐ **B1.8b** Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 AU calculation - Input lysate quantification). If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing. Appendix 3 can be used to take notes of the volumes used for sample lysis and relative A260 absorption.

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 **Tissues Lysis Buffer** (not included - Immagina catalog no. #IBT0032; for resuspension volumes check the guidelines in section A.2.2 - Input lysate preparation and quantification -Table 2) supplemented as per instruction in the section “Before starting the experiment – Lysis Buffer Supplementing” & Table 3. Please note that Tissues Lysis buffer contains CHX (20 µg/mL).
- ☐ **B1.3c** Centrifuge at max speed (20,000 g) for 2 min at 4°C to remove tissue and membrane debris and collect the supernatant.
- ☐ **B1.4c** Centrifuge again the supernatant for 5 min at max speed (20,000 g) at 4°C and collect the supernatant. Keep on ice for 20 min.
- ☐ **B1.5c** Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 A.U. 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. Appendix 3 can be used to take notes of the volumes used for sample lysis and relative A260 absorption.

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) aliquots present in the kit are concentrated and are NOT intended to be used without proper dilution. This kit includes two aliquots of RsP: you can resuspend only one and keep the other at -80°C until needed.

Before starting the experiment thaw on ice one of the aliquots of 200 µL 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 300 µL aliquots as this approach allows you to conduct one experiment with 6 samples per aliquot.

Beads functionalization steps:

The amount of beads to be used for beads functionalization depends on the number of samples that you mean to process and their concentration (expressed in A.U.). The protocol is intended for pulldown with 0.6 A.U. of input material per reaction. If the total input is between 0.05 and 0.6 A.U., you can follow the protocol without adjustments. For total quantities lower than 0.05 A.U., please consider preparing the sample again increasing the number of cells. If this is not possible, please refer to Appendix 4.

Please remember that this protocol is intended for multiplexing of 6 samples, so we suggest working with 6 samples in parallel for beads functionalization and digestion.

Table 5 reports the volumes of components to be used in the following steps for one single sample (N=1). Please multiply the volumes times the number of samples you are using (N). Volumes for N=6 are also reported in Table 5.

Please, always functionalize at least beads for 3 samples in one single tube (N>3). Because washing volumes of WB are independent from the number of samples, consider that if you functionalize beads for N<3, you will not be able to process 36 samples.

To ensure an effortless and thorough process we suggest printing the checklist in Appendix 5, fill it with your specific volumes/number of samples and mark each step completed during the manipulation.

Reagent	N=1	N=6	Needed in Step
RiboLace magnetic beads (RmB) v2-0	96 µL x N	576 µL	B2.3
OH-buffer (OH)	170 µL x N	1000 µL	B2.4
Nuclease-free water	**1000 µL**	**1000 µL**	B2.5
B-Buffer (BB)	170 µL x N	1000 µL	B2.6
Diluted RiboLace Smart Probe (RsP)	50 µL x N	300µL	B2.8
mPEG	5.2 µL x N	31.2 µL	B2.11
Nuclease-free water	**1000 µL**	**1000 µL**	B2.12
W-buffer (WB)	**1000 µL**	**1000 µL**	B2.13
W-buffer (WB)	100 µL x N	600 µL	B2.14

Table 5. *Components' volumes to use in Step B2.*

- ☐ 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 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 **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 **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 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 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 **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 W-buffer (**WB**).
- ☐ **B2.15** 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. You can use Appendix 7 as a guide for the digestion process.

Please remember that this protocol is intended for multiplexing 6 samples, so we suggest working with 6 samples in parallel for beads functionalization and digestion.

⚠ *If after sequencing you notice that the readlength distribution of your samples is not showing a major peak around 30-35 nt, you may consider increasing the Nux amount for digestion of your samples. Please refer to Appendix 8 for expected readlength distribution.*

Reagent	0.05 < A.U. < 0.6	Needed in Step
Sample	x µL (≤150 µL)	B3.1
W-buffer (WB)	up to 150 µL	B3.1
Nux Enhancer (NE)	0.6 µL	B3.2
Diluted Nux (dNux)	3 µL dNux	B3.4
SUPERaseIn	1 µL	B3.5
WB to be added after digestion	150	B3.6

Table 4. *Components' volumes to use in Step B3.*

☐ **B3.1** Start with a total volume of lysate corresponding to **maximum** 0.6 A.U. (see Section A2.3 for calculation) diluted in W-buffer (**WB**) to the final volume of 150 µL.

- If you could not read the absorbance of your samples at Nanodrop, please refer to Appendix 4.
- If you are using 150 µL of lysate to reach the minimum A.U., DO NOT add additional WB for the digestion (Step B3.1). Total volume for digestion must be 150 µL.

☐ **B3.2** Add 0.6 µL of **Nux Enhancer (NE)**.

☐ **B3.3** Dilute 2 µL of **Nuclease** (**Nux**) by adding 18 µL W-buffer (**WB**). Pipet up and down 5 times to mix well the diluted Nux solution (dNux). The 20 µL of dNux prepared will be enough to process 6 samples in one single experiment. If you plan to process only 3 samples, you can dilute 1 µL Nux by adding 9 µL W-buffer (WB).

☐ **B3.4** Digest the sample in a 1.5 mL tube for 45 min at 25 °C with 3 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 µL of **SUPERaseIn** for 10 min on ice.

☐ **B3.6** **Add 150 µL of WB** to the digested lysate, for a **final volume of 300 µL**. Mix by gentle inversion. Total volume at the end of step B3 must be 300 µL.

Step B4. RIBOSOMES PULLDOWN

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

☐ **B4.1** Add the **300** µL of **digested cell lysate** to the functionalized beads (to avoid dilution, discard the supernatant of the beads just 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 Zymo 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 Zymo 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 Zymo 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 10 µL of **G1F Buffer** (**G1F**) directly to the column matrix and wait 1 min.
- ☐ **B5.12** Centrifuge for 30 seconds at 12,000 g at RT.
- ☐ **B5.13** The extracted RNA is present in the flow-through. Keep the Eppendorf with the flow through.
- ☐ **B5.14** With Nanodrop, measure the absorbance of each sample at 260 nm (set up the “nucleic acid” function of the Nanodrop), using 1 µL of G1F Buffer (G1F) as blank.

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

To start with library preparation, the suggested quantity of extracted RNA is **≥ 50 ng** per sample. When deciding from how much RNA to start for library preparation, please consider that after the pooling of the samples, the input material for the final library must not exceed 1.5 µg. For example, multiplexing 6 samples you can start from a minimum of 50 ng per sample up to a maximum of 250 ng per sample (250 ng * 6 = 1.5 µg). **The kit is intended to generate 6 libraries composed of 6 multiplexed samples each (36 samples total).** If you wish to generate multiplexed libraries from less than 6 samples each, please consider that **the number of**

multiplexed samples must be ≥ 3 (See Summary Table 5). Using less than 6 samples per libraries will result in the processing of less than 36 samples in total (i.e., 6 libraries of 3 samples each = 18 samples total).

☐ If you have between 25 ng and 49 ng of extracted RNA in your sample, it is still possible to start with the preparation of the library by adding 0.02 ng of a "spike-in" (check Appendix 9) or by increasing the starting material combining multiple pulldowns for the same sample (please check Appendix 4).

☐ It is important to start from the same amount of material for each sample multiplexed, in order to ensure comparable number of reads for each sample. For the same reason, we suggest multiplexing in the same library samples with similar translational states.

- **If you have less than 24 ng 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.**

Samples to Multiplex	Minimum amount per sample	Maximum amount per sample
6	**50 ng**	**250 ng**
5	60 ng	300 ng
4	75 ng	375 ng
3	100 ng	500 ng

Table 5. *Minimum and maximum RNA amounts per sample to start the library pooling should be carefully considered. To process all 36 samples, it's essential to multiplex 6 samples into each of the 6 libraries. Using a 3x6 multiplexing combination will allow you to process maximum 18 samples, as a maximum of 6 multiplexed libraries is possible.*

C. RPF's LIBRARY PREPARATION

ALL-IN-ONE RiboLace Multiplexing 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)	#IBT0152	200 µL	-20°C	vial	Red
C1	L1 enzyme (L1)	#IBT0162	40 µL	-20°C	vial	Red
C1	ATP 10 mM	#IBT0173	200 µL	-20°C	vial	Red
C1/C2/C3	RNA Clean & Concentrator™-5	Additionally Required Material	**2columns/ sample**			
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	40 µL	-20°C	vial	Blue
C2	GTP	#IBT0201	30 µL	-20°C	vial	Blue
C2	MnCl2	#IBT0211	30 µL	-20°C	vial	Blue
C2b	Barcoded Linker a (L_a)	#IBT0223	20 µL	-80°C	vial	
C2b	Barcoded Linker b (L_b)	#IBT0224	20 µL	-80°C	vial	
C2b	Barcoded Linker c (L_c)	#IBT0225	20 µL	-80°C	vial	
C2b	Barcoded Linker d (L_d)	#IBT0226	20 µL	-80°C	vial	
C2b	Barcoded Linker e (L_e)	#IBT0227	20 µL	-80°C	vial	
C2b	Barcoded Linker f (L_f)	#IBT0228	20 µL	-80°C	vial	
C2.3	G1F Buffer	#IBT0102	1.5 mL	4 °C	vial	Clear
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	75 µL	-20°C	vial	Green
C4	L4 enzyme (L4)	#IBT0281	15 µL	-20°C	vial	Green
C4	DTT	#IBT0291	20 µL	-20°C	vial	Green
C4	dNTP	#IBT0301	20 µL	-20°C	vial	Green

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
C4	AR Enzyme (AR)	#IBT0311	10 µL	-20°C	vial	Green
C5	L5 enzyme (L5)	#IBT0322	800 µL	-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	500 µL	4°C	vial	Clear
C1*	3P-RNA 1 µM (RNA)	#IBT0362	20 µL	-80°C	vial	Clear

Step C1. 5' PHOSPHORYLATION

NOTE: We suggest starting the library preparation with a minimum of 50 ng of extracted RNA per sample and a total of 1.5 µg of pooled RNA (See Table 5). It is important to start from the same amount of material for each sample multiplexed, in order to ensure comparable number of reads for each sample. For the same reason, we suggest multiplexing in the same library samples with similar translational states and ideally from the same organism. This protocol has been optimized for the ligation and pooling of **6** samples. In this way you will be able to prepare 6 libraries composed of 6 samples each (36 samples). The kit, however, will allow you to multiplex between 3 and 6 samples. Multiplexing less than 3 samples per library will reduce the total number of samples that can be processed.

☐ C1.1 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 B5.14	x
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 10,000-16,000 g for 30 seconds, unless otherwise specified.

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

☐ C1.5 Add 100 µL adjusted Zymo 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. BARCODED ADAPTOR LIGATION AND POOLING

This protocol has been optimized for the ligation and pooling of **6 samples**. In this way you will be able to prepare 6 libraries composed of 6 samples each (36 samples). However, the kit allows to multiplex between 3 and 6 samples. Multiplexing less than 6 samples per library will reduce the total number of samples that can be processed (for example: 3 samples x 6 pooled libraries = 18 samples), since the reagents are intended for 6 library prep reactions.

Each sample in a library must have a unique barcoded linker for its unambiguous identification. You were provided 6 different barcoded linkers (L_a, L_b, L_c, L_d, L_e, L_f). Please be careful in using one different barcoded linker for each of your samples. The barcoded linkers can be combined as you please. However, the barcoded linkers have been optimized for being used with 6 samples, so if you are working with less than 6 samples you may select suboptimal combinations of barcodes.

Please, take note of the corresponding sample-barcoded linker combinations. You can use Appendix 10 for your annotations.

According to your starting quantity of RNA, use the amount of linker indicated in the following table (Section C2.1). For example, if you started with 3 samples of 50 ng each, use 0.5 µL of barcoded linker, selecting a unique barcode for each sample.

☐ **C2.1** Please note Mix the following reagents in a 0.2 mL nuclease-free PCR tube. The table reports the volumes to add for each single sample:

	50-59 ng	60-90 ng	91-250 ng	251-350	351-500
RNA (from Step C1.12)	7 µL	7 µL	7 µL	7 µL	7 µL
Buffer L2	1 µL	1 µL	1 µL	1 µL	1 µL
GTP	0.5 µL	0.5 µL	0.5 µL	0.5 µL	0.5 µL
$MnCl_2$	0.6 µL	0.6 µL	0.6 µL	0.6 µL	0.6 µL
L2 enzyme	1 µL	1 µL	1 µL	1 µL	1 µL
Linker L_a/b/c/d/e/f	**0.5 µL**	**0.75 µL**	**1 µL**	**1.2 µL**	**1.6 µL**

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

☐ **C2b POOLING:** Depending on how many samples you started with, pool together the reactions (minimum = 3, maximum = 6 samples).

☐ **C2b.1** Add **G1F buffer up to 110 µL final volume (i.e., if 6 samples are pooled together: 10 µL * 6 = 60 µL samples add 50 µL of G1F). Resuspend well with a P200 pipet until solution appears completely clear.**

☐ **C2b.2** Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 10,000-16,000 x g for 30 seconds, unless otherwise specified. ***CAREFUL: the volumes in this purification are different from the other ones!!***

☐ **C2b.3** Prepare adjusted Zymo RNA Binding Buffer by mixing 110 µL of buffer and 110 µL of ethanol (95-100%).

☐ **C2b.4** Add the **220 µL** adjusted Zymo RNA Binding Buffer (from step 7.5) to the sample and mix.

- ☐ **C2b.5** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**
- ☐ **C2b.6** Add 330 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.
- ☐ **C2b.7** Add 400 µL Zymo RNA Prep Buffer to the column and centrifuge. Discard the flow-through.
- ☐ **C2b.8** Add 700 µL Zymo RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
- ☐ **C2b.9** Add 400 µL Zymo RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
- ☐ **C2b.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.
- ☐ **C2b.11** Add **10 µL** of **nuclease-free water** directly to the column matrix, wait 1 minute at RT and centrifuge. Save the flow-through.

- **From this point on, you will be working with only ONE SAMPLE.**

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 C2b.11)	**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 10,000-16,000 g for 30 seconds, unless otherwise specified.

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

☐ C3.6 Add the 100 µL adjusted Zymo RNA Binding Buffer (from step 8.5) 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 Zymo RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

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

☐ C3.11 Add 400 µL Zymo RNA Wash Buffer to the column and centrifuge.

☐ C3.12 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.

☐ C3.13 Add **12 µL** of **nuclease-free water** to the column matrix, wait 1 minute at RT and centrifuge. 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 at 50 °C for 40 min, then heat-inactivate at 70°C for 10 min.

☐ **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 –20 °C for **one week.**

Step C5. PCR AMPLIFICATION – PCR 1

☐ **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
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 400 µL of 70% 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 70% 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 over drying 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. Transfer the supernatant into a new tube.

⚠ **SAFE STOPPING POINT (store at -20°C)**

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).

⚠ **SAFE STOPPING POINT (store at -20°C)**

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 ~210-230 bp (see Fig. 3). 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 210-230 bp peak, you need to purify the libraries from gel before proceeding with sequencing.

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

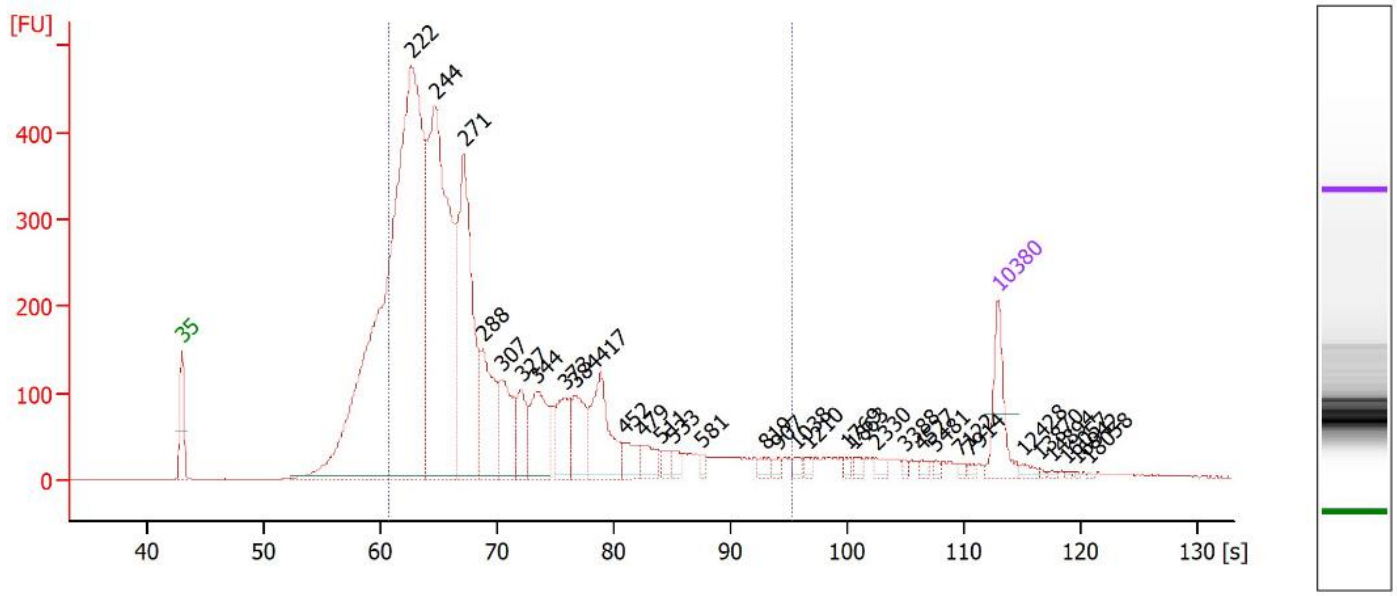

Figure 3. Example electropherogram libraries results. *Typical electropherogram for a library prepared with an immortalized cell line. The library was analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The electropherogram needs to present at least one major peak between 210 to 230. Tin this example, the peak at 222 bp corresponds to the size of RPFs, while the shorter peak around 190 bp corresponds with the size of adaptor dimers.*

D. SEQUENCING

Step D1. HOW TO SEQUENCE – DO IT YOURSELF - Minimal sequencing requirements:

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, NovaSeqX, 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.

Libraries can be sequenced both in Single-End (SE) and Pair-End (PE) mode, with length ≥ 150 bp. We suggest Pair-End (PE) sequencing with a length of 150 bp and a depth of 50 million reads/sample. For a library composed of 6 multiplexed samples, it would be 300 million reads per library (90Gb).

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.

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.

Expected Illumina sequencing output.

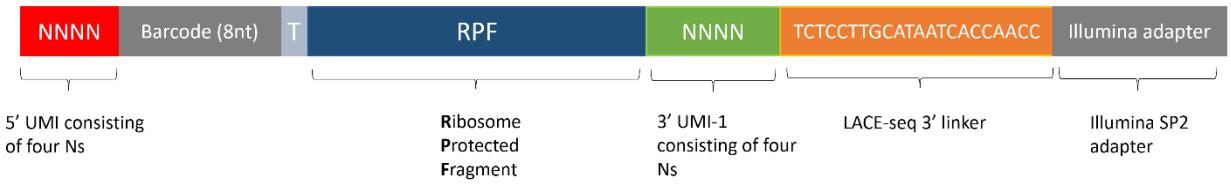

Figure 4. Expected Illumina sequencing output. *Example of a read generated.*

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 thirteen precedes the start of the RPF. The sequence content of a high-quality library has a T peak in position thirteen in 90-100% of the reads (Figure 5).

<u>To check T peak use fastqc command:</u>

fastqc –nogroup --outdir outputdir input.fastq

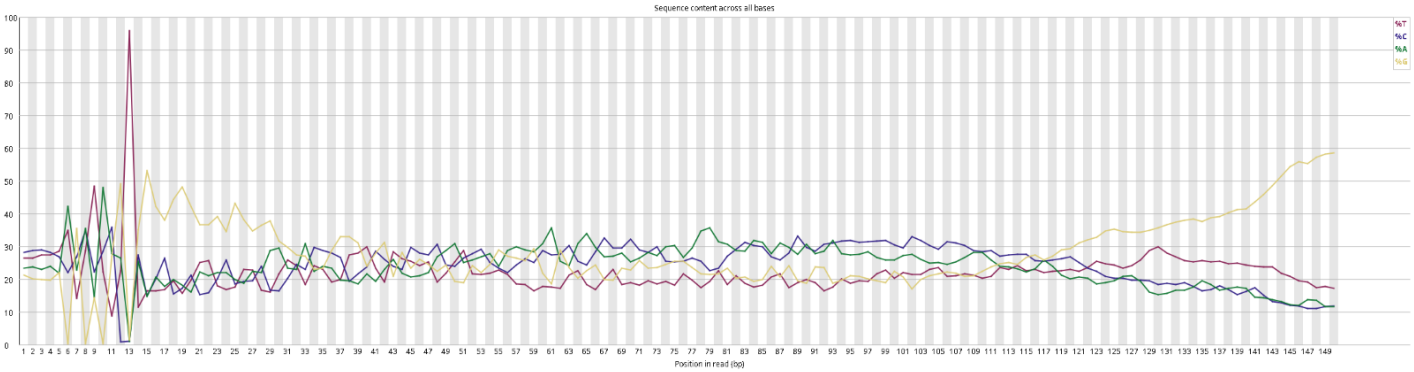

Figure 5. Sequence content across all bases. *The sequence content of a high-quality library has a T peak in position thirteen in 90-100% of the reads.*

Workflow overview

There are 5 main steps in the analysis pipeline:

E1. Software installation
E2. Trimming/UMI extraction /Barcodes extraction
E3. Bowtie2 for contaminant removal
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: Trimming/UMI extraction/Barcodes extraction

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.

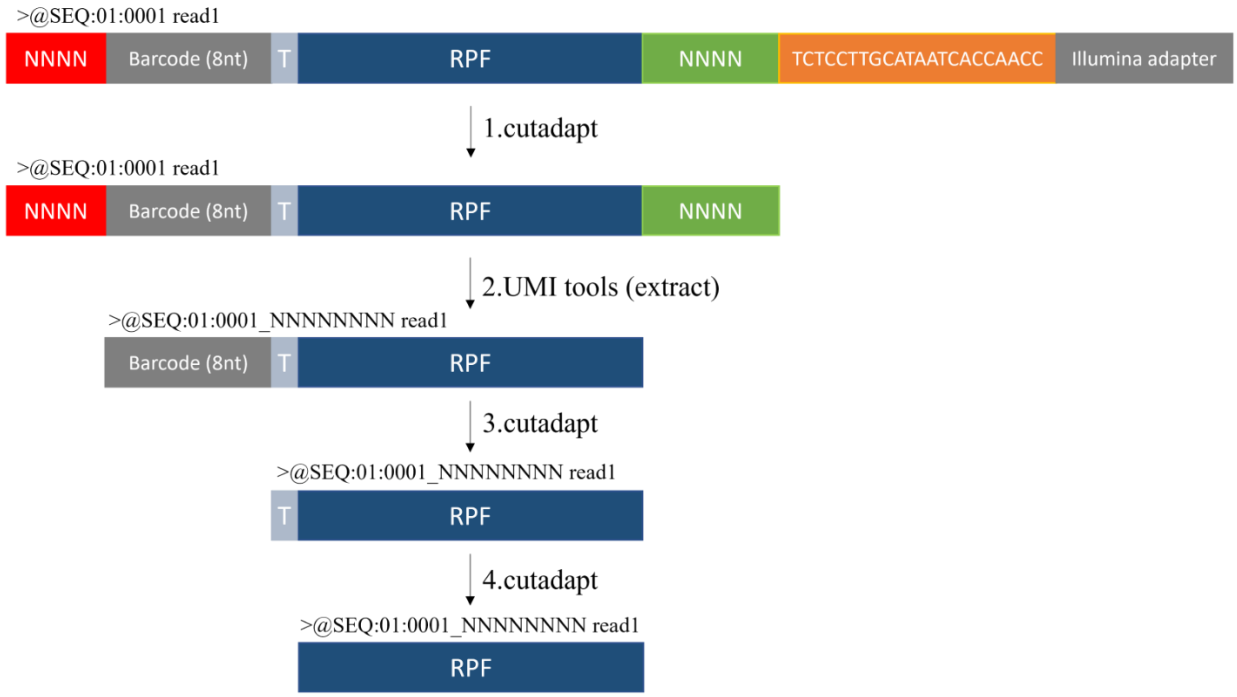

Figure 6. UMI extraction and trimming step schematic. *The RPF extraction is done in 4 steps: linker removal, UMI extraction, barcodes extraction, T removal.*

E2.1: cutadapt

First the Linkers (L_a-f) ire 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 barcodes

Barcodes (in fasta format) are removed with cutadapt and corresponding FASTQ files are generated for each unique barcode/sample.

The list of input barcodes for barcodes.fasta are:
>L_a
GTACCTTG
>L_b
TAATGCCG
>L_c
CTCCTAGA
>L_d
GATACTAG
>L_e
TAGTACAG
>L_f
ACGATACT

cutadapt --cores N –minimum-length 4 -e 0.2 –discard-untrimmed -g ^file: barcodes.fasta -o samplename_{name}.fastq.gz extract.fastq

Parameter	Definition
--cores N	specify number of threads in computer for this job (Depends on the computer)
--minimum-length 4	Reads are retained if they are longer than 4 nt.
-e 0.2	This sets the error tolerance used when searching for adapters.
--discard-untrimmed	Reads in which *no* adapter is found are discarded
-g ^file: barcodes.fasta	Removal of barcodes present in barcodes.fasta
-o samplename_{name}.fastq.gz	The output file name follows the format samplename_{name}.fastq.gz where samplename is a user-specified string and {name} is a placeholder that is dynamically replaced with the actual name of the barcode.
extract.fastq	The input file name

E2.4: cutadapt

The T preceding the RPF is then removed:

cutadapt --cores N -g ^T --discard-untrimmed -o trim2.fastq samplename_{name}.fastq.gz

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
samplename_{name}.fastq.gz	The input file name must be the same as the output file name in step E2.3

Step E3: Bowtie2 for contaminants removal

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: STAR 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 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.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 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 utilized (µL)	AU/mL (after blank subtraction)	AU/µL	V for 0.6 AU max (µL)
1						
2						
3						
4						
5						
6						

Table 6. *Sample Lysis Output Summary*

App 4. Low Amount of Starting Material – Troubleshooting Guidelines

Day one of this protocol ends with the pull-down of the RNA fraction containing the RPF fragments 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 50 ng of pull-down RNA per sample -the more, the better. This amount is required to compensate for the losses occurring during the multiple purification steps required to produce a library that can be sequenced.

In this protocol there are two checkpoints: the total A.U. measured after lysis at Nanodrop and the amount of RNA retrieved after ribosomes pulldown.

1) LOW AMOUNT OF A.U. TROUBLESHOOTING:

- **If your total A.U. < 0.05**:
  The protocol is intended for samples with a TOTAL A.U. between 0.05 and 0.6. If your total A.U. is less than 0.05, we suggest you prepare your samples again increasing the input material and/or scaling down the volume of the lysis buffer.

- **If you can not read an absorbance ≈ 1 A260** at the Nanodrop:
  The amount of starting material and the suggested volumes of supplemented lysis buffer should ensure that you read an absorbance of the lysate that is in the range of 1 A260 nm at the Nanodrop. If you CAN NOT read an absorbance ≈ 1 A260, we suggest you prepare your samples again increasing the input material and/or scaling down the volume of the lysis buffer.

- **If you can not prepare your samples again** increasing the A.U.:
  If you are working with very precious samples and you can not start again, you should still follow the protocol for beads functionalization for A.U. between 0.05 and 0.6 and use your whole lysate for digestion. Please take into account that in this way the protocol can lead to suboptimal results, and you may obtain less than 50 ng of RNA after pulldown.

2) LOW AMOUNT OF RNA AFTER PULLDOWN (< 50 ng) TROUBLESHOOTING:

The amount of RNA obtained after ribosome pulldown is highly dependent on cell/tissue type, cell/tissue level of translation, and the input A.U.

- **In the case of RNA extraction < 25 ng after pulldown**:
  The suggested approach is either restarting the workflow from the beginning with a higher amount of starting material/scaling the volume of supplemented lysis buffer, or you can combine the RNA extracted from multiple pulldowns from the same sample. Please note that this will reduce the number of different samples that can be processed with the kit.

- **If you get between 25 and 49 ng of RNA after pulldown** and do not wish to restart the procedure from the beginning nor to combine different pulldowns, you can directly proceed with the library preparation adding a “spike-in”. Please refer to Appendix 9.

App.5 Beads functionalization checklist

Reagent	N=1	N=___	Needed in	Step-by-Step Checklist
			B2.1	□ Place RmB v2-1 at RT for 30 min
			B2.2	□Vortex 30'
RiboLace magnetic beads (RmB)	96 µL	____	B2.3	□Add "___" Beads in 1.5 / 2 mL Tube □Place on magnet □REMOVE Supernatant
OH-buffer (OH)	170 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)	170 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)	50 µL	____	B2.8	□Remove from magnet □Resuspend in diluted RsP: "___" µL
			B2.9	□Incubate 1h shaking 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	5.2 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 □Repeat wash 2nd time
W-buffer (WB)	100 µL	____	B2.14	□Place on magnet □REMOVE Supernatant □Resuspend in "____"µL W-Buffer
			B2.15	□Aliquot equal volumes in N tubes

Table 7. *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.

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

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 8. *Sample beads functionalization summary.*

App.7 Nuclease digestion checklist

Table 9 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 0.6 AU max (µL)	V of W-Buffer up to 150 (µL)
1				
2				
3				
4				
5				
6				

Table 9. *Summary of the volumes to utilize for the dilution of lysate Step B3.*

Table 10 is intended as a guideline to follow for digesting the lysate.

Reagent	0.6 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 150 µL**
Nux Enhancer (NE)	0.6 µL	B3.2	□ Add 0.6 µL of NE to the lysate
Nux (Nux) + W-Buffer		B3.3	□ Dilute 2 µL of Nux in 18 µL W-buffer to create diluted Nux (dNux) / 1 µL of Nux in 9 µL W-buffer
Diluted Nux (dNux)	3 µL	B3.4	□ Add 3 µL of dNux to the lysate □ Incubate 45 min at 25°C
SUPERase•In	1 µL	B3.5	□ Stop the reaction by adding 1 µL of SUPERase•In into the lysate □ Incubate for 10 min on ice
W-Buffer	**150** µL	B3.6	□ Add 150 µL of WB to the lysate (**final volume = 300 µL**)

Table 10. *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 Readlength distribution

The quantity of Nuclease (Nux) to utilize for lysing the sample is essential to ensure good quality of data. A first glimpse of a successful digestion can result from the observation of the “Readlength distribution output”. Indeed, when the Nux is utilized correctly for the specimen of interest (Fig.7A), the reads of the sequenced libraries will display a sharp 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 suboptimal amount of Nux is used (Fig.7B), the digestion is not efficient, and the boundaries of the RNA protected by the ribosome are not sharp resulting in longer reads that can halt the downstream analysis and in-frame calculation.

If your reads after sequencing look like Fig.7B, we suggest repeating the experiment increasing the Nux amount.

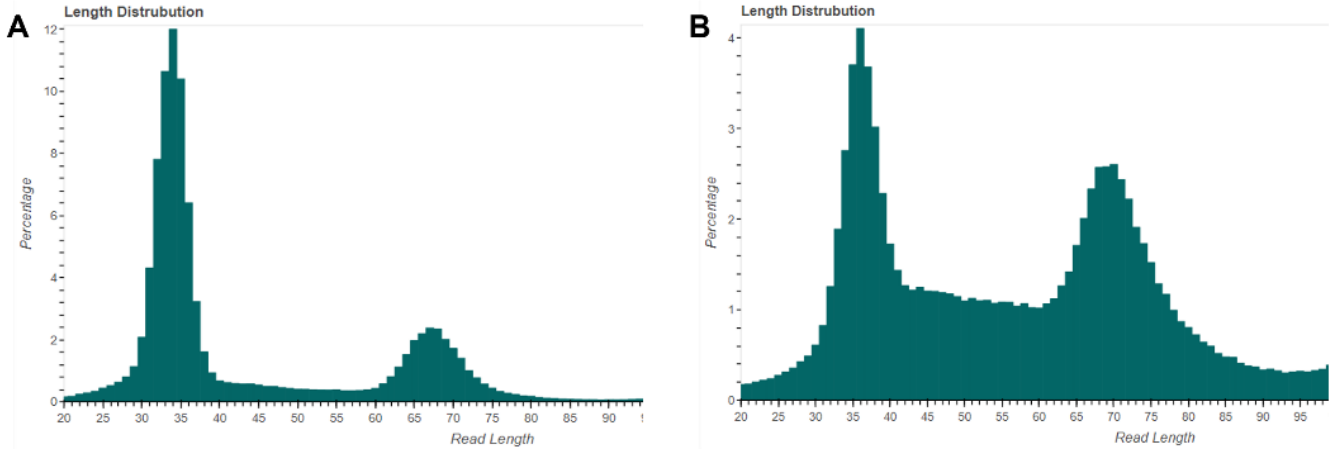

Fig.7 *Example of read length distribution obtained by sequencing libraries originated from A) sample digested with the right amount of Nux or B) underdigested sample.*

App.9 Spike-in Strategy:

Should you not have the possibility to repeat the pull-down, but you have between 25 ng and 49 ng 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.8). 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 library generation. 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)
- ☐ Quantify the 3P-RNA 1 µM (10 ng/µL) using a Qubit™ microRNA Assay Kit.
- ☐ Dilute the 3P-RNA 1 µM in water to 0.04 ng/µL final concentration.
- ☐ Add 0.5 µL of Spike-in (corresponding to 0.02 ng of spike-in Fig.8) to the total amount of RNA after pull-down.
- ☐ Start the Phosphorylation (Step C1) using the spiked-in RNA using the standard protocol for 35-50 ng.
- ☐ Follow up the normal protocol for 50-59 ng from Step C2.

Spike-in RNA exogenous sequence

5′-CTGAGAAAGTAGAGCAAGAAGAAATAGAGC-3′

Fig.8 *Spike-in RNA exogenous sequence.*

App.10 Barcoded Adaptors ligation checklist

Table 11 is intended as a guideline to follow for ligation with barcoded linkers.

Sample number	Sample name	Amount of RNA (ng)	Barcoded linker chosen	µL of barcoded Linker
1				
2				
3				
4				
5				
6				

Table 11. *Components needed for Ligation Reaction, with each sample corresponding to a unique barcoded linker choosing among L_a, L_b, L_c, L_d, L_e, L_f.*

Table 12 is intended as a guideline to follow for pooling multiple samples.

Sample to pool	Sample volume (µL)	Volume after pooling (µL)	Final volume before Zymo	µL of G1F to add
N = 6	10	N x 10 = 60	110	110 – (N x 10) = 110 – 60 = **50**
N = ___	10	___ x 10 = ___	110	110 – (__ x 10) = 110 - ___ =

Table 12. *In the first line it is indicated how to calculate the volume of WB when multiplexing 6 samples (N = 6). Please fill the second row with N = your number of multiplexed samples to calculate the volume of WB to add before Zymo purification.*

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.

In order to perform TE analysis, you need to start from a lysate of **AT LEAST 1 A.U. total**.

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 (from RiboLace section). If the required volume to reach 0.4 AU is more than 50 µL, **do not exceed 100 µL of sample** and do not dilute it in WB.
- ☐ **TE1.2** Add 3 volumes of the Zymo RNA Binding Buffer (ZBB), i.e. 150 µL ZBB + 50 µL sample.
- ☐ **TE1.3** Incubate the solution at RT for 5 min with shaking at 600 rpm.
- ☐ **TE1.4** Add an equal volume of EtOH 95-100%, i.e., 200 µL EtOH 95-100% for 200 EtOH 95-100% sample + ZBB, 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 Zymo RNA Prep Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.

- ☐ **TE1.7** Add 700 µl Zymo RNA Wash Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **TE1.8** Add 400 µl Zymo RNA Wash Buffer to the column and centrifuge for 30 seconds at 12,000 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, wait 1 minute, and centrifuge for 30 seconds at 12,000 g at RT.
- ☐ **TE1.11** 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 I 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 reconstituted DNAse I every 10 µg of extracted RNA.
- ☐ **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 12,000 g at RT. Discard the flow through.
- ☐ **TE1.19** Add 400 µl Zymo RNA Prep Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **TE1.20** Add 700 µl Zymo RNA Wash Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. Discard the flow-through.
- ☐ **TE1.21** Add 400 µl Zymo RNA Wash Buffer to the column and centrifuge for 30 seconds at 12,000 g at RT. 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. Wait 1 minute at RT and centrifuge for 30 seconds at 12,000 g at RT.
- ☐ **TE1.24** 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.9).

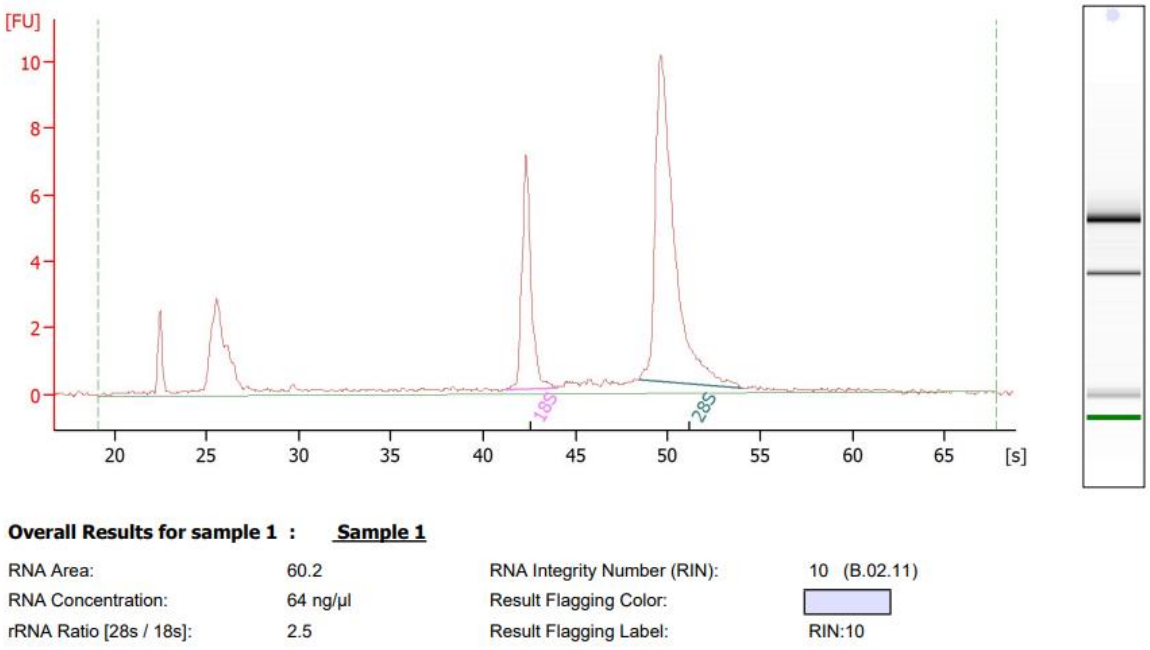

Figure 9. Example electropherogram RNA integrity. *Typical electropherogram for a total RNA prepared with an immortalized cell line. The RNA was analyzed on an Agilent 2100 Bioanalyzer using the Eukaryote Total RNA Nano Kit. The electropherogram needs to present an RNA Integrity Number (RIN value) between 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 (50-100M reads PE150 per sample) 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.

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