RiboSeq Gel free

A faster path from sample to sequencing with more insights into ribosomes footprints and without gel extraction steps.

Product	Catalogue no	Rxns.
RiboSeq Gel Free	#GF001	12

Shipping: Blue Ice and Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: RiboSeq Gel free contains all reagents to perform ribosome profiling from cells/tissues to final Next Generation Seqeuncing library without the need of RNA size-selection and PAGE-gel extraction steps. The kit includes components for 12 reactions. The kit is suitable for Illumina platforms (MiSeq, NovaSeq 6000, 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
RiboSeq Gel free 4°C components	1 box	4°C
RiboSeq Gel free -20°C components	1 box	-20°C
RiboSeqGel free -80°C components	1 bag	-80°C
iUDIs plate	1 box	-20°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
- Nanodrop ND-1000 UV-VIS Spectrophotometer
- Microcentrifuge and non-stick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- Automatic wheel (rotator)
- Magnetic stand for 1.5mL tube
- RNA Clean & Concentrator™-5 (Zymo catalog. no. R1015 or R1016)
- AMPure XP for PCR Purification (Beckman Coulter catalog no. A63881)
- PCR Clean-up column kit (i.e., NucleoSpin® Macherey-Nagel catalog no 740609)
- Agilent 2100 Bioanalyzer
- Agilent High Sensitivity DNA Kit (Agilent Tech. catalog no. 5067-4626)

Optional material:

- 15% TBE-Urea polyacrylamide gel (e.g. BioRad catalog no. 450-6053 or Thermo Scientific catalog no. EC6885BOX)
- Gel Loading Buffer II (Denaturing PAGE) (Thermo Scientific catalog no. AM8546G)
- Ultra-low range molecular weight marker (i.e., Thermo Scientific catalog no. 10597012 or similar)
- SYBR Gold (Thermo Scientific, catalog no. S11494)

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 must be taken into account when programming experiments with the IMMAGINA– RiboSeq Gel free kit.

Please start the LACEseq NGS library only if you have at least 1.5 μg of total RNA after ribosomes isolation with RiboLace (step 5.13). If you have between 0.5 μg and 1.5 μg of total RNA (see step 7) please add 0.2 ng of spike-in RNA (0.5 μL) to successfully finalize the library. If you have less than 0.5 μg of total RNA, the sample does not have sufficient active ribosomes to start the library with. Be aware that - depending on the actual amount of ribosome protected fragment - the spike-in sequence will account for 1% - 20% of your total raw reads output. The sequence of the spike-in is reported at the end of the protocol. Please add 10% PhiX in the sequencing run if you are using the spike-in.

If needed, RiboSeq Gel free kit 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. In any case, if possible, 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 using the provided IMMAGINA lysis buffer (Cat nr. #GF001-7) or IMMAGINA Tissue lysis buffer (Cat. nr. #RL001-2, not included please purchase separately). Both lysis buffers have to be supplemented as indicated in Table 1 page 7 immediately before use. The use of different lysis buffers is strongly discouraged because it may interfere with the efficiency of ribosomes pull-down.

The kit has been optimized for a cell lysate input between 0.4 - 0.9 total AU (Abs260 nm). The Abs260nm should be measured using Nanodrop (selecting Nucleic Acid function) with the supplemented lysis buffer (Table 1, page 6) as blank. Briefly, use 1.5 µL of supplemented lysis buffer for blank subtraction and then quantify 1.5 µL of lysed specimen. If the instrument does not allow to utilize the supplemented lysis buffer as blank, please use water instead. After blank subtraction, quantify both the supplemented lysis buffer and the lysed specimen, then subtract the absorption calculated at 260 nm for both for the following quantifications steps.

Example:

- ☐ Lysis buffer Abs260nm = 7 AU
- ☐ Specimen Abs260nm = 17 AU
- ☐ Absorbance value of lysate = 17 – 7 = 10 AU

After proper quantification of the lysate, you should consider it as concentration in AU/mL. Thus, to calculate the volume of lysate to utilize, divide the concentration for 1000 to obtain AU/µL. The

volume calculated as in the example below has to be used for the pulldown experiment in Step 3, and for the proper quantity of reagents during beads functionalization in Step 2.

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

Please consider starting with the maximum amount of material (0.9 AU) to maximise the possibility of pulling down sufficient material for library preparation. This is particularly important, especially if specimen presents a low translational rate.

Reagent Recommendations

RiboSeq Gel Free kit allows the user to change the amount of reagents per pull-down according to the lysate amount of input. As general suggestion, always use the maximum AU input in the suggested range if possible. 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 lower translational levels such as primary cells or tissues with a low rate of protein synthesis (e.g., mouse spinal cord).

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 0.9 AU.

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
RNA extracted after Step 5	>3 µg

Optional Nuclease optimization (for Step 3)

The quantity of Nuclease (Nux) to utilize for lysing the sample could be optimized before proceeding with the pulldown. The kit contains a concentrated vial of Nux (#GF001-9) that is intended to be diluted before using, and that need to be added to the lysate sample in a fix quantity, depending on the amount of AU as starting material. This quantity is suitable for the majority of cell lines; however, it can be modulated depending on the needs and the type of specimen. To optimize this quantity, after lysing the sample in Step 1, start with 0.4 AU as starting material and W-buffer up to a final volume of 300 µL. Perform a titration assay, by adding to each reaction different quantities of Nux, below and example:

Starting lysate	Quantity of diluted Nux (Step. 3.3)	Sample
0.4 AU	0	NT
0.4 AU	0.4 µL (AU x 1)	A
0.4 AU	2 µL (AU x 5)	B
0.4 AU	20 µL (AU x 50)	C

- ☐ Digest the samples for 45 min at 25°C.
- ☐ Stop digestion with 1 µL of SUPERaseIn for 10 min on ice.
- ☐ Add 30 µL SDS 10% and 5 µL of Proteinase K and incubate at 37°C for 75 min.
- ☐ Add 310 µL of Acid Phenol:Chloroform:Isoamyl Alcohol.
- ☐ Vortex and centrifugate at 14,000 x g for 5 min.
- ☐ Keep the aqueous phase and transfer it into a new vial.
- ☐ Add 500 µL Isopropanol and 2 µL GlycoBlue.
- ☐ Mix and incubate a RT for 3 min, then store at -80°C for 2 hours.
- ☐ Pellet the RNA by centrifugation (20000g) for 30 min at 4°C.
- ☐ Remove the supernant and wash the pellet once with 70% cold ethanol. Centrifuge for 5 min at 20000g, 4°C.
- ☐ Resuspend the pellet in 10 µL of Nuclease Free Water.
- ☐ Extracted RNA need to be run on a 15% TBE-urea gel.
- ☐ 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.
- ☐ Prepare samples: add Gel Loading Buffer II to 1.5 µg of RNA (1:1 volume).
- ☐ Use an ultra-low range molecular weight marker as reference.
- ☐ Load the samples and the Marker on 15% TBE-urea polyacrylamide gel and run the gel for 1 h at 200V until the bromophenol blue band reaches the bottom of the gel.
- ☐ Stain the gel for 5 minutes with SYBR Gold in TBE and visualize the RNA using a UV-Transilluminator.

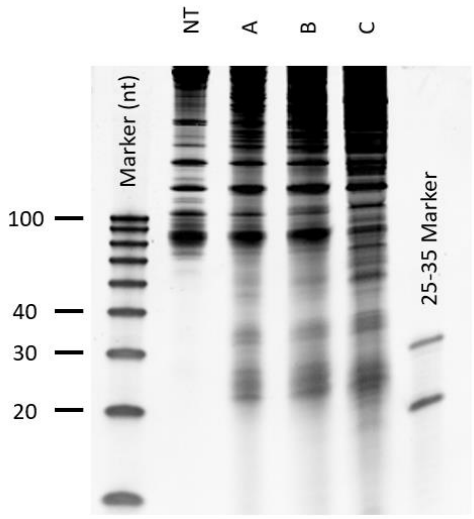


Fig.1 Example of RNA extracted after nuclease titration run on 15% TBE-Urea gel.

As depicted in the figure 1 above, not digested sample do not present the typical enrichment of fragments at 25-35 nt. Under-digested sample (A) do not display a high enrichment of RPFs, while over-digested sample (C) shows a smear of signal on the gel. In this example, quantity of Nux used in sample B (AU x 5) need to be utilized for all the reactions.

Optional intermediate checkpoint: PAGE visualization of the RPF after Ribosome pulldown (end of Step 5)

The RNA recovered at the end of Step 5 should be quantified by Nanodrop before proceeding with Step 6. This RNA contains the ribosomes protected fragments (RPFs) that are needed to prepare the library. If the quantity of RNA extracted is more than the 1.5 µg needed for the library preparation, we strongly suggest running 1 µg of the extracted RNA on a 15% TBE-urea gel following the protocol below to check the presence of RPFs. In case the RPFs are not visible on the gel, please contact our tech support (techsupport@immaginabiotech.com) before proceeding with library preparation. If you do not have at least 2.5 µg of extracted RNA (1.5 µg for library and 1 µg for running the gel), prioritize the library preparation and avoid running this optional intermediate checkpoint, keeping 1.5 µg for library preparation.

It is possible to start the library preparation in Step 6 with less than 1.5 µg of extracted RNA, however the final outcome cannot be guaranteed. **If a lower amount is utilized, please add the spike-in and scale down proportionally the Linker MC+ in step 6** multiplying for the RNA utilized for 0.67 (e.g., for 0.75 µg of RNA= 0.67*0.75 = 0.5 µL of Linker MC+ to utilize). If you start with more than 1.5 µg, use always 1 µl of linker MC+.

Protocol for optional intermediate checkpoint PAGE visualization of the RPF after Ribosome pulldown

- ☐ As a checkpoint, 1 µg of the extracted RNA could be run on a 15% TBE-urea gel.

- ☐ 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.
- ☐ Prepare samples: add Gel Loading Buffer II to 1 µg of RNA obtained from Step 5 (1:1 volume).
- ☐ Use an ultra-low range molecular weight marker as reference.
- ☐ Load the samples and the Marker on 15% TBE-urea polyacrylamide gel and run the gel for 1 h at 200V until the bromophenol blue band reaches the bottom of the gel.
- ☐ Stain the gel for 5 minutes with SYBR Gold in TBE and visualize the RNA using a UV-Transilluminator.

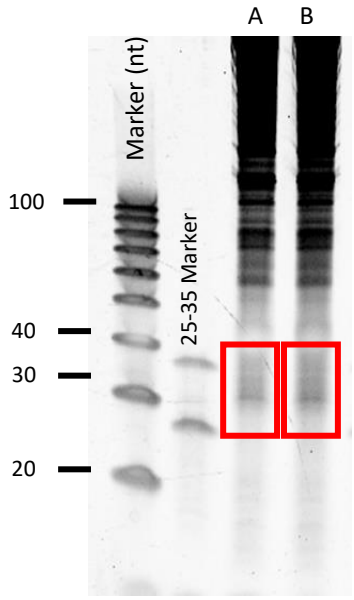
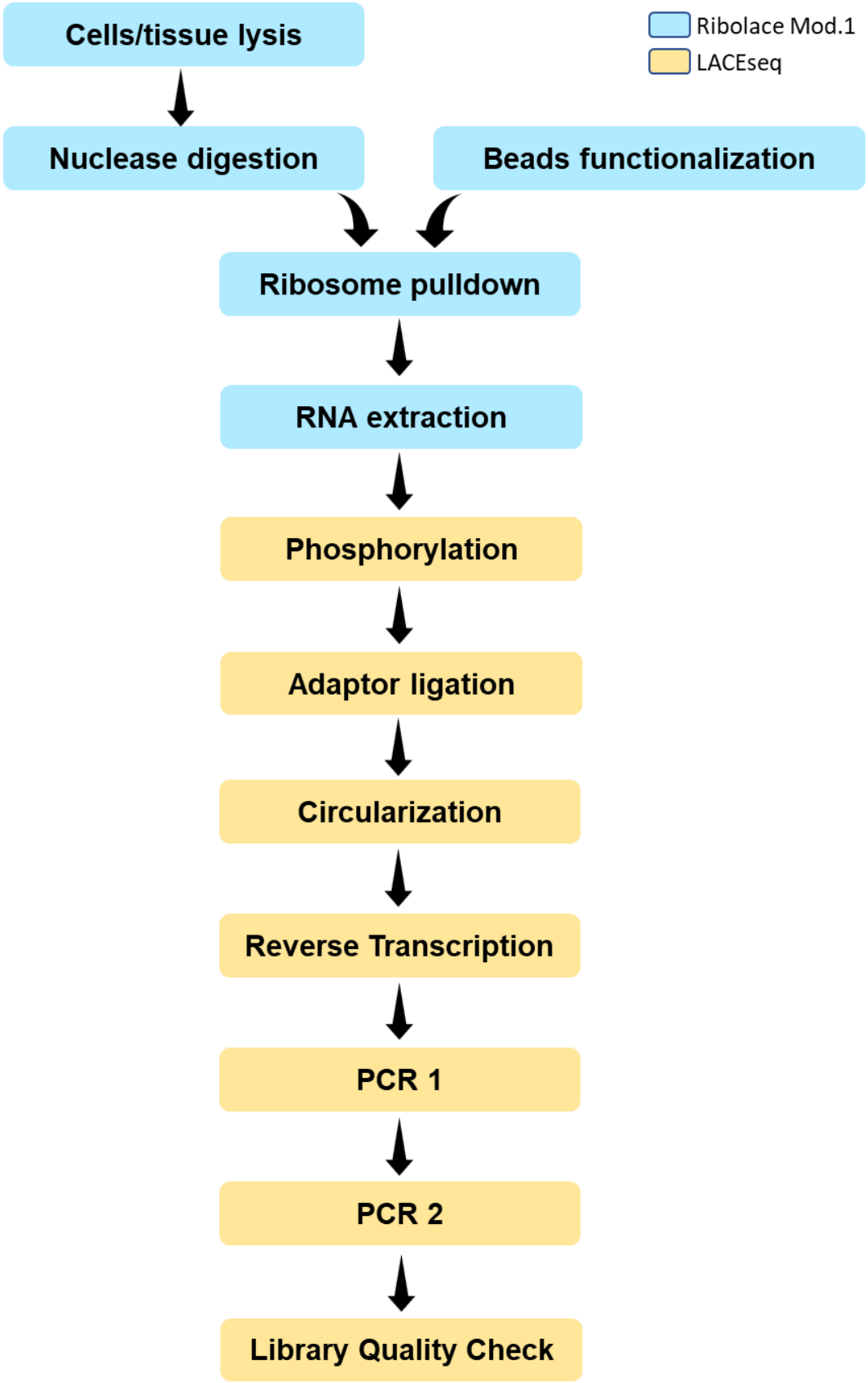


Fig.2 Example of RNA extracted after pulldown run on 15% TBE-Urea gel

- ☐ Please note that a signal between 25 nt and 35 nt should be visible. Bands present in the red square belong to the 80S ribosomes protected fragments (RPFs) and their presence in the gel indicates a proper ribosome pulldown with the RiboSeq gel free kit.



Overview of the RiboSeq Gel free kit workflow.

Pulldown of active Ribosomes

RiboSeq Gel free components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial cap colour
B-Buffer (BB)	#GF001-2	10 mL	4°C	Bottle	--
W-buffer (WB)	#GF001-3	50 mL	4°C	Bottle	--
High affinity ribosome beads (hiRB)	#GF001-4	1.8 mL	4°C	Vial	clear
OH-buffer (OH)	#GF001-5	5 mL	4°C	Bottle	--
Lysis buffer (LB)	#GF001-7	3 x 1.9 mL	-20°C	Vial	clear
RiboLace smart probe (RsP)	#GF001-8	195 µL	-20°C	Vial	clear
Nuclease (Nux)	#GF001-9	19.5 µL	-20°C	Vial	clear
mPEG	#GF001-10	100 µL	-20°C	Vial	clear
Nux Enhancer (NE)	#GF001-11	12 µL	-20°C	Vial	clear
G1F Buffer (G1F)	#GF001-32	150 µL	4°C	Vial	clear

Note:

Step 2 (page 13) and Step 3 (page 15) can be performed in parallel.

⚠ Before starting the experiment

RiboLace smart probe dilution: add 812.5 µL of B-buffer to the RiboLace smart probe vial previously thawed on ice. After use, it is suggested to aliquot the diluted mix, and store the aliquots at -80°C to avoid more than two freeze-thaw cycles. The aliquots need to take into account the amount to utilize in Step 2.8. As a suggestion, creating aliquots of 168 µL will allow to perform 3 experiments with each vial when following the protocol using 0.4-0.6 AU lysate input, or 2 experiments using 0.6-0.9 AU input.

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), DNase I (5U/mL final concentration), and RiboLock RNase Inhibitor (200 U/mL final concentration) (Table 1).

Table 1. *Recipe for the supplementation of the provided lysis buffer or tissues lysis buffer.*

Final volume	Lysis buffer (LB)	Sodium deoxycholate (SDC)	DNase I	RiboLock RNase Inhibitor
Stock concentration		10%	1 U/µL	40 U/µL
300 µL	267 µL	30 µL	1.5 µL	1.5 µL

We suggest creating fresh supplemented lysis buffer just before performing the Lysis Step, supplementing a mix for multiple samples for reproducibility (e.g., three samples to lyse in 300 µL each, prepare 900 µL by supplementing 801 µL of LB with 90 µL of SDC 10%, 4.5 µL of DNAse I and 4.5 µL of RiboLock).

Please check if, after adding Sodium deoxycholate a whiteish and cloudy solution appears. If so, please do not proceed with the lysis of the sample and contact our tech support (techsupport@immaginabiotech.com).

Please consider lysing your specimen according to the following table:

Table 2. *Quantity of lysis buffer depending on specimen amount.*

Specimen	Quantity	Lysis buffer	Volume of supplemented LB (µL)
Cell	0.3 – 1 million cells	#GF001-7	50 µL
Cell	1 – 5 million cells	#GF001-7	150 µL
Cell	> 5 million cells	#GF001-7	300 µL
Tissue	< 10 mg	#RL001-2	500 µL
Tissue	> 10 mg	#RL001-2	800 µL

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 – but it is not mandatory - to increase the efficiency of the ribosomes' affinity purification. If you are working with human or mouse tissues, please note that both Lysis buffer and W-buffer contain CHX (10 ng/mL). CHX treatment could induce accumulation of ribosomes within the first 10 codons.
- ☐ **1.2a** After incubation, place the cells on ice and wash them with **cold PBS** containing CHX (20 µg/mL).
- ☐ **1.3a** Remove all residual PBS with a pipette. It is crucial that all the PBS is removed before proceeding with the lysis to avoid diluting the lysis buffer.
- ☐ **1.4a** Perform the lysis directly adding the complete **supplemented lysis buffer** (Table 1) to each cell dish and scrape vigorously (a proper mechanical scraping is important for good 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 at 4°C.
- ☐ **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 supplemented lysis buffer previously prepared (Table 1) as blank.

Suspension Cells lysis

- ☐ **1.1b** Treat the cells with 10 µg/mL of CHX for 5 min at 37°C before lysis. 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 wash with **cold PBS** containing CHX (20 µg/mL).
- ☐ **1.3b** Collect and centrifuge at 950g for 5min at 4°C. Remove the supernatant and resuspend in complete **lysis buffer** (Table 1)
- ☐ **1.4a** Lysate cells by passing them through a G26 needle ~20 times (please note that if the volume is below 50 µL, using the syringe will lead to the loss of specimen, as a possibility you could pipette up and down ~20 times avoiding creating bubbles).
- ☐ **1.5b** Pellet the nuclei and cell debris by centrifugation at 20000 g for 5 min at 4°C.
- ☐ **1.6b** Transfer the supernatant to a new tube. Leave on ice for 20 min.
- ☐ **1.7b** 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.

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 with 800 µL of **Tissues Lysis Buffer** (not included - IMMAGINA catalog no. #RL001-2) supplemented as in Table 1. Pease note that both Tissues 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 at 4°C to remove tissue and membrane debris and collect the supernatant.

☐ **1.4c** Centrifuge again the supernatant for 5 min at max speed (20000 g) at 4°C 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 tissues 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 possible amount.

Table 3. *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	Needed in Step
High affinity ribosome beads (hiRB)	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	1000 µL	1000 µL	2.5
B-Buffer (BB)	180 µL x N	270 µL x N	2.6
Diluted RiboLace Smart Probe (RsP)	54 µL x N	77.5 µL x N	2.8
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 **High affinity ribosome beads** (**hiRB**) from 4°C and place the tube at RT for at least 30 min.

☐ 2.2 Vortex the High affinity ribosome beads (hiRB) tube thoroughly for > 30 sec.

☐ 2.3 Put the requested volume of High affinity ribosome beads (hiRB) (see Table 3 for volume) in a new 1.5 mL tube. Place the tube on a magnet to separate the High affinity ribosome beads (hiRB). Remove the supernatant.

☐ 2.4 Remove the tube from the magnet and wash the High affinity ribosome beads (hiRB) with **OH-buffer (OH)** (see Table 2 for volume) for 5 min shaking at 1400 rpm at RT. Remove the supernatant.

☐ 2.5 Wash with **nuclease-free water** (see Table 3 for volume) by shaking for 2 min at 1400 rpm at RT, place the tube on the magnet and remove the supernatant. If High affinity ribosome beads (hiRB) are binding to the plastic tube, you can add Triton X-100 to a final concentration of 0.1%.

☐ 2.6 Wash the High affinity ribosome beads (hiRB) with **B-buffer** (**BB**) (see Table 3 for volume), shaking for 3 min at 1400 rpm at RT. Repeat the wash once again with the same volume indicated in Table 3. Place the tube on the magnet for at least 1 min and remove the supernatant.

If High affinity ribosome beads (hiRB) are binding to the plastic tube, you can add Triton X-100 to a final concentration of 0.1%.

☐ **2.7** Keep at least 2 µL of diluted RiboLace smart probe (**RsP, see "Before starting the experiment",** page 9) for security check point (see grey box below).

☐ **2.8** Resuspend the High affinity ribosome beads (hiRB) with diluted RiboLace smart probe (**RsP**).

☐ **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 remove 3 µL of the supernatant (unbound probe) for security check point (see below). Keep the remaining volume in the vial.

☐ **2.11** Add **mPEG** to the tube (see Table 3 for volume), mix in a shaker at 1400 rpm 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 3 for volume) for 2 min with shaking at 1400 rpm at RT.

☐ **2.13** Wash the High affinity ribosome beads (hiRB) two times with **W-buffer** (**WB**) (see Table 3 for volume) for 2 min with shaking at 1400 rpm at RT. Remove the supernatant.

☐ **2.14** Resuspend the High affinity ribosome beads (hiRB) with W-buffer (**WB**) (see Table 3 for volume), and equally divide the functionalized beads in individual tubes according to the (N) number of samples you are processing.

Remove the W-buffer (WB) only before adding the cell lysate *(*Step 4) to avoid drying the beads.

⚠Security Check Point

CHECK PROPER BEADS FUNCTIONALIZATION

Comparing the difference in the absorbance measured at A 270 nm (Nanodrop ND-1000) for the unbound probe (collected in Step 2.10) and the staring solution of the diluted RiboLace smart probe (**RsP**) (collected in Step 2.7) allows an estimation of the binding efficiency. In particular, ~ 10-50 % absorbance reduction in the unbound probe compared to the starting solution is expected.

STEP 3. NUCLEASE TREATMENT

Table 4 *Components' volumes to use in Step 3*

Lysate input / Reagent	0.4 < A.U < 0.6	0.61 < A.U < 0.9	Needed in Step
W-buffer (WB)	Up to 300 µL	Up to 450 µL	3.1
Nux Enhancer (NE)	0.6 µL	0.9 µL	3.2
Diluted 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) diluted in W-buffer (**WB**) to the final volume as indicated in Table 4.

☐ **3.2** Add **Nux Enhancer (NE)** (See Table 4 for volume).

☐ **3.3** In a 0.2 mL vial, dilute 1.5 µL of **Nuclease** (**Nux**) by adding 98.5 µL W-buffer (**WB**). Pipet up and down 5 times to mix well the diluted Nux solution. If you are dealing with not conventional samples (e.g., immortalized cell lines) or if your specimen presents some changes that might hamper to correct protein translation, you could perform a titration curve with the Nux prior performing your experiment. This step is not mandatory but could assess the proper quantity of Nux to add in your specific sample (see: Optional Nuclease optimization Note at page 5).

☐ **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 according to Table 4. Trash the remaining diluted Nux solution.

☐ **3.5** Stop digestion with **SUPERaseIn** (see Table 4 for volume) for 10 min on ice.

STEP 4. ADD RIBOLACE & WASHING

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 (3-10 rpm) at 4°C.

⚠**4.3** Remove the tubes from the wheel. **DO NOT CENTRIFUGATE** but allow the entire solution with the beads to settle at the bottom of the tube. Pull down the beads by gently hand 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** Remove the supernatant. Carefully wash the beads twice with 1000 µL W-buffer (**WB**). Do not remove the samples from the magnet. Carefully add the WB on the opposite side of the Eppendorf to where the beads are present. Carefully remove the supernatant without disturbing the beads.

⚠**4.6** Remove completely the W-buffer (**WB**) before removing the beads from the magnet. Proceed immediately with Step 5 without drying the beads for too long to avoid cracking them.

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

STEP 5. RNA EXTRATION

- ☐ **5.1** Extract the RNA by directly adding 200 µL of the Zymo Binding Buffer (**ZBB***) to the beads pipetting up and down.
- ☐ **5.2** Transfer the beads suspension to a new nuclease-free 1.5 mL tube.
- ☐ **5.3** Incubate the beads suspension at RT for 5 min with shaking at 600 rpm.
- ☐ **5.4** After the incubation, place the tube on a magnet and collect the supernatant, transferring it to a new nuclease-free 1.5 mL tube. Discard the beads.
- ☐ **5.5** Add 200 µL of EtOH 95-100% (see Table 5 for volume) mixing the solution by pipetting.
- ☐ **5.6** Transfer the mixture to the **Zymo-Spin™ Column*** and centrifuge for 30 seconds at 12000 g at RT.
- ☐ **5.7** Add 400 µl **RNA Prep Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ **5.8** Add 700 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow-through.
- ☐ **5.9** Add 400 µl **RNA Wash Buffer*** to the column and centrifuge for 1 minute at 12000 g at RT to ensure complete removal of the wash buffer.
- ☐ **5.10** Carefully, transfer the column into a new RNase-free tube.
- ☐ **5.11** Add 12 µL of **G1F Buffer** (**G1F**) directly to the column matrix and centrifuge for 30 seconds at 12000 g at RT.
- ☐ **5.12** The extracted RNA is present in the flow-through.
- ☐ **5.13** With Nanodrop, measure the absorbance of each sample at 260 nm (setup the “nucleic acid” function of the Nanodrop), using 1 µL of G1F Buffer (G1F) as blank. In order to start with library preparation, you need at least 1.5 µg of total RNA. If you have between 0.5 µg and 1.5 µg of total RNA in your sample, please add the spike-in RNA according to step 6. **Do not start the library if you have less than 0.5 µg of total RNA.**

⚠ ***The reagents are part of the RNA Clean & Concentrator™-5 kit (Zymo catalog. no. R1015 or R1016)**

Library preparation of ribosome protected fragments.

Ribosome Profiling Gel free components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial Cap color
Buffer BL1 (BL1)	#GF001-12	100 µL	-20°C	vial	Red
L1 enzyme (L1)	#GF001-13	14 µL	-20°C	vial	Red
ATP (10 mM)	#GF001-14	80 µL	-20°C	vial	Red
Buffer L2 (BL2)	#GF001-15	60 µL	-20°C	vial	Blue
L2 enzyme (L2) (Mix A)	#GF001-16	14 µL	-20°C	vial	Blue
MnCl2	#GF001-17	30 µL	-20°C	vial	Blue
GTP	#GF001-18	20 µL	-20°C	vial	Blue
Linker MC+	#GF001-19	30 µL	**-80°C**	vial	Blue
Buffer L3 (BL3)	#GF001-20	30 µL	-20°C	vial	Yellow
Enzyme L3 (L3)	#GF001-21	15 µL	-20°C	vial	Yellow
PEG 8000 (PEG)	#GF001-22	300 µL	-20°C	vial	Yellow
ATP (1 mM)	#GF001-35	80 µL	-20°C	vial	Clear
Primer L4 (PL4)	#GF001-23	15 µL	-20°C	vial	Green
Buffer L4 (BL4)	#GF001-24	60 µL	-20°C	vial	Green
L4 enzyme (L4)	#GF001-25	13 µL	-20°C	vial	Green
dNTPs	#GF001-26	15 µL	-20°C	vial	Green
DTT	#GF001-27	20 µL	-20°C	vial	Green
AR Enzyme (AR)	#GF001-33	10 µL	-20°C	vial	Green
L5 enzyme (L5)	#GF001-28	1.3 mL	-20°C	vial	Clear
Fw PCR1 (F1)	#GF001-29	13 µL	-20°C	vial	Clear
Rev PCR1 (R1)	#GF001-30	13 µL	-20°C	vial	Clear
TR buffer (TR)	#GF001-34	0.5 mL	4°C	vial	Clear
RNA Spike- in 1 µM	#GF001-36	20 µL	-80°C	vial	Clear

STEP 6. 5' PHOSPHORYLATION

☐ **Note:** If you start step 6.1 with an amount lower than 1.5 µg (between 0.5 µg and 1.5 µg of total RNA) there is the possibility to add a total amount of **0.2 ng of spike-in** to your RNA in the reaction of step 6.1. Please quantify the spike-in (1 µL) using a Qubit™ microRNA Assay Kit and dilute the spike-in to a final concentration of 0.4 ng/µL. Thus, add 0.5 µL of diluted spike-in to the sample in table 6.1.

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

Buffer L1	5µL
ATP (10 mM)	5 µL
L1	1 µL
RNA from step 5.12	1.5 - 5 µg
H_2O	Up to 50 µL

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

☐ 6.3 Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 10,000-16,000 g for 30 seconds, unless otherwise specified.

☐ 6.4 Prepare adjusted RNA Binding Buffer by mixing 50 µL of buffer and 50 µL of ethanol (95-100%).

☐ 6.5 Add 100 µL adjusted RNA Binding Buffer (from step 6.4) to the sample and mix.

☐ 6.6 Transfer the mixture to the Zymo-Spin™ Column and centrifuge. Keep the flow-through: Small RNAs (17-200 nt) are in the flow-through!

☐ 6.7 Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ 6.8 Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

☐ 6.9 Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

☐ 6.10 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 new RNase-free tube.

☐ 6.11 Add 7 µL of **nuclease-free water** directly to the column matrix and centrifuge.

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

STEP 7. LIGATION

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

RNA (from Step 6)	7 µL
Buffer L2	1 µL
GTP	0.5 µL
MnCl2	0.6 µL
L2 enzyme	1 µL
Linker MC+	1 µL

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

☐ **7.3** Add 40 µL **nuclease-free water.**

☐ **7.4** Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 10,000-16,000 x g for 30 seconds, unless otherwise specified.

☐ **7.5** Prepare adjusted RNA Binding Buffer by mixing 50 µL of buffer and 50 µL of ethanol (95-100%).

☐ **7.6** Add the 100 µL adjusted RNA Binding Buffer (from step 7.5) to the sample and mix.

☐ **7.7** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!

☐ **7.8** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ **7.9** Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

☐ **7.10** Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

☐ **7.11** 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.

☐ **7.12** Add 9 µL of **nuclease-free water** directly to the column matrix and centrifuge.

STEP 8. CIRCULARIZATION

☐ **8.1** Mix the following reagents in a 0.2 mL nuclease-free PCR tube **(NOTE: use the 1 mM ATP vial (clear cap colour), not the 10 mM ATP vial (red cap colour):**

RNA (from Step 7)	**9 µL**
Buffer L3	2 µL
ATP (1mM)	1 µL
PEG8000*	8 µL
Enzyme L3	1 µL

*Please note that PEG8000 is a very viscous solution. Carefully pipette and check that the right amount is in your tip. Possibly add it as first reagent in the Eppendorf.

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

☐ **8.3** Add 30 µL **nuclease-free water.**

☐ **8.4** Purify the reaction through the **RNA Clean & Concentrator™-5 kit.** Perform all steps at room temperature and centrifugation at 10,000-16,000 g for 30 seconds, unless otherwise specified.

☐ **8.5** Prepare adjusted RNA Binding Buffer by mixing 50 µL of buffer and 50 µL of ethanol (95-100%).

☐ **8.6** Add the 100 µL adjusted RNA Binding Buffer (from step 8.5) to the sample and mix.

☐ **8.7** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!

☐ **8.8** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ **8.9** Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

☐ **8.10** Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

☐ **8.11** 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.

☐ **8.12** Add 12 µL of **nuclease-free water** directly to the column matrix and centrifuge.

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

STEP 9. REVERSE TRASCRIPTION

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

Circular RNA (from step 8)	**12 µL**
dNTPs	1 µL
Primer L4	1 µL

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

☐ **9.3** Add the following reagents to the annealed RNA-primer mix:

Buffer L4	4 µL
DTT	1 µL
L4 enzyme	1 µL

☐ **9.4** Incubate for 40 min at 50 °C, then heat-inactivate for 10 min at 70 °C.

☐ **9.5** Transfer on ice for at least 1minute.

☐ **9.6** Add 0.5 µL of AR Enzyme (AR).

☐ **9.7** Incubate the solution at 37°C for 1h, then at 80°C for 20 minutes.

⚠ **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 10. PCR AMPLIFICATION – PCR 1

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

cDNA (from step 9)	20 µL
L5 enzyme (L5)	50 µL
F1	0.8 µL
R1	0.8 µL
H_2O	28.4 µL

☐ **10.2** 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
8 Cycles	98°C	30 secs
	61°C	30 secs
	72°C	10 secs
Hold	4°C	∞

☐ **10.3** Purify the PCR reaction by adding 160 µL of **Agencourt AMPure XP** beads to each sample and mix well by pipetting the entire volume up and down at least 10 times.

☐ **10.4** Incubate at room temperature for 5 minutes to let the library bind to the beads.

☐ **10.5** Place the tubes on the magnetic rack until the solution is completely clear. While the tubes are still sitting on the magnetic separation device, discard the supernatant with a pipette.

☐ **10.6** Keep the tubes on the magnetic rack. Wash the beads by adding 300 µL of 75% ethanol to each sample without disturbing the beads.

☐ **10.7** Wait for 30 seconds and use a pipette to carefully remove the supernatant containing contaminants. The library will remain bound to the beads during the washing process.

☐ **10.8** Repeat washing step with 75% ethanol once, keeping the beads on the magnet.

☐ **10.9** Let the beads pellet dry on the magnetic rack at room temperature for ~2–4 minutes. **Avoid to over dry the beads (pellet cracked) as this will significantly decrease elution efficiency.**

☐ **10.10** Remove the tubes from the magnetic rack and add 50 µL of nuclease-free water to cover the pellet. Mix thoroughly by pipetting up and down to ensure complete bead dispersion. Incubate at room temperature for at least 3 minutes to rehydrate.

☐ **10.11** Place the sample tubes on the magnetic rack for 2 minutes or longer, until the solution is completely clear.

☐ **10.12** Transfer the supernatant (about 50 µL) from each tube to a clean tube and proceed with the next step.

STEP 11. PCR AMPLIFICATION – PCR 2

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

PCR1 (from step 10.12)	49 µL
L5 enzyme (L5)	50 µL
LACEseq UDIs (10 µM)	1 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 Cycles	98°C	30 secs
	60°C	30 secs
	72°C	10 secs
Hold	30 secs	∞

☐ **11.3** Purify the PCR reaction by using NucleoSpin Gel and PCR CleanUp kit (or equivalent) follow the standard protocol in Section 5.1 of the manufacture manual. Elute each sample in 21 µl of TR buffer (TR).

STEP 12. LIBRARY QUALITY CHECK

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

☐ **12.2** 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. 3). Additional peaks might be observed at about 170-190 bp that are originated from adapter dimers. If the peaks areas are higher than 50% of the principal 200 bp peak, you need to purify the libraries from gel before proceeding with sequencing.

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

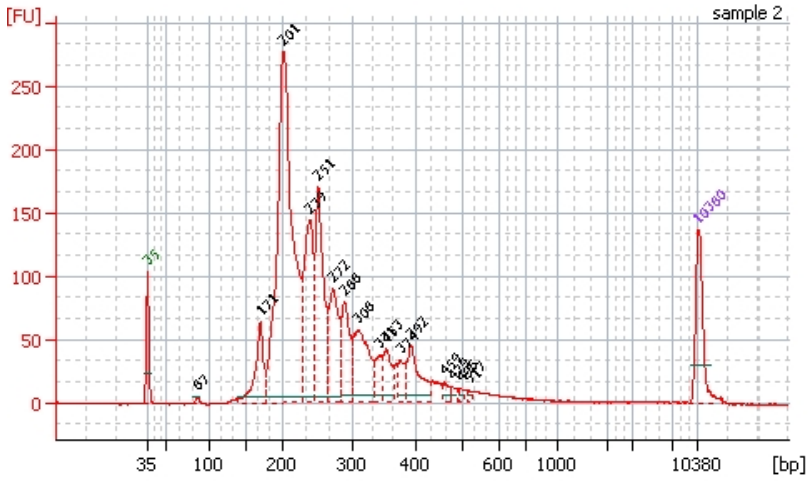


Figure 3. ***Example electropherogram results for RiboSeq Gel Free libraries.*** *Typical electropherogram for a library prepared with the Control Lysate. Library was analysed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The peak at 201 bp corresponds to the size of RPFs plus, while the peaks at 170-190 bp correspond with the size of adaptor dimers.*

Spike-in RNA exogenous sequence (20 µL, 0.4 ng/uL):

5′-CTGAGAAAGTAGAGCAAGAAGAAATAGAGC-3′

STEP 13. HOW TO SEQUENCE

The libraries produced are suitable for Illumina platforms MiSeq, NovaSeq 6000, and NextSeq 550/1000/2000.

We suggest sequencing 100 bp SE with deepness between100 and 120 M reads/sample. If you would like to observe rare translational events, such as uORF, and ribosome readthrough, we suggest you sequence 200 M reads/sample. Please note that, if you are willing to visualize trisomes, longer reads are required, thus in this case we suggest sequencing 150 bp SE between 100 and 120 M reads.

If it is possible, please utilize a sequencer with pattern flow cells such as NovaSeq 6000.

For Novaseq 6000 we suggest entering for XP protocol with the pool concentrated 0.47 nM, while for standard protocol 0.7 nM. In general, we prefer adding 3.5% quantity of PhiX.

For MiSeq you should load the libraries pool concentrated 12 pM.

For NextSeq 2000 we suggest loading the library at 500 pM, with a 10% spike in of PhiX.

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Notes: