Active Ribo-Seq with RiboLace

Product	Catalog no	Rxns.
RiboLace Mod. 1	#RL001_Mod.1_12	12

Shipping: Blue Ice and Dry ice

Storage Conditions: store components according to storage conditions reported on labels and on Page 6 of 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.

The kit is compatible with the PAGExt RPF gel extraction kit (Cat. no. #KGE002_12) and with the LACEseq RiboSeq library preparation kit (LACEseq Cat. no. #LS001_12).

Suitable for: Eukaryotic cell lines and tissues

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

Kit contents	Qty.	Storage
RiboLace Mod.1 4°C components	1 box	4°C
RiboLace Mod.1 -20°C components	1 box	-20°C
RiboLace Mod.1 -80°C components	1 bag	-80°C

Additionally Required Materials

- 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-Aldrich 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

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 Abs260nm) 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 - RiboLace Mod.1. 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

Technical notes

(i) Step2 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.

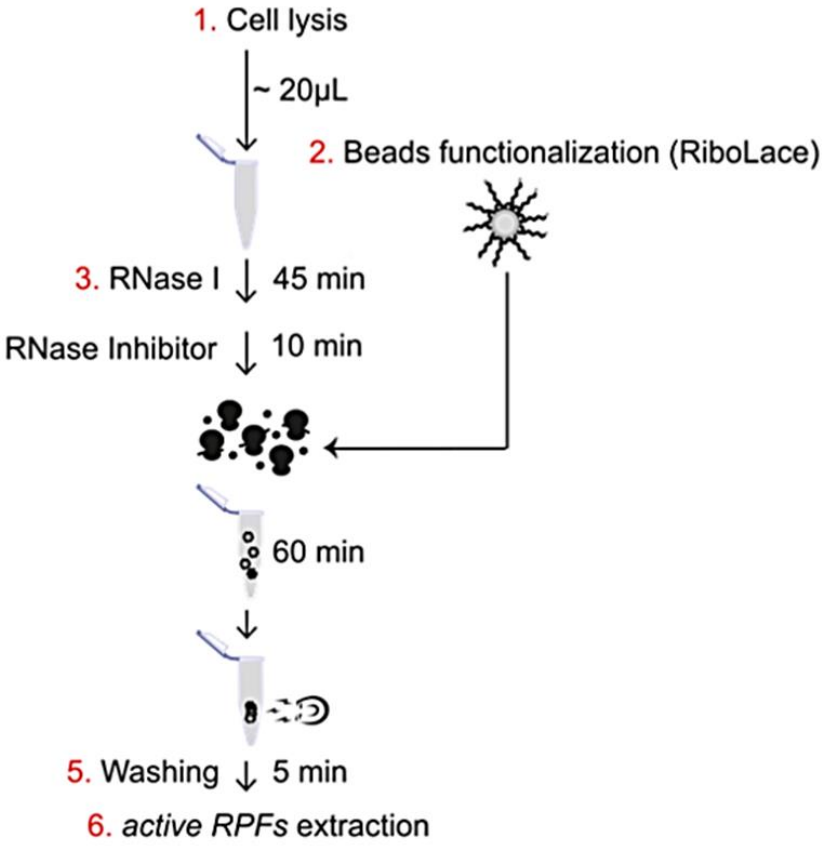

Figure 2. ***Overview of the RiboLace kit workflow***

Pull-down of active Ribosomes

RiboLace_Mod.1 components list

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)** solution 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**.

☐ **5.10** Proceed with RPFs PAGE Purification using PAGExt kit (Cat. no ##KGE002_12)

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