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 as indicated on datasheet

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.

Reagents provided

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: 5 months from the delivery date

Active Ribo-Seq with RiboLace

RiboLace kit code number.....

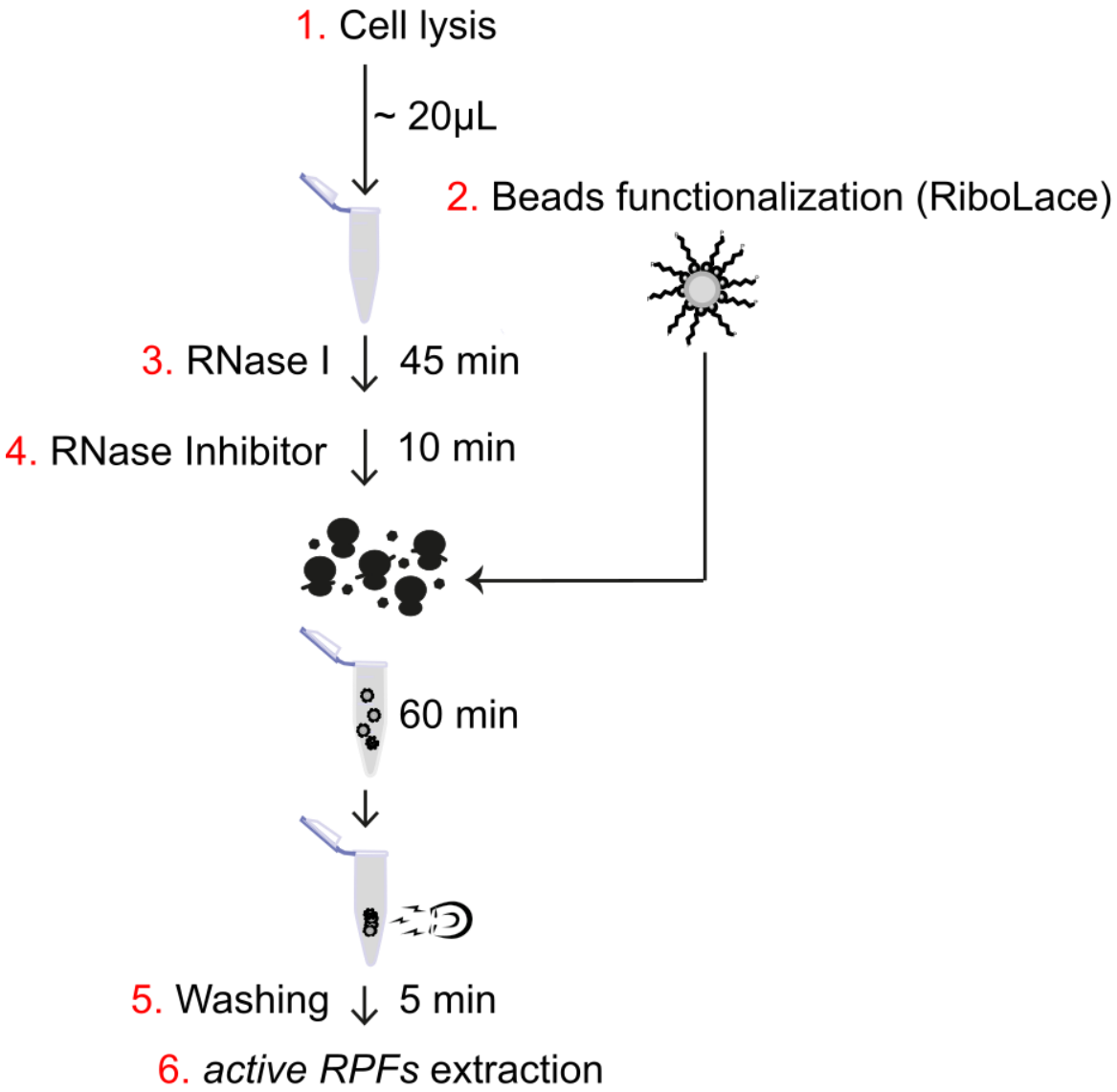
Number of samples (N).....

Reagents and equipment to be supplied by user:

- 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	265 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 beds 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
- *….continued on Page 3…..*

...step 2.Beads Functionalization

- 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 in (B) 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

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

The yield is typically between 4 and 10 ng of RPFs for each RiboLace pulldown.

- 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
  - PAGE Purify RPFs following Step 1 of RiboLace Module 2 or other gel extraction protocols and quantify the RPFs using a Qubit RNA HS Assay Kit or a Qubit™ microRNA Assay Kit

- Prepare RPFs library using IMMAGINA RiboLace Ribo-Seq - Module 2 or other protocols.