LACEseq

NGS library preparation kit for ribosome profiling suitable for Illumina sequencing.

Product	Catalog no	Rxns.
LACEseq	LS001-12	12

Shipping: Dry ice

Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: LACEseq kit is designed for quick (1-day) high quality library preparation of small RNA bearing the 3'-Phosphate signatures. This product is particularly selected for performing libraries from Ribosome Protected Fragments (RPFs) isolated using RiboLace kits (#RL00S-04, # RL00P-12) and or other protocols. The kit includes components for 12 reactions, and it is suitable for Illumina platforms (MiSeq, NovaSeq 6000, NextSeq1000/2000).

Suitable for: Eukaryotic cell lines and tissues

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

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Kit storage info

	Qty.	Storage
-20°C components	1 box	-20°C
-80°C components	1 bag	-80°C

Table 1. *Kit composition (in boxes and bag) and storage temperature.*

Additionally Required Materials

- RNAse-free water
- Nanodrop ND-1000 UV-VIS Spectrophotometer
- Microcentrifuge and non-stick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- 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)

INTRODUCTION

LaceSeq™, an Immagina proprietary technology, has been developed to elevate both the efficiency and simplicity of library preparation. The nucleases commonly used for the generation of the RPFs, leave a phosphate at the 3' extremity of the digested RNA fragments. Unlike the standard library preparation protocols that remove this molecular signature, LaceSeq™ exploits it to specifically target the RPFs and drive their preferential uptake into the NGS library.

After the initial selective ligation between the RPF 3'P end and our Linker, a second specific intra-molecular ligation is initiated. The kinetics of the second ligation ensures lower incorporation of RNA contaminants. The original proof-of-concept of the technology was published on NAR in 2021 with the circAID name.

The structure of the LaceSeq™ Linker has also been optimized to support downstream processes of NGS sequencing and Data analysis. Specific Unique Molecular Identifiers (UMI) are present in the linker, allowing the identification of PCR duplication products. Illumina adapters and Unique Dual Indexes (iUDIs) required for multiplexing (to be purchase separately), are added after circularization and Reverse Transcription via a two-step PCR amplification.

It's important to note that the LACEseq kit do not includes the iUDIs plate and that a set can be purchased together with the kit. We offer four different sets of iUDIs primers, 12 Forward and 12 Reverse per each set (Cat. no. #UDI0Z1-12, Z13-24, Z25-36 or Z37-48).

IMMAGINA’s mission is to develop unique and smart enabling technologies to break down the walls in translational studies. Please visit our website https://immaginabiotech.com/ for a complete overview of our products & services and our proprietary technologies.

WORKFLOW OVERVIEW

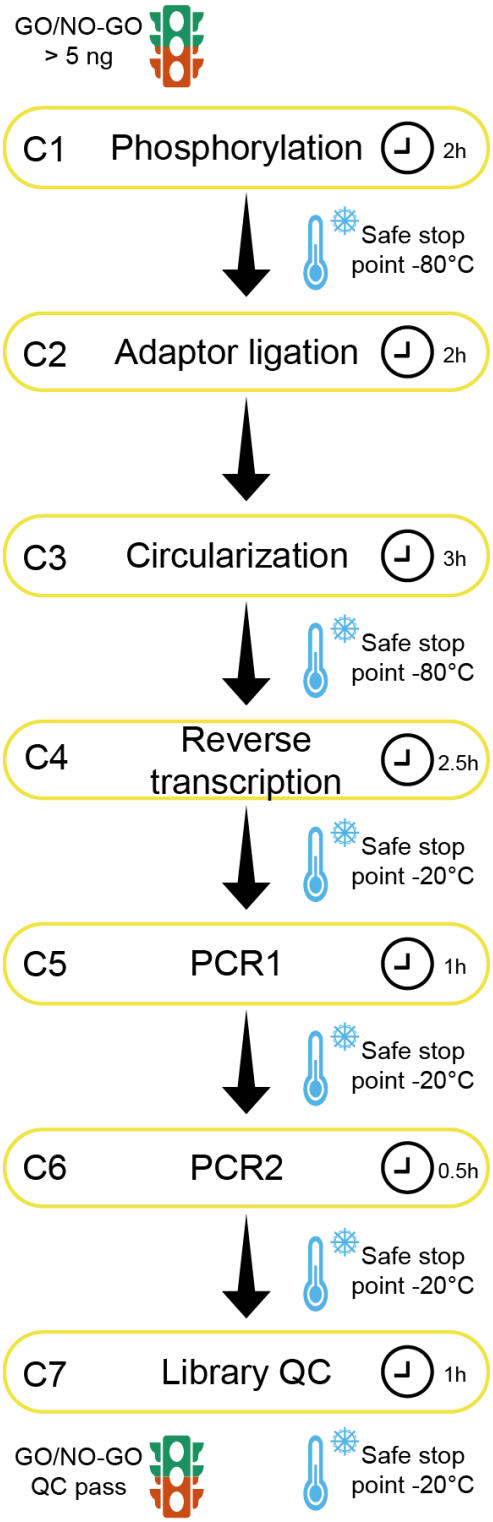

Fig.1 Overview of the LACEseq Kit workflow. In each box, steps are present in the left corner, while the time to complete the step is listed in the right corner (clock picture). Safe stopping points (thermometer pictures), and GO/NO-GO conditions (traffic lights) are placed right at the bottom of the corresponding steps.

Optimal Workflow Recommendations

- ⚠ Please avoid running more than 6 samples in parallel. Longer manipulation time may introduce an unwanted variability between the first and the last samples.
- Allocate at least 2 days for the completion of the entire workflow.
- If possible, please perform a preliminary lysis experiment to set the lysis volume following the suggested AU operational range (see section A3).
- The Beads Functionalization and the Nuclease Digestion (Steps B2 and B3) can be performed in parallel, to shorten the protocol length.
- This protocol has been optimized to perform the library preparation starting with at least 5 ng of RPF after pulldown and PAGE extraction.
- The protocol provides guidelines for different RPFs amount recovered after PAGExt extraction, from 5 to 40 ng.
- A set of tables is available in the Appendixes to allow for a step-by-step approach while running the experiment. To improve user experience, we suggest printing them and having them available during the actual manipulations.

C. RPF's LIBRARY PREPARATION

LACEseq components and additional required materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
C1	Buffer BL1 (BL1)	#IBT0151	100 µL	-20°C	vial	Red
C1	L1 enzyme (L1)	#IBT0161	15 µL	-20°C	vial	Red
C1	ATP 10 mM	#IBT0171	100 µL	-20°C	vial	Red
C1/C2/C3	RNA Clean & Concentrator™-5	Additionally Required Material				
C1/C2/ C3/C5	Nuclease Free Water	Additionally Required Material				
C2	Buffer L2 (BL2)	#IBT0181	60 µL	-20°C	vial	Blue
C2	L2 enzyme (L2)	#IBT0191	15 µL	-20°C	vial	Blue
C2	MnCl2	#IBT0211	30 µL	-20°C	vial	Blue
C2	GTP	#IBT0201	20 µL	-20°C	vial	Blue
C2	Linker MC (1 µM) (MC)	#IBT0221	30 µL	**-80°C**	vial	Blue
C3	Buffer L3 (BL3)	#IBT0231	50 µL	-20°C	vial	Yellow
C3	L3 Enzyme (L3)	#IBT0241	15 µL	-20°C	vial	Yellow
C3	PEG 8000 (PEG)	#IBT0251	300 µL	-20°C	vial	Yellow
C4	Primer RT_T (RT_T)	#IBT0261	20 µL	-20°C	vial	Green
C4	Buffer L4 (BL4)	#IBT0271	75 µL	-20°C	vial	Green
C4	L4 enzyme (L4)	#IBT0281	15 µL	-20°C	vial	Green
C4	dNTPs	#IBT0301	20 µL	-20°C	vial	Green
C4	DTT	#IBT0291	20 µL	-20°C	vial	Green
C5	L5 enzyme (L5)	#IBT0321	1.5 mL	-20°C	vial	clear
C5	Fw PCR1 (F1)	#IBT0331	20 µL	-20°C	vial	clear
C5	Rev PCR1 (R1)	#IBT0341	20 µL	-20°C	vial	clear
C6	TR buffer (TR)	#IBT0351	0.5 mL	4°C	vial	clear
C1*	3P-RNA 1 µM (RNA)	#IBT0361	20 µL	-80°C	vial	clear

* 3P-RNA 1 µM (RNA) is utilized as control RNA sample for the library preparation (see Appendix.10)

Step C1. 5' PHOSPHORYLATION

NOTE: Input for the library is an RPFs amount between 5 and 40 ng (quantified by Qubit™ microRNA Assay Kit after gel extraction). In case of less RPFs amount, combine multiple ribosomes' pulldowns for the same sample.

Both the reagents of the library preparation and the PCR cycles need to be tailored according to the RPFs input amount you utilize. We strongly suggest starting the library preparation with all the amount of RPFs that you extracted (from 5 to 40 ng) to maximize the output of each reaction and to obtain good libraries.

☐ **C.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
RPFs (between 5 to 40 ng)	10 µL
H_2O	29 µL

☐ **C1.2** Incubate the reaction for 1h at 37 °C in a thermal cycler.

☐ **C1.3** 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.

☐ **C1.4** Prepare adjusted RNA Binding Buffer by mixing N x 50 µL of buffer and N x 50 µL of ethanol (95-100%).

☐ **C1.5** Add 100 µL adjusted RNA Binding Buffer (from step C1.4) to each sample and mix.

☐ **C1.6** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ **C1.7** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ **C1.8** Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

☐ **C1.9** Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

☐ **C1.10** 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.

☐ **C1.11** Add 7 µL of **nuclease-free water** directly to the column matrix and centrifuge. Save the flow-through.

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

Step C2. ADAPTOR LIGATION

NOTE: When dealing with RPF quantities falling between two predefined categories (e.g., if you have 13 ng), follow these guidelines for selecting the appropriate linker volume:

- ☐ If your RPF quantity is between the lower and upper limits of a category (e.g., 10 ng and 20 ng), choose the lower value of the linker (in the 13-ng example, use the linker for 10 ng, which is 0.5 µL).
- ☐ When working with intermediate quantities (e.g., 7 ng or 14 ng), opt for a linker volume below the midpoint. For instance, use 0.25 µL for 7 ng and 0.5 µL for 14 ng.
- ☐ Once your RPF quantity reaches or exceeds the halfway point of a category (e.g., 9 ng or 18 ng), consider utilizing the upper limit of the linker volume. For example, use 0.5 µL for 9 ng and 1 µL for 18 ng.
  These guidelines will help ensure precise and consistent linker volume selection based on your RPF quantities.

☐ **C2.1** Mix the following reagents in a 0.2 mL nuclease-free PCR tube:

	RPFs amount (25 – 35 nt)			
	5-7 ng	**8-14 ng**	**15-24 ng**	**25-40 ng**
RNA (from Step 7)	6 µL	6 µL	6 µL	6 µL
Buffer BA	1 µL	1 µL	1 µL	1 µL
GTP	0.5 µL	0.5 µL	0.5 µL	0.5 µL
$MnCl_2$	0.6 µL	0.6 µL	0.6 µL	0.6 µL
Enzyme Mix A	1 µL	1 µL	1 µL	1 µL
Linker MC 1µM	0.25 µL	0.5 µL	1 µL	2 µL
H_2O	0.75 µL	0.5 µL	-	-

☐ **C2.2** Incubate the reaction for 1h at 37 °C in a thermal cycler.

☐ **C2.3** Add 40 µL **nuclease-free water.**

☐ **C2.4** 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.

☐ **C2.5** Prepare adjusted RNA Binding Buffer by mixing 50 µL of buffer and 50 µL of ethanol (95-100%).

☐ **C2.6** Add the 100 µL adjusted RNA Binding Buffer (from step 7.5) to the sample and mix.

☐ **C2.7** Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ **C2.8** Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ **C2.9** Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

- ☐ **C2.10** Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
- ☐ **C2.11** Add 400 µL RNA Wash Buffer to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer.
- ☐ **C2.12** Carefully, transfer the column into a RNase-free tube.
- ☐ **C2.13** Add 9 µL of **nuclease-free water** directly to the column matrix and centrifuge. Save the flow-through.

Step C3. CIRCULARIZATION

☐ C3.1 Prepare a 1 mM **ATP** solution by diluting the ATP stock in **nuclease-free water** (e.g. 1 µL ATP + 9 µL nuclease-free water). Pipet up and down to mix well the solution. Assemble the following reaction in a 0.2 mL nuclease-free PCR tube**:**

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 the first reagent in the Eppendorf.

☐ C3.2 Incubate the reaction for 2h at 25 °C in a thermal cycler.

☐ C3.3 Add 30 µL **nuclease-free water.**

☐ C3.4 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.

☐ C3.5 Prepare adjusted RNA Binding Buffer by mixing 50 µL of buffer and 50 µL of ethanol (95-100%).

☐ C3.6 Add the 100 µL adjusted RNA Binding Buffer (from step 8.5) to the sample and mix.

☐ C3.7 Transfer the mixture to the Zymo-Spin™ Column and centrifuge. **Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!**

☐ C3.8 Add 150 µL of ethanol and mix. Transfer the mixture to a new column and centrifuge. Discard the flow-through.

☐ C3.9 Add 400 µL RNA Prep Buffer to the column and centrifuge. Discard the flow-through.

☐ C3.10 Add 700 µL RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

☐ C3.11 Add 400 µL RNA Wash Buffer to the column and centrifuge for 1 minute to ensure complete removal of the wash buffer.

☐ C3.12 Carefully, transfer the column into a RNase-free tube.

☐ C3.13 Add 12 µL of **nuclease-free water** directly to the column matrix and centrifuge. Save the flow-through.

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

Step C4. REVERSE TRANSCRIPTION

☐ **C4.1** For the generation of single-strand cDNA, combine the following reagents:

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

☐ **C4.2** Incubate the circular RNA-primer mix at 70°C for 5 minutes and then transfer on ice for at least 1 minute.

☐ **C4.3** Add the following reagents to the annealed RNA-primer mix:

Buffer L4	4 µL
DTT	1 µL
L4 enzyme	1 µL

☐ **C4.4** Incubate for 40 min at 50 °C, then heat-inactivate for 10 min at 80 °C.

⚠**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 C5. PCR AMPLIFICATION – PCR 1

☐ **Note:** the cycles of Step C5 – PCR 1 and Step C6 – PCR 2 depend on the starting RPFs that you used in Step C1. The number of cycles is important for avoiding overamplification, please refer to the following table for the correct number of cycles to utilize.

Table 4 *Number of cycles of PCR to use in Step C5 (PCR1) and C6 (PCR2)*

	RPFs amount (25 – 35 nt)					
	5 ng	**6-9 ng**	**10-14 ng**	**15-19**	**20-34 ng**	**35-40 ng**
PCR 1 cycles	9	9	8	7	7	6
PCR 2 cycles	7	6	6	6	5	5

☐ **C5.1** Combine the following reagents (for reaction) in a final volume of 100 µL:

cDNA (from step C4.4)	20 µL
L5 enzyme (L5)	50 µL
F1	0.8 µL
R1	0.8 µL
H_2O	28.4 µL

☐ **C5.2** 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
6-9 Cycles*	98°C	30 secs
	61°C	30 secs
	72°C	10 secs
Hold	4°C	∞

*Please refers to Table 4 for correct number of cycles.

☐ **C5.3** Transfer the reaction mix into a new 1.5 mL tube.

☐ **C5.4** Purify the PCR reaction by adding 160 µL of **Agencourt AMPure XP** beads **(Warm the beads at Room Temperature before use)** to each sample and mix well by pipetting the entire volume up and down at least 10 times.

☐ **C5.5** Incubate at room temperature for 5 minutes to let the library bind to the beads.

☐ **C5.6** 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.

☐ **C5.7** Keep the tubes on the magnetic rack. Wash the beads by adding 300 µL of 75% ethanol to each sample without disturbing the beads.

- ☐ **C5.8** 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.
- ☐ **C5.9** Repeat the washing step with 75% ethanol once, keeping the beads on the magnet.
- ☐ **C5.10** 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.**
- ☐ **C5.11** 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.
- ☐ **C5.12** Place the sample tubes on the magnetic rack for 2 minutes or longer until the solution is completely clear.

Step C6. PCR AMPLIFICATION – PCR 2

☐ **C6.1** Combine the following reagents for reaction (final volume 100 µL):

PCR1 (from step C5.12)	49 µL
L5 enzyme (L5)	50 µL
LACEseq UDIs (10 µM)	1 µL

☐ **C6.2** 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
5-7 Cycles*	98°C	30 secs
	60°C	30 secs
	72°C	10 secs
Hold	30 secs	∞

*Please refers to Table 4 for correct number of cycles.

☐ **C6.3** Purify the PCR reaction by using NucleoSpin Gel and PCR CleanUp kit (or equivalent) and following the manufacturer's standard protocol (Section 5.1 of the manual). Elute each sample in 21 µl of TR buffer (TR).

Step C7. LIBRARY QUALITY CHECK

The libraries originated contains adaptor dimers together with the library originated from the RPFs. We suggest extracting the library peak after running it on a Page gel by size selection procedure. A possibility is to perform this step using IMMAGINA PAGE Gel Extraction Kit (cat no. KGE00-12) which allows for more stringent selection of libraries than bead-based selection.

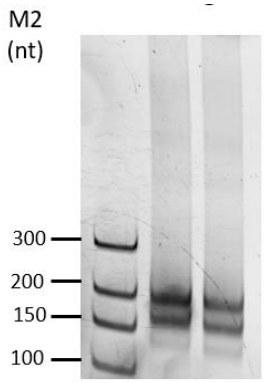

Figure 2. Example of library run on gel before size selection. Typical run on a 10% TBE gel of a Library produced with the LACEseq kit. Library peak is 200-220 nt, while the adaptor dimer is 170-190 nt.

In any case, we recommended to evaluate the library by Agilent 2100 Bioanalyzer using the **Agilent High Sensitivity DNA Kit** both before and after gel run to check the presence of the library and the adaptor dimer content.

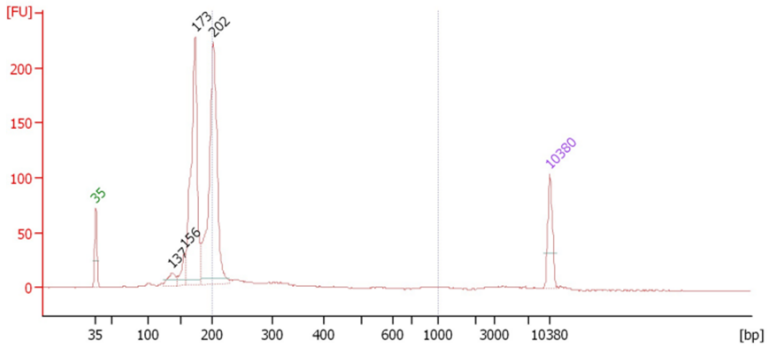

Figure 3. Example electropherogram libraries results before size selection. Typical electropherogram for a library prepared with RPFs obtained from an immortalized cell line with RiboLace kit. The library was analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The electropherogram presents a peak between 200 to 220. In this example, the peak at 202 bp corresponds to the size of RPFs, while the peaks at 173 bp correspond with the size of adaptor dimers**.**

After extraction of the library from gel with the PAGExt kit, only the sharp peak between 200-220 should be recorded via Agilent 2100 Bioanalyzer. For more information, please visit PAGExt webpage at https://immaginabiotech.com/product/pagext

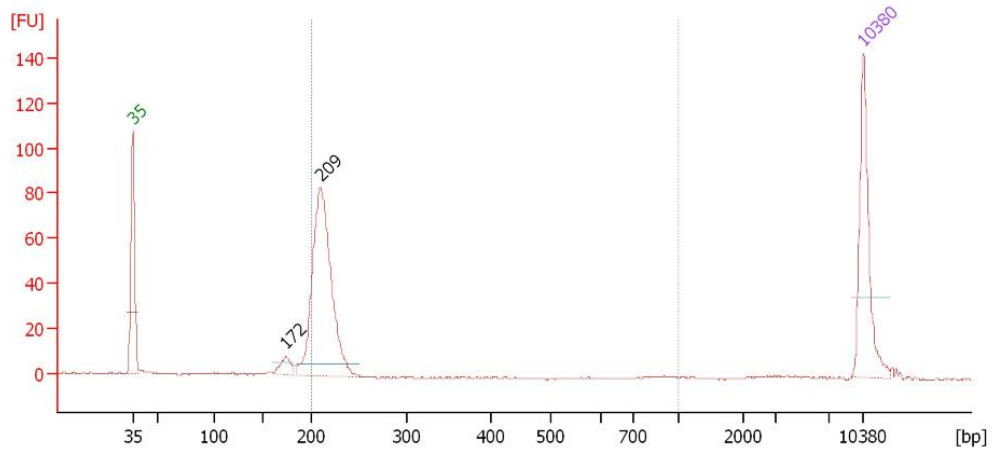

Figure 4. Example electropherogram libraries results after gel run and size selection. Typical electropherogram for a library prepared with RPFs obtained from an immortalized cell line with RiboLace kit. The library was analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The electropherogram presents a peak between 200 to 220. In this example, the peak at 209 bp corresponds to the size of RPFs, while the peaks at 172 bp correspond with the size of adaptor dimers**.**

D. SEQUENCING

Step D1. HOW TO SEQUENCE – DO IT YOURSELF

The libraries produced are suitable for Illumina platforms, we strongly suggest the use of sequencing platforms based on pattern flow cells such as MiSeq, NovaSeq 6000, and NextSeq 1000/2000. Although compatible, we do not advise the use of open-flow cells such as NextSeq 550, which in our experience yield less satisfactory sequencing runs.

Sequencing lengths shorter than 100 bp should be avoided, although theoretically sufficient, shorter read length may lead to loss of reads during computational analysis. We suggest Single-End sequencing run with depth between 100 and 120 Million reads/sample, and if you would like to observe rare translational events, such as uORF, and ribosome readthrough, to increase the depth to 200 M reads/sample.

To visualize RPFs deriving from the footprint of ribosome disome and trisome the sequencing length must be increased to at least 150 bp or 200bp.

In common practice, Single-End sequencing is less frequently used than Pair-End sequencing, and some facilities are more prone to performing PE sequencing. It is possible to sequence our library in PE (with a length of 150bp) but with a depth of 200 - 240 Million reads, this is because only the Forward Reads are retained for the Data analysis.

For Novaseq 6000 using the XP protocol, we suggest an entry concentration of the pool of 470 pM, while for standard protocol of 700 pM. In general, we prefer adding a 3.5% quantity of PhiX.

For MiSeq the loading concentration of the library pool should be 12 pM, while for NextSeq 1000/2000 we suggest loading the library at 500 pM, with a 10% spike-in of PhiX.

Step D2. HOW TO SEQUENCE – OUTSOURCE TO IMMAGINA

Supporting the whole RiboSeq workflow requirements, IMMAGINA provides sequencing services for RiboSeq NGS libraries, RNAseq libraries, and the required downstream data analysis:

- a basic package for the trimming of the sequencing data, the alignment of the reads and the counts,
- a package for the calculation of the Translation Efficiency,
- a Premium package for the development of ad-hoc analysis.

You can contact us at info@immaginabiotech.com to discuss your needs in detail.

APPENDIX

App.1 Library Preparation: Positive control

In the kit is present a positive control reaction for library preparation. The 3P-RNA 1 µM (RNA) is an exogenous RNA fragment of known sequence, with a length comparable to the RPF's and a 3'P extremity. Mimicking an RPF molecule allows to carry on the multiple reaction and purification steps required for the production of the library.

Please quantify 1 µL of the 3P-RNA 1 µM (RNA) using a Qubit™ microRNA Assay Kit and use a volume equal to 10 ng of it to start the library preparation from Step C1. 5' PHOSPHORYLATION.

During gel run in Step C8. LIBRARY EXTRACTION, two bands of similar quantity should be visible as depicted in the image (Fig.2 page 16), while after extraction of the library, only the sharp peak between 200-220 should be recorded via Agilent 2100 Bioanalyzer (Fig.3 page 16).

In any case, we recommended to evaluate the library by Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit both before and after gel run to check the presence of the library and the adaptor dimer content (Fig.4 page 17).

Contacts

Info

info@immaginabiotech.com

Sale support (quoting, ordering, and order status update)

orders@immaginabiotech.com

Technical service (technical inquiries and quality complaints)

techsupport@immaginabiotech.com

Viale Dell'industria, 47, 38057, Pergine Valsugana (TN), ITALY

www.immaginabiotech.com

+39 04611787270

Notes: