Ribosome Profiling All-In-One Set

from your sample to sequencing

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

Shipping: Blue Ice and Dry ice

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

Shelf Life: 12 months

Description: Ribosome profiling All-In-One set contains all reagents to perform ribosome profiling from cell lysis to sequencing. This set includes RiboLace Module 1, LaceSeq, PAGE Extraction Gel and UDIs for 12 reactions. This kit is suitable for Illumina platforms (MiSeq, NovaSeq, HiSeq 2000/2500, NextSeq550/1000/2000).

Suitable for: Eukaryotic cell lines and tissues

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

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

Additionally Required Materials

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

Recommendations

Sample Recommendations

Please note that the success of the experiment is strongly affected by the translational state of your biological samples. Two lysates similarly concentrated (i.e., similar Abs 260 nm) could have different amounts of translating ribosomes due to a different efficiency in protein synthesis. For example, immortalized cells are known to have higher rates of translation than primary cells. In addition, treatments such as drugs and transfection reagents could affect ribosome activity. Thus, the rate of protein synthesis of each sample has to be taken into account when programming experiments with the IMMAGINA - Ribosome Profiling All-In-One. In any case, always use the maximum available AU input in the suggested range.

Input lysate preparation and quantification

Cells and tissues should be lysed following Step 1 of this manual with IMMAGINA cell lysis buffer (Cat nr. #RL001-1, provided) or with IMMAGINA Tissue lysis buffer (Cat. nr. #RL001-2, to be purchased separately). Both lysis buffers have to be supplemented as indicated in Table 1 Pag 5 immediately before use. Using lysis buffers others than those provided is strongly discouraged because it can interfere with the efficiency of ribosomes pull-down.
The kit has been optimized for input between 0.1 - 0.3 total AU (Abs260 nm) of cell lysate. The Abs260nm should be measured using Nanodrop (selecting Nucleic Acid function) with the supplemented lysis buffer (Table 1, Pag 5) as blank.

Examples:

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

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

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

- □ To start with 0.1 AU use: 0.1 AU/0.004 AU/µL = 25 µL of lysate
- □ To start with 0.3 AU use: 0.6 AU/0.004 AU/µL = 75 µL of lysate

Using the Positive Controls

If you are using this kit for the first time, we strongly recommend performing the positive control reaction for library preparation. The positive control (CTRL, Cat. Nr. #LS001-1) is an RNA fragment with a 5'OH and 3'P (1 µM). For library preparation of the positive control, use 1 µl of the RNA control (10 ng) and follow protocol from Step 7.1.

Technical notes

(i) Step 2 can be performed in parallel to Step 1 and/or Step 3.
(ii) SDS 10% must be pre-warmed before starting the experiment and can be stored at RT.

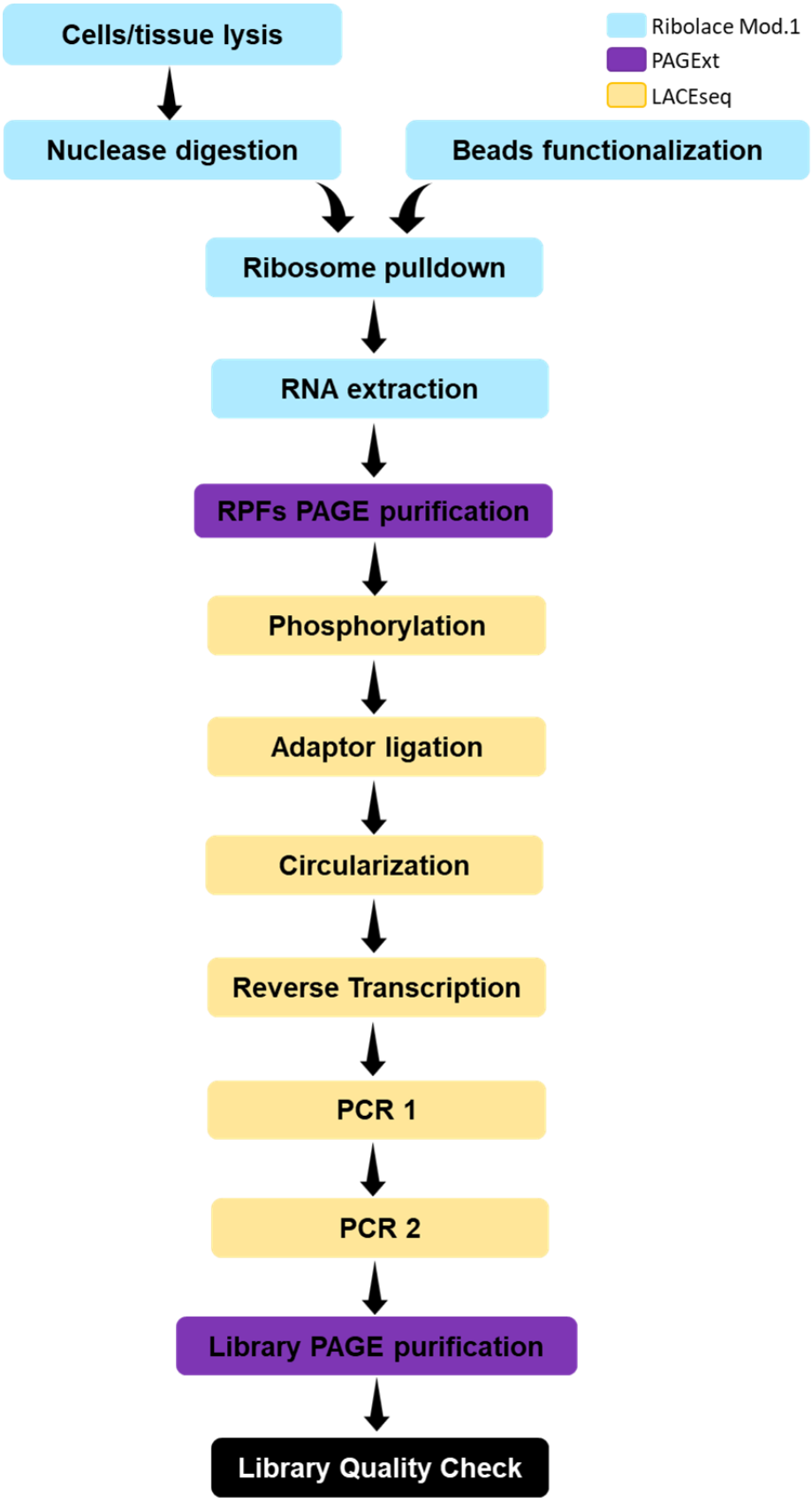


Figure 2. ***Overview of the Ribosome Profiling All-In-One Set workflow.***

Pull-down of active Ribosomes

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

Kit component	Cat. nr.	Volume	Storage	Type	Vial
SDS 10% (SDS)	#RL001-9	0.5 mL	RT	Vial	clear
B-Buffer (BB)	#RL001-3	5 mL	4°C	Bottle	--
W-buffer (WB)	#RL001-4	25 mL	4°C	Bottle	--
RiboLace magnetic beads (RmB) v2-0	#RL001-25	1.2 mL	4°C	Vial	clear
OH-buffer (OH)	#RL001-14	5 mL	4°C	Bottle	--
Proteinase K (K)	#RL001-17	65 µL	4°C	Vial	clear
Lysis buffer (LB)	#RL001-1	2x 1.9 mL	-20°C	Vial	clear
RiboLace smart probe (RsP)	#RL001-5	76 µL	-20°C	Vial	clear
Nuclease (Nux)	#RL001-7	19.5 µL	-20°C	Vial	clear
mPEG	#RL001-22	39 µL	-20°C	Vial	clear
Stabilizing Nux Solution (SS)	#RL001-24	5 µL	-20°C	Vial	clear
25-35 Marker (25-35 M)	#RL001-26	13 µL	-80°C	Vial	clear

Before starting the experiment

RiboLace smart probe dilution(1): add 316.5 µL of B-buffer to the RiboLace smart probe vial previously thaw on ice. After use, it is suggested to aliquot the mix, and store the aliquots at -80°C to avoid more than two freeze-thaw cycles.

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

Table 1. *Recipe for the supplementation of the provided Lysis Buffer. 300 µL is the suggested volume for a 10-cm dish or 5 million cells pellet. For other size of dishes/wells or number of cells, use a proportional volume.*

Final volume	Lysis buffer	Sodium deoxycholate (10%)	DNase I	RiboLock
300 µL	265 µL	30 µL	1.5U	60 U

STEP 1. CELL LYSIS

Adherent Cells lysis

☐ **1.1a** Treat the cells with 10 µg/mL of **cycloheximide (CHX)** for 5 min at 37°C before lysis. We recommend using cells at 70-80% confluence. *CHX treatments is suggested to increase the efficiency of the ribosomes' affinity purification, but it is not mandatory. If you are working with human or mouse tissues, please note that both Lysis buffer and W-buffer contain CHX (10 ng/mL)*

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

☐ **1.3a** Remove all residual PBS with a pipette.

☐ **1.4a** Perform the lysis directly adding the supplemented **Lysis Buffer** (Table 1) to each cell dish and scrape vigorously (a proper mechanical scraping is important for efficient lysis!).

☐ **1.5a** Collect the cell lysate in a 1.5 mL microcentrifuge tube and pellet the nuclei by centrifugation at 20000 g for 5 min.

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

☐ **1.7a** With Nanodrop, check the absorbance of the cell lysate at 260 nm (setup the "nucleic acid" function of the Nanodrop), using 1 µL of the complete lysis buffer previously prepared as blank (Table 1).

Suspension Cells lysis

☐ **1.1b** Treat the cells with 10 µg/mL of **cycloheximide** for 5 min at 37°C before lysis. We recommend using cells at 70-80% confluence. COMMENTS: *CHX treatments is suggested to increase the efficiency of the ribosomes' affinity purification, but it is not mandatory. If you are working with human or mouse tissues, please note that both Lysis buffer and W-buffer contain CHX (10 ng/mL)*

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

☐ **1.3b** Collect and centrifuge at 950g for 5 min at 4°C. Remove the supernatant and resuspend in complete **Lysis Buffer** (Table 1)

☐ **1.4b** Lysate cells by passing them through a G26 needle (~10 times) without generating bubbles.

☐ **1.5b** Pellet the nuclei by centrifugation at 20000 g for 5 min.

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

☐ **1.7b** With Nanodrop, check the absorbance of the cell lysate at 260 nm with supplemented lysis buffer (Table 1) as blank subtraction.

Tissues lysis

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

☐ **1.2c** Resuspend up to 10 mg of tissue powder with 800 µL of Tissues Lysis Buffer (not included - IMMAGINA cat. nr. #RL001-2) supplemented as in Table 1. Pease note that both Lysis buffer and W-buffer contain CHX (100 ng/mL and 10 ng/mL respectively)

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

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

☐ **1.5c** With Nanodrop, check the absorbance of the cell lysate at 260 nm (setup the "nucleic acid" function of the Nanodrop), using 1 µL of the supplemented lysis buffer previously prepared (Table 1) as blank.

STEP 2. BEADS FUNCTIONALIZATION

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

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

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

☐ **2.3** Put 90 µL of **RiboLace magnetic beads (RmB) v2-0** in a new 1.5 mL tube. Final volume = 90 µL x N (N = number of sample). Place the tube on the magnet to separate the RiboLace magnetic beads (RmB) v2-0. Remove supernatant.

☐ **2.4** Remove the tube from the magnet and wash the **RiboLace magnetic beads (RmB**) v2-0 for 5 min with an equal volume (= 90 µL x N) of **OH-buffer (OH)**, then remove the supernatant.

☐ **2.5** Wash with 900 µL of **Nuclease-free water**, place the tube on the magnet and remove the supernatant. If RiboLace magnetic Beads v2-0 (RmB) are binding to the plastic tube, you can add 0.1% final Tx100.

☐ **2.6** Wash the beads in a final volume (90 µL x N) of **B-buffer (BB)**, 3 min, two times in total. Place the tube on the magnet for at least 1 min and remove the supernatant. If RiboLace magnetic beads v2-0 (RmB) are binding to the plastic tube, you can add 0.1% final Tx100.

☐ **2.7** Resuspend the beads in a volume (30 µL x N) of **RiboLace smart probe (RsP)** previously diluted[(1)].

☐ **2.8** Save on ice 2 µL of **RiboLace smart probe (RsP**) diluted for security point (see below).

☐ **2.9** Incubate for 1h at RT in a shaker at 1400 rpm. Do not allow beads to sediment.

During the incubation, we suggest starting the Nuclease treatment (STEP. 3).

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

☐ **2.11** Passivate with **mPEG** adding a volume (3 µL x N) to the tube, mix in a shaker at RT for 15 min. Do not allow the beads to precipitate.

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

☐ **2.13** Wash the RiboLace magnetic beads v2-0 (RmB) two times with 500 µL **W-buffer** (**WB**).

☐ **2.14** Resuspend the RiboLace magnetic beads v2-0 (**RmB**) in a 200 µL of **W-buffer (WB)**, and equally divide the functionalized beads in individual tubes according to the (N) number of samples. **Do not remove the W-buffer until Step 4.1. Do not let the beads to try dry.**

⚠ Security Check Point

CHECK PROPER BEADS FUNCTIONALIZATION

Comparing the absorbance at 270 nm (Nanodrop ND-1000) of the unbound probe to **RiboLace smart probe** (**RsP**) starting solution allows an estimation of the binding efficiency (~ 10-50 % absorbance reduction is expected).

STEP 3. NUCLEASE TREATMENT

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

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

☐ **3.3** In a 0.2 mL vial, pipet 1.5 µL of **Nuclease** (**Nux**) and add 98.5 µL **W-buffer** (**WB**). Pipet up and down 5 times to mix well the diluted Nux solution.

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

Diluted Nux µL = A.U x 5.

Trash the remaining diluted Nux solution.

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

STEP 4. RIBOLACE PULL-DOWN

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

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

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

☐ **4.3** Take out the tubes from the wheel. **DO NOT CENTRIFUGATE**, pull down the beads by gentle handle shaking. Place the tubes on ice and put them on a magnet at 4°C.

☐ **4.4** Keep working on ice and separate the beads with a magnet. **DO NOT REMOVE THE BEADS FROM THE MAGNET and NEVER TOUCH THE BEADS IN THE NEXT WASHING STEPS.**

☐ **4.5** Carefully wash the beads two times with 500 µL **W-buffer** (**WB)**.

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

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

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

STEP 5. ACTIVE RPFs EXTRATION

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

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

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

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

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

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

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

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

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

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

☐ **5.9** Resuspend the pellet in 5 µL of **Nuclease Free Water**.

STEP 6. PAGE PURIFICATION OF THE RIBOSOME PROTECTED FRAGMENTS

RiboSeq All In One Set components needed in this part:

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

⚠ ***Pre-run the gel at 200 V for 30 min in TBE prepared with nuclease-free water. Clean well the gel wells with a syringe to remove UREA residuals before loading the samples.***

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

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

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

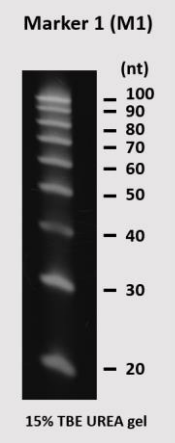
☐ **6.4** Denature the samples, 25-35 Marker and marker M1 for 90 s at 80 °C. Place the tubes immediately on ice.

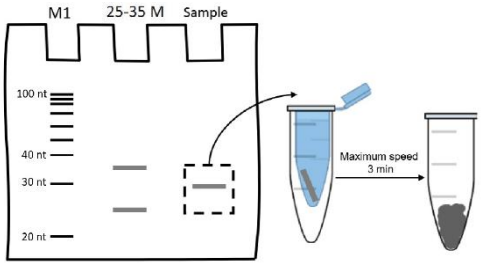
☐ **6.5** Load the samples, 25-35 Marker, and Marker 1 on 15% TBE-urea polyacrylamide gel and run the gel at 200V until the bromophenol blue band reaches the bottom of the gel.

☐ **6.6** Stain the gel with SYBR Gold and visualize the RNA using a UV-Transilluminator.

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

⚠ ***The RPFs are ~25-35 nts in length. The 25-35 Marker, is a mix of two oligos 25 nt and 35 nt in length. It can be used as a size marker.***





☐ **6.8** Place each gel slice in a pierced tube (provided) and nest the pierced small tube inside a 1.5-ml RNase-free Microcentrifuge tube (provided). Spin at maximum speed for 3 min at 4°C. Transfer carefully any remaining gel debris from the pierced tube, discard the pierced tube and keep the 1.5 ml microcentrifuge tube.

☐ **6.9** Add 400 µL of RNA Extraction Buffer (REB), close the vial with the provided cup, incubate the tubes for 1 hour at - 80°C, thaw them at RT and then place the samples on a wheel in slow motion (3 rpm), at RT overnight.

☐ **6.10** With a 1 mL cut-tip, add the gel slurry to the provided filter tube and spin at 650g for 3 min at 4°C to remove the gel debris. Transfer the eluted solution to a new tube.

☐ **6.11** Add 700 µL of isopropanol and 1.5 µL GlycoBlue to the eluted sample.

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

☐ **6.13** Thaw the samples on ice and pellet the RNA by centrifugation (20000g) for 30 min at 4°C.

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

☐ **6.15** Remove the supernant and resuspend the pellet in 11 µL TR buffer.

☐ **6.16** Quantify the RPFs (1 µL) using a Qubit™ microRNA Assay Kit.

LIBRARY PREPARATION OF RIBOSOME PROTECTED FRAGMENTS

RiboSeq All In One Set components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial
Buffer BPK	#LS001-1	80 µL	-20°C	Vial	Red
PK enzyme (PK)	#LS001-2	13 µL	-20°C	Vial	Red
ATP (10 nM)	#LS001-3	80 µL	-20°C	Vial	Red
Buffer BA	#LS001-4	20 µL	-20°C	Vial	Blue
Enzyme Mix A (Mix A)	#LS001-5	13 µL	-20°C	Vial	Blue
MnCl2	#LS001-6	10 µL	-20°C	Vial	Blue
GTP	#LS001-7	10 µL	-20°C	Vial	Blue
Linker MC (1 µM)	#LS001-8	26 µL	-80°C	Vial	Blue
Buffer BLB	#LS001-9	30 µL	-20°C	Vial	Yellow
Enzyme Mix B (Mix B)	#LS001-10	13 µL	-20°C	Vial	Yellow
PEG 8000	#LS001-11	200 µL	-20°C	Vial	Yellow
RT_T Primer (RT_T)	#LS001-12	13 µL	-20°C	Vial	Green
Buffer BRT	#LS001-13	60 µL	-20°C	Vial	Green
RT enzyme (RT)	#LS001-14	13 µL	-20°C	Vial	Green
dNTPs	#LS001-15	13 µL	-20°C	Vial	Green
DTT	#LS001-16	15 µL	-20°C	Vial	Green
Amplification mix (AM)	#LS001-17	1.3 mL	-20°C	Vial	Clear
Fw PCR1 (F1)	#LS001-18	12 µL	-20°C	Vial	Clear
Rev PCR1 (R1)	#LS001-19	12 µL	-20°C	Vial	Clear
Control (CTRL)	#LS001-22	10 µL	-80°C	Vial	Clear

Note:

Input RPFs amount: ≥ 5 ng (quantified by Qubit after gel extraction). In case of less RPFs amount, combine multiple ribosomes' pulldowns for the same sample.

STEP 7. 5' PHOSPHORYLATION

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

Buffer BPK	5µL
ATP (10 mM)	5 µL
PK	1 µL
RNA from step **6.16**	10 µL
H2O	29 µL

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

☐ **7.3** Purify the reaction through the **RNA Clean & Concentrator™-5 kit**, following the protocol for small RNAs and performing the final elution in a volume of 6 µL of **Nuclease-free water**. For more information, see Appendix 1.

STEP 8. LIGATION

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

	RPFs amount (25 – 35 nt)			
	5 ng	**10 ng**	**20 ng**	**40 ng**
RNA (from Step 7.3)	6 µL	6 µL	6 µL	6 µL
Buffer BA	1 µL	1 µL	1 µL	1 µL
GTP	0.5 µL	0.5 µL	0.5 µL	0.5 µL
$MnCl_2$	0.6 µL	0.6 µL	0.6 µL	0.6 µL
Enzyme Mix A	1 µL	1 µL	1 µL	1 µL
Linker MC 1µM	0.25 µL	0.5 µL	1 µL	2 µL
H_2O	0.75 µL	0.5 µL	-	-

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

☐ **8.3** Add **Nuclease-free water** up to 50 µL final volume, then purify the reaction through the **RNA Clean & Concentrator™-5 kit**, following the protocol for small RNAs and performing the final elution in a volume of 8 µL of **Nuclease-free water**. For more information, see Appendix 1.

STEP 9. CIRCULARIZATION

☐ **9.1** Prepare a 1 mM **ATP** solution by diluting the ATP stock (10 nM) in **Nuclease-free water** (e.g. 1 µL ATP + 9 µL nuclease-free water). Pipet up and down to mix well the solution. Assemble the following reaction in a 0.2 mL nuclease-free PCR tube:

RNA (from Step 8.3)	8 µL
Buffer BLB	2 µL
ATP (1mM)	1 µL
PEG8000	8 µL
Enzyme Mix B	1 µL

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

☐ 9.3 Add **Nuclease free water** up to 50 µL final volume, then purify the reaction through **RNA Clean & Concentrator™-5 kit**, following the protocol for small RNAs and performing the final elution in a volume of 10 µL of **Nuclease-free water**.

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

STEP 10. REVERSE TRASCRIPTION

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

Circular RNA (from step 9.3)	10 µL
dNTPs	1 µL
RT_T Primer	1 µL
H2O	Up to 14 µL

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

☐ 10.3 Add the following reagents to the annealed RNA:

Buffer BRT	4 µL
DTT	1 µL
RT enzyme	1 µL

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

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

STEP 11. PCR AMPLIFICATION – PCR 1

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

cDNA (from Step 10.4)	20 µL
Amplification Mix	50 µL
F1	0.8 µL
R1	0.8 µL
H2O	28.4 µL

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

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

** we recommend performing a pilot experiment and reducing or increasing the number of PCR cycles, as necessary, to achieve adequate library yields.*

☐ **11.3** Purify PCR samples by using 1.6x volume **Agencourt AMPure XP beads** following manufacturer's instructions. Elute the sample in 40 µL of **Nuclease-free water**. The volume of Agencourt AMPure XP beads for a given reaction can be derived from the following equation:

Avoid over dried of the beads (pellet cracked) as this will significantly decrease elution efficiency

STEP 12. PCR AMPLIFICATION – PCR 2

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

PCR1 **(from Step 11.3)**	40 µL
Amplification Mix	50 µL
LACEseq UDIs (10 µM)	1 µL
H2O	9 µL

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

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

** we recommend performing a pilot experiment and reducing or increasing the number of PCR cycles, as necessary, to achieve adequate library yields.*

☐ **12.3** Use Agencourt XP beads (1.6x ratio) or NucleoSpin Gel and PCR CleanUp kit to purify the entire 100-µl PCR reaction. *Agencourt XP beads: follow manufacturer's instructions and elute*

the sample in 40 µL of nuclease-free water. Nucleospin Gel columns: follow the standard protocol in Section 5.1 of the manufacture manual. Elute each sample in 20 µl of nuclease-free water.

STEP 13. PAGE PURIFICATION OF LIBRARIES

RiboSeq All In One Set components needed in this part:

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

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

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

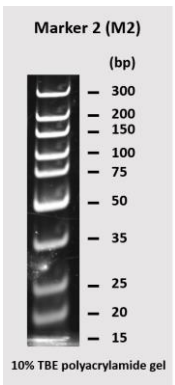
☐ **13.3** Load the samples and marker on a 10% TBE polyacrylamide gel (split the sample total volume into 2 adjacent lanes) and run the gel for 50 min at 200V. If the loading dye contains xylene cyanol, run the gel till the xylene cyanolreaches the bottom of the gel.

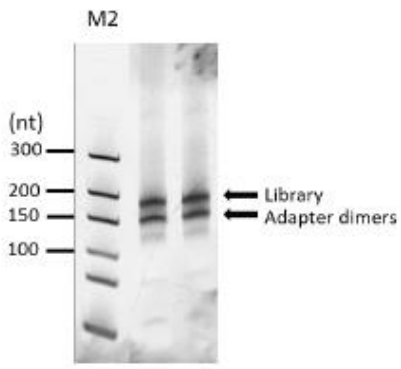
☐ **13.4** Stain the gel with SYBR Gold and visualize the libraries using a UV-Transilluminator.

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

☐ **13.6** Place gel slice in a pierced tube (provided) and nest the pierced small tube inside a 1.5-ml RNase-free Microcentrifuge tube (provided). Spin at maximum speed for 3 min at 4°C. Transfer carefully any remaining gel debris from the pierced tube, discard the pierced tube and keep the 1.5 ml microcentrifuge tube.

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





☐ **13.8** With a 1 mL cut-tip, transfer the liquid and gel slurry into a spin filter (provided) and spin at 650g for 3 min at 4°C to remove the gel debris. Transfer the eluted solution to a new 1.5 ml tube;

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

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

☐ **13.11** Thaw the samples on ice and pellet the RNA by centrifugation (20000g) for 30 min at 4°C.

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

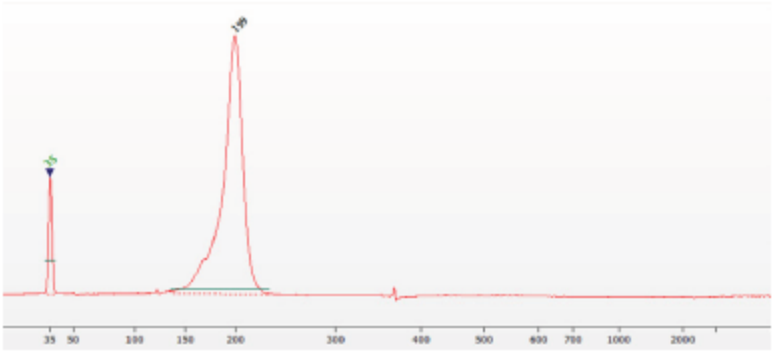
☐ **13.13** Remove the supernant and resuspend the pellet in 11-15 µL TR buffer. Proceed with Library Quality Check

STEP 14. LIBRARY QUALITY CHECK

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

☐ **14.2** Use the library profile results to determine whether each sample is suitable for sequencing. Successful library production should yield a major peak at ~200 bp (see Fig. below). The peaks observed at ~170 and 189 bp comes from adapter dimers (up to 50% is acceptable for sequencing).

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



Example electropherogram results LACEseq libraries after PAGE size selection.

APPENDIX 1: Zymo column purification for smRNA (RNA Clean & Concentrator™-5)

- ☐ Perform all steps at room temperature and centrifugation at 10,000-16,000 x g for 30 seconds, unless otherwise specified.
- ☐ Prepare adjusted RNA Binding Buffer (as needed). Mix an equal volume of buffer and ethanol (95-100%). Example: Mix 50 µl buffer and 50 µl ethanol.
- ☐ Add 2 volumes of the adjusted buffer to the sample and mix. Example: Mix 100 µl adjusted buffer and 50 µl sample.
- ☐ Transfer the mixture to the Zymo-Spin™ Column and centrifuge. Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!
- ☐ Add 1 volume ethanol and mix. Example: Add 150 µl ethanol to 150 µl sample. Transfer the mixture to a new column and centrifuge. Discard the flow-through.
- ☐ Add 400 µl RNA Prep Buffer to the column and centrifuge. Discard the flow-through.
- ☐ Add 700 µl RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
- ☐ Add 400 µl RNA Wash Buffer to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer. Carefully, transfer the column into a RNase-free tube (not provided).
- ☐ Add DNase/RNase-Free Water (for the elution volume see the specification at each Step) directly to the column matrix and centrifuge.

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Notes: