RiboLace XL

Fast and flexible solution to isolate active ribosomes for ribosome profiling experiments

Product	Catalog no	Rxns.
RiboLace XL	#RL001_XL	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 XL contains all reagents to isolate active ribosomes by affinity purification and magnetic separation. Thanks to additional beads, control and probes compared with standard RiboLace (Cat. no. RL001_Mod1) this product is suggested for the experimental designs which include challenging samples, like ones with low translational rates.

The kit is compatible with the PAGExt RPF gel extraction kit (Cat. no. #KGE-002) and with the LACEseq RiboSeq library preparation kit (LACEseq Cat. no. #LS-001).

Suitable for: Eukaryotic cell lines and tissues

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

Kit contents	Qty.	Storage
RiboLace XL 4°C components	1 box	4°C
RiboLace XL -20°C components	1 box	-20°C
RiboLace XL -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 XL.

RiboLace XL allows the user to fine tune the amount of reagents per pull-down according to the cell type and/or the efficiency of global protein synthesis.

RiboLace XL includes a control cell pellet as a reference for lysis and pull-down efficiency.

Input lysate preparation and quantification

Cells and tissues should be lysed following Step 1 of this manual with IMMAGINA cell lysis buffer (Cat nr. #RL001XL-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 6 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 0.4 - 0.9 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 6) 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 0.4 AU use: 0.4AU/0.01 AU/µL = 40 µL of lysate
  - ☐ To start with 0.6 AU use: 0.6AU/0.01 AU/µL = 60 µL of lysate
  - ☐ To start with 0.9 AU use: 0.9AU/0.01 AU/µL = 90 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 0.4 AU use: 0.4AU/0.004 AU/µL = 100 µL of lysate
  - ☐ To start with 0.6 AU use: 0.6AU/0.004 AU/µL = 150 µL of lysate
  - ☐ To start with 0.9 AU use: 0.9AU/0.004 AU/µL = 225 µL of lysate

Reagent Recommendations

RiboLace XL allows the user to change the amount of reagents per pull-down according to the lysate input amount. An input in the range 0.4-0.6 AU (Abs260nm) is suggested for samples with high translational levels such as immortalized cell lines (e.g MCF7, HeLa, K562) or tissues with high rate of protein synthesis (e.g. mouse liver). An input in the range 0.61- 0.9 AU (Abs260nm) is instead suggested for samples with low translational levels such as primary cells or tissues with a low rate of protein synthesis (e.g. mouse spinal cord). Always use the maximum AU input in the suggested range if you can.

When considering the input range to use, please note that cell treatments such as drugs, and transfections could negatively affect translation and ribosome activity.

In case of unknown translational levels, start with 0.6-0.9 AU.

If you are not able to start with at least 0.4 AU of cell lysate, please use less and scale down the reagents accordingly.

Practical Example

Input	5 million K562 cells
Supplemented lysis buffer (Table 1) volume	300 µL
Abs 260 nm read by Nanodrop	10 AU
Pull-down of active ribosomes conditions	0.6 AU, 60 µL of lysate, 3 µL of Nux
RPFs quantified by Qubit MicroRNA Assay	~30 ng

Using the Positive Control Pellet

If you are using this kit for the first time, we strongly recommend to perform a positive reaction with the provided Control Cell Pellet (Cat. Nr. #RL001XL-0). This positive control is a cell pellet of about 10 million immortalized mammalian cells. It should be stored at - 80°C and used within one month from the kit delivery. This Control Cell Pellet has to be lysed following the Step 1 of this protocol (paragraph for Suspension Cells) with 300 µL of Lysis buffer supplemented as in Table 1 . The Abs 260 obtained should be in the range of 7-10 AU.

For ribosome pull-down use 0.6 AU of the obtained Control cell lysate diluted in W-buffer to a final volume of 300 µL. Proceed following the protocol for samples in the range 0.4-0.6 A.U. The RPFs recovery yield (measured by Qubit microRNA kit after PAGE) should be higher than 5 ng total.

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.

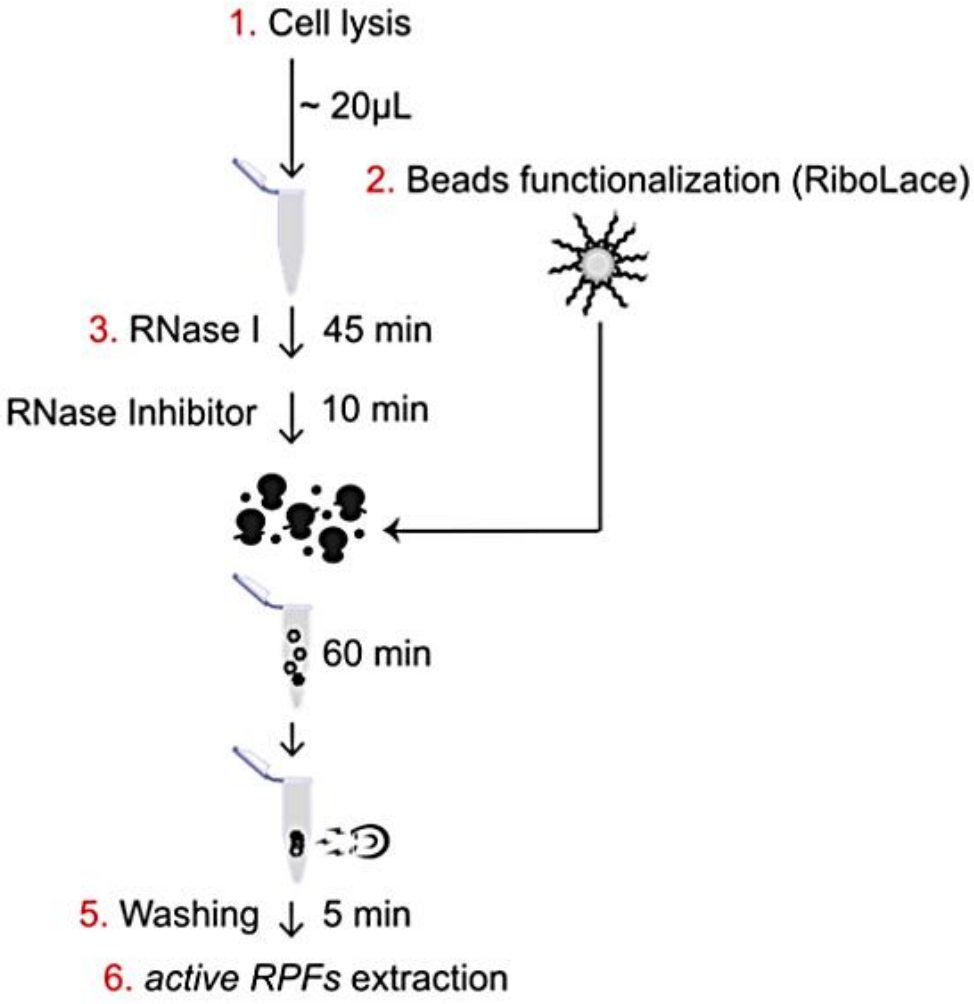

Figure 1. ***Overview of the RiboLace XL kit workflow***

Pull-down of active Ribosomes

RiboLace XL components list:

Kit component	Cat. nr.	Volume	Storage	Type	Vial
SDS 10% (SDS)	#RL001XL-9	0.5 mL	RT	Vial	clear
B-Buffer (BB)	#RL001XL-3	5 mL	4°C	Bottle	--
W-buffer (WB)	#RL001XL-4	50 mL	4°C	Bottle	--
RiboLace magnetic beads (RmB) v2-0	#RL001XL-25	1.8 mL	4°C	Vial	clear
OH-buffer (OH)	#RL001XL-14	5 mL	4°C	Bottle	--
Proteinase K (K)	#RL001XL-17	130 µL	4°C	Vial	clear
Lysis buffer (LB)	#RL001XL-1	2x 1.9 mL	-20°C	Vial	clear
RiboLace smart probe (RsP)	#RL001XL-5	195 µL	-20°C	Vial	clear
Nuclease (Nux)	#RL001XL-7	19 µL	-20°C	Vial	clear
mPEG	#RL001XL-22	100 µL	-20°C	Vial	clear
Stabilizing Nux Solution (SS)	#RL001XL-24	12 µL	-20°C	Vial	clear
25-35 Marker (25-35 M)	#RL001XL-26	13 µL	**-80°C**	Vial	clear
Positive Control Pellet (+CP)	#RL001XL-0	200 µL	**-80°C**	Vial	clear

Before starting the experiment

RiboLace smart probe dilution(1): add 812.5 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. For other size of dishes/wells, 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!**

NOTE: Lysate input amount in the range 0.4-0.6 AU is usually enough for immortalized cell lines (e.g. MCF7, HeLa, K562) or tissues with high rate of protein synthesis (e.g mouse liver). On the contrary and as an example, lysate input amount in the range 0.61-0.9 AU is needed to get a good yield from tissues with a low rate of protein synthesis (e.g mouse spinal cord) or primary cells. If you do not know the translational state of your sample start with the maximum amount you can.

Table 2. *Components' volumes to use in Step 2. N = number of reactions*.

Lysate input / Reagent	0.4 < A.U < 0.6	0.61 < A.U < 0.9	Step
RiboLace magnetic beads (RmB) v2-0	96 µL x N	144 µL x N	2.3
OH-buffer (OH)	180 µL x N	270 µL x N	2.4
Nuclease-free water	1800 µL	1800 µL	2.5
B-Buffer (BB)	180 µL x N	270 µL x N	2.6
RiboLace Smart Probe (RsP)	54 µL x N	77.5 µL x N	2.7
mPEG	5,4 µL x N	7.5 µL x N	2.11
Nuclease-free water	1000 µL	1000 µL	2.12
W-buffer (WB)	1000 µL	1000 µL	2.13
W-buffer (WB)	105 µL x N	105 µL x N	2.14

☐ **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 the requested volume of **RiboLace magnetic beads** (**RmB**) v2-0 (see Table 2 for volume) in a new 1.5 mL tube. 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 **OH-buffer (OH)** (see Table 2 for volume), then remove the supernatant.

☐ **2.5** Wash with **Nuclease-free water** (see Table 2 for volume), 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 RiboLace magnetic Beads v2-0 (**RmB**) with **B-buffer** (**BB**) (see Table 2 for volume), 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 RiboLace magnetic beads v2-0 (**RmB**) with **RiboLace smart probe** (**RsP**)**,** previously prepared [(1)] (see Table 2 for the volume to add to the beads).

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

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

During the incubation, we suggest to start 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 the beads adding **mPEG** to the tube (see Table 2 for volume) and 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 **nuclease-free water**, (see Table 2 for volume).

☐ **2.13** Wash the RiboLace magnetic beads v2-0 (**RmB**) two times with **W-buffer** (**WB**) (see Table 2 for volume)

☐ **2.14** Resuspend the RiboLace magnetic beads v2-0 (**RmB**) with W-buffer (**WB**) (see Table 2 for volume), 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

Table 3 *Components' volumes to use in Step 3*

Lysate input / Reagent	0.4 < A.U < 0.6	0.61 < A.U < 0.9	
W-buffer (WB)	Up to 300 µL	Up to 450 µL	3.1
Stabilizing Nux Solution (SS)	0.6 µL	0.9 µL	3.2
Nuclease (Nux)	µL = A.U x 5	µL = A.U x 5	3.4
SUPERaseIn	1,0 µL	1,5 µL	3.5

☐ **3.1** Start with a total volume of lysate corresponding to 0.4 - 0.9 A.U (260 nm) (see pag 3 for calculation) and add **W-buffer** (**WB**) to the final volume as indicated in Table 3.

☐ **3.2** Add **Stabilizing Nux Solution (SS)** (See Table 3 for volume) 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 Table 3. Trash the remaining diluted Nux solution.

☐ **3.5** Stop digestion with µL **SUPERase•In** (see Table 3 for volume) for 10 min on ice.

STEP 4. RIBOLACE PULL-DOWN

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

Lysate input / Reagent	0.4 < A.U < 0.6	0.61 < A.U < 0.9	
W-buffer (WB)	1000 µL	1000 µL	4.5
W-buffer (WB)	300 µL	400 µL	4.6

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 (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 W-buffer (**WB)** (see Table 4 for volumes).

☐ **4.6** Remove the beads from the magnet and resuspend them with µL W-buffer (**WB)** (see Table 4 for volumes).

☐ **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.***

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

Lysate input / Reagent	0.4 < A.U < 0.6	0.61 < A.U < 0.9	
SDS 10%	30 µL	40 µL	5.1
Proteinase K (K)	7,5 µL	10 µL	5.1
Acid Phenol:Chloroform:Isoamyl Alcohol	337.5 µL	450 µL	5.2
NaCl 2M in DEPC water	20 µL	20 µL	5.4
Isopropanol	750 µL	1000 µL	5.6
GlycoBlue.	2 µL	2 µL	5.6
Nuclease free water	10 µL	10 µL	5.9

☐ **5.1** Add **SDS 10%** (**SDS**) and **proteinase K** (**K**) (see Table 5 for volumes) to the beads suspension, and incubate at 37 °C in a water bath for 75 min.

☐ **5.2** Add **Acid Phenol:Chloroform:Isoamyl Alcohol** (see Table 5 for volume).

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

☐ **5.4** If there is no phase separation, add **NaCl 2M in DEPC water** (see table 5 for volumes) and repeat the centrifugation).

- ☐ **5.5** Keep the aqueous phase and transfer it into a new vial.
- ☐ **5.6** Add **isopropanol** (see Table 5 for volumes) and **GlycoBlue** (see Table 5 for volumes).
- ☐ **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 < 0.4 A.U)
- ☐ **5.8** Pellet the RNA by centrifugation (20000g) for 30 min at 4°C.
- ☐ **5.9** Resuspend the pellet in 10 µL of **nuclease free water**.
- ☐ **5.10** Proceed with RPFs PAGE Purification using PAGExt kit (Cat. no #KGE-002)

Contacts

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