IMMAGINA
• BioTECHNOLOGY •
The Ribosome Company

Active Ribo-Seq with RiboLace

Product	Catalog no	Rxns.
RiboLace Ribo-Seq - Module 1	#RL001_mod1	9

Shipping: Dry ice and 4°C

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: RiboLace Ribo-Seq kit module 1 is used for 1-day extraction of ribosome protected fragments (RPFs) from ribosomes in active translation. Suitable also for samples with low amounts of input material.

List of components

Product (label)	Catalog no	Store Conditions	Quantity
RiboLace Technology (RiboLace Ribo-Seq kit)	#RL001_mod1	according to manual	1kit - 9 rxns
SDS 10%	#RL001-9	RT	1.9 mL
B-Buffer (BB)	#RL001-3	4°C	10 mL
W-buffer (WB)	#RL001-4	4°C	20 mL
RiboLace magnetic beads (RmB) v2.0	#RL001-25	4°C	0.95 mL
OH-buffer (OH)	#RL001-14	4°C	5 mL
Proteinase K (K)	#RL001-17	4°C	50 µL
Lysis buffer (LB)	#RL001-1	-20°C	6 mL
RiboLace smart probe (RsP)	#RL001-5	-20°C	60 µL
Nuclease (Nux)	#RL001-7	-20°C	15 µL
mPEG	#RL001-22	-20°C	30 µL
Stabilizing Nux Solution (SS)	#RL001-24	-20°C	5 µL

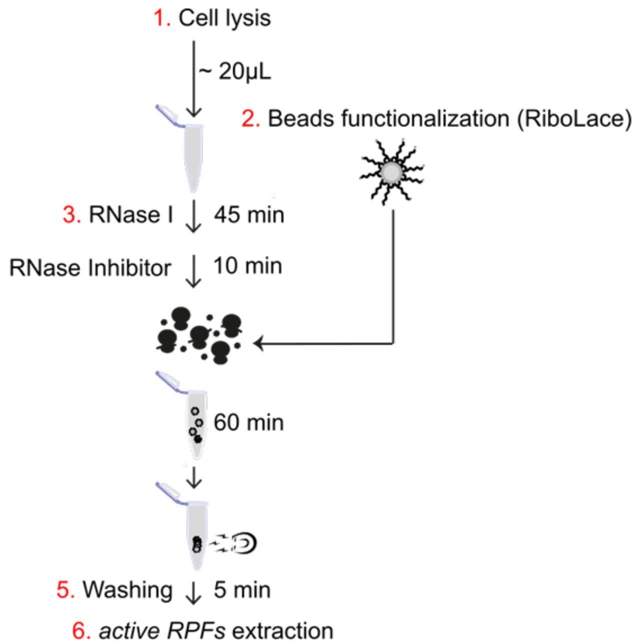
Shelf life: 12 months from the delivery date.

Additional Materials Required:

- 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 and DEPC water
- Acid-phenol:chloroform (Ambion catalog no. AM9720)
- Nanodrop ND-1000 UV-VIS Spectrophotometer
- GlycoBlue (Ambion catalog no. AM9515)
- Isopropanol (Sigma catalog no. 278475)
- Microcentrifuge and nonstick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- Automatic wheel (rotator)
- Magnetic stand for 1.5mL tube
- Qubit Fluorometer
- Qubit™ microRNA Assay Kit or RNA HS Assay Kit

Work always in an RNase-free environment.

Principle and Procedure



Before starting the experiment

RiboLace smart probe dilution[1]: add 250 µL of B-buffer to the RiboLace smart probe vial previously thaw on ice. After use, it is suggested to aliquot the mix (3 x 100 µL), and store the aliquots at -80°C to avoid more than two freeze-thaw cycles.

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

Example for a 10 cm dish				
Optimal final volume	Lysis buffer	Sodium deoxycholate (10%)	DNase I	RiboLock
0.3 mL	267 µL	30 µL	1.5U	60 U

Technical notes: (i) Step1 and Step2 can be performed in parallel. (ii) Pre-warm SDS 10% before starting the experiment. (iii) use a cut-tip 1 mL to recover gel debris after gel extractions.

Day 1 (Step 1 and Step 2)

Step 1. Cell Lysis - sample prep

Cells lysis

- To block ribosomes on the mRNA and to reduce ribosome dissociation when the RiboLace binds, it is suggested to treat the cells with at least 10 μg/mL of cycloheximide for 5 min at 37°C before lysis. We recommend using cells at 70-80% confluence.
- After incubation, place the cells on ice and wash them with cold PBS containing CHX (20 μg/mL).
- Remove all residual PBS with a pipette.
- Perform the lysis directly adding the lysis buffer to each cell dish and scrape vigorously (a proper mechanical scraping is important for good lysis!).
- Collect the cell lysate in a 1.5 mL microcentrifuge tube and pellet the nuclei by centrifugation at 20000 g for 5 min.
- Transfer the supernatant to a new tube and keep it on ice for 20 min.
- With Nanodrop, check the absorbance of the cell lysate at 260 nm with lysis buffer as blank subtraction (using the “nucleic acid” function of the Nanodrop).

Tissues lysis

- Pulverize the tissue under liquid nitrogen with mortar and pestle.
- Recover the powder in a 1.5 mL tube.
- Resuspend with 800 μL of tissues lysis buffer (not included- IMMAGINA catalog no. #RL001-2).
- Centrifuge at max speed (20000 g) for 2 min to remove tissue and membrane debris and collect the supernatant.
- Centrifuge again the supernatant for 5 min at max speed (20000 g) and collect the supernatant Keep on ice for 20 min.
- With Nanodrop, check the absorbance of the cell lysate at 260 nm with lysis buffer as blank subtraction (using the “nucleic acid” function of the Nanodrop).

Step 2. Beads Functionalization

DO NOT LET THE BEADS DRY OUT AT ANY POINT!

- Remove the RiboLace magnetic beads from 4°C and place the tube at RT.
- Vortex the RiboLace magnetic beads tube for > 30 sec.
- Put 90 μL of beads 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 beads. Remove supernatant.
- Remove the tube from the magnet and wash the beads for 5 min with an equal volume (90 μL x N) of OH-buffer, then remove the supernatant.
- Wash with 900 μL of nuclease-free water, place the tube on the magnet and remove the supernatant. If beads are binding to the plastic tube you can add 0.1% final Tx100.
- Wash the beads in a final volume (90 μL x N) of B-buffer, 3 min, two times in total. Place the tube on the magnet for at least 1 min and remove the supernatant. If beads are binding to the plastic tube you can add 0.1% final Tx100.
- Resuspend the beads in a volume (30 μL x N) of RiboLace smart probe (previously prepared[1], see above).
- Incubate for 1h at RT in a shaker at 1400 rpm. Do not allow beads to sediment.
- After the incubation, place the tube on a magnet and take out 3 μL of the supernatant (unbound probe) for security point (page 3). Leave the rest in the vial.
- Collect 1 μL of RiboLace smart probe (previously prepared[1], see above) for security point.
- Passivate with mPEG adding a volume of 3 μL x N to the tube, mix in a shaker at RT for 15 min. Do not allow the beads to precipitate.
- Place the tube on a magnet for 2–3 min, discard the supernatant and wash with 500 μL of nuclease-free water.
- Wash the beads 2 times with 500 μL of W-buffer, resuspend them in 200 μL of W buffer and equally divide the functionalized beads in individual tubes according to the (N) number of samples. Do not dry the beads. Remove the buffer only before adding the cell lysate *(see Step 5 on page 4)*.

Security Point: **CHECK PROPER BEADS FUNCTIONALIZATION**
Comparing the absorbance of the unbound probe at A 270 nm (Nanodrop ND-1000) to the 1 mM RiboLace smart probe starting solution allows an estimation of the binding efficiency (~ 10 % absorbance reduction is expected).

Examples of volumes (μL) to use				
Number of replicates	Beads	Diluted smart probe	Cell lysate in W-buffer	SS solution
1	90	30	150	0.3
3	270	90	450	0.9

Day 1 (Step 3-4-5-6) Pull-down and active RPFs extraction

Step 3 - 4

RNase I & SUPERaseIN treatments

- Start with a total volume of lysate corresponding to 0.03 - 0.3 a.u (260 nm) diluted in W-buffer in a final volume of 150 µL.
  Example: if A260 = 10 a.u/mL = 0.01 a.u/µL = 30 µL needed.
- Add 0.3 µL of SS solution.
- In a 0.2 mL vial, pipet 1.5 µL of Nux and add 98.5 µL W-buffer. Pipet up and down 5 times to mix well the diluted Nux solution.
- Digest the sample in a 1.5 mL tube for 45 min at 25 °C with the diluted nuclease prepared before using a volume according to the formula:

  Diluted Nux µL = A.U (0.3 - 0.03) x 5.

  Trash the remaining diluted Nux.
- Stop digestion with 0.5 µL SUPERaseIn for 10 min on ice.

Step 5

Add RiboLace & washing

We strongly recommend to pull down the beads by gentle hand shaking instead of centrifuge. Do not collect residual beads on the cup of the tube

- Add the digested cell lysate to the beads (to avoid dilution, discard the supernatant of the beads before adding the cell lysate).
- Incubate for 70 min, on a wheel in slow motion (3 rpm) at 4°C.
- 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.
- 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.**
- Carefully wash the beads two times with 500 µL of W-buffer.
- Remove the beads from the magnet and resuspend them in W-buffer to a final volume of 200 µL. Transfer the suspension to a new nuclease-free 1.5 mL tube.

Step 6

Active RPFs extraction

⚠ *Warning: remember to add acid phenol:chloroform:isoamyl alcohol to the beads*

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

- Add 20 µL SDS 10% (final 1%) and 5 µL of proteinase K, and incubate at 37 °C in a waterbath for 75 min.
- Extract RNA by acid-phenol:chloroform. Protocol example:
  - Add an equal volume of acid phenol:chloroform:isoamyl alcohol.
  - Vortex and centrifugate at 14,000 x g for 5 min.
  - If there is no phase separation, add 20 µL of NaCl 2M (in DEPC water) and repeat the centrifugation.
  - Keep the aqueous phase and transfer it into a new vial.
  - Add 500µL of isopropanol and 2 µL of GlycoBlue.
  - 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 < 0.1 a.u.)
  - Pellet the RNA by centrifugation (20000g) for 30 min at 4°C.
  - Resuspend the pellet in 5 µL of Nuclease-Free Water.

Step 7
RPFs PAGE Purification

All the buffers, filters and plastics required for this step are included in RiboLace Mod. 2 (#RL001_Mod.2) or PAGExt IMMAGINA kits (#KGE-002).

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 to load the samples.

- Prepare samples: add 5 µL of Gel Loading Buffer II to 5 µL of RPFs obtained from 1-3 rxns of RiboLace Ribo-Seq kit module 1 or other protocols.
- Prepare M1 marker: mix 1 µL M1, 4 µL nuclease-free water and 5 µL of Gel Loading Buffer II.
- (Optional) Prepare RNA control: mix 2 µL IMMAGINA RNA control, 3 µL nuclease-free water and 5 µL Gel Loading Buffer II.
- Denature the samples, RNA control or marker M1 for 90 s at 80 °C. Place the tubes immediately on ice.
- Load the samples, RNA control, and marker on 15% TBE-urea polyacrylamide gel and run the gel at 200V until the bromophenol blue band reaches the bottom of the gel.
- Stain the gel with SYBR Gold and visualize the RNA using a Transilluminator.
- Size select the ribosome protected fragments (RPF) between 25-nt and 35-nt according to the the marker M1 and/or RNA control (see Fig. 1). Also, excise the “RNA control” oligo bands. You can use the “RNA control” as a reference sample for all next steps of the library prep.
- Place each gel slice in a provided 1.5 mL blue-cup tubes. Spin at maximum speed for 3 min at 4°C. Collect the gel debris and discard the empty blue cupped tubes.
- Add 400 µL of Buffer I (#RL001-10 or #KGE002-2), 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.
- With a 1 mL cut-tip, add the gel slurry to the provided filter and spin at 650g for 3 min at 4°C to remove the gel debris. Transfer the eluted solution to a new tube.
- Add 700 µL of isopropanol and 1.5 µL GlycoBlue to the eluted sample.
- Store at - 80°C for 2h (fast procedure) or overnight (safe procedure).
- Thaw the samples on ice and pellet the RNA by centrifugation (20000g) for 30 min at 4°C and air-dry the pellet.
- Resuspend the pellet in 11 µL TR buffer.
- Quantify the RPFs using a Qubit RNA HS Assay Kit or a Qubit™ microRNA Assay Kit.
- Prepare RPFs library using IMMAGINA RiboLace Mod. 2, LACEseq or other protocols.

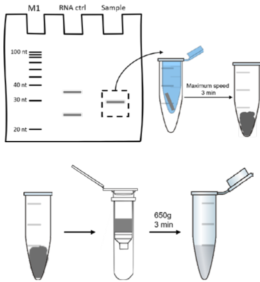


Fig. 1

The yield depends on the translational state of the sample. It is typically between 4 and 10 ng of RPFs for each RiboLace pulldown.

Related products

Product	Catalog no	Rxns.
RiboLace_Mod.2	#RL001_mod2	9
LACEseq	#LS-001	9
Tissue Lysis Buffer	#RL001_2	9 (20 mL)
PAGExt	#KGE-002	18

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Notes: