LACEseq ribosome profiling kit for Illumina

Product	Catalog no	Rxns.
LACEseq	#LS-001_12	12

Shipping: Dry ice

Storage Conditions: store components according to storage conditions reported on labels and on this manual

Shelf Life: 12 months

Description: LACEseq kit is designed for quick (1-day) high quality library preparation of Ribosome Protected Fragments (RPFs) isolated using RiboLace Ribo-Seq kit module 1 or other protocols. This kit is suitable for Illumina platforms (MiSeq, NovaSeq, HiSeq, NextSeq550).

Suitable for: Eukaryotic cell lines and tissues

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

Kit contents	Qty.	Storage
LACEseq -20°C components	2 box	-20°C
LACEseq -80°C components	1 Alu-bag	-80°C

Additionally Required Materials

- o RNAse free water
- o Acid-phenol:chloroform (Ambion catalog no. AM9720)
- o GlycoBlue (Ambion catalog no. AM9515)
- o Isopropanol (Sigma catalog no. 278475)
- o Microcentrifuge and nonstick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- o Automatic wheel (rotator)
- o Magnetic stand for 1.5mL tube
- o Qubit Fluorometer
- o Qubit™ dsRNA Assay Kit
- o AMPure XP for PCR Purification (Beckman Coulter catalog no. A63881)
- o NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel catalog no 740609.10/.50/.250
- o Agilent 2100 Bioanalyzer
- o High-Sensitivity DNA chip (Agilent Tech. catalog no. 5067-4626)

Optional:

- o PAGExt Gel extraction kit (IMMAGINA catalog no. #KGE-002)
- o UDI’s Plate (IMMAGINA catalog no. #LS-UDI-002-24)

Recommendations

Sample Recommendations

- **Input RNA amount**: ≥ 5ng of ribosome protected fragments (quantified after gel extraction)
- **Input RNA quality**: high RNA purify and integrity is recommended in order to ensure optimal downstream results

Using the Positive Controls

If you are using this kit for the first time, we strongly recommend performing the positive control reaction for library preparation. The positive control (CTRL, Cat. Nr. #LS001-1) is an RNA fragment with a 5'OH and 3'P (1 µM). For library preparation of the positive control, use 1 µl of the RNA control (10 ng) and follow protocol.

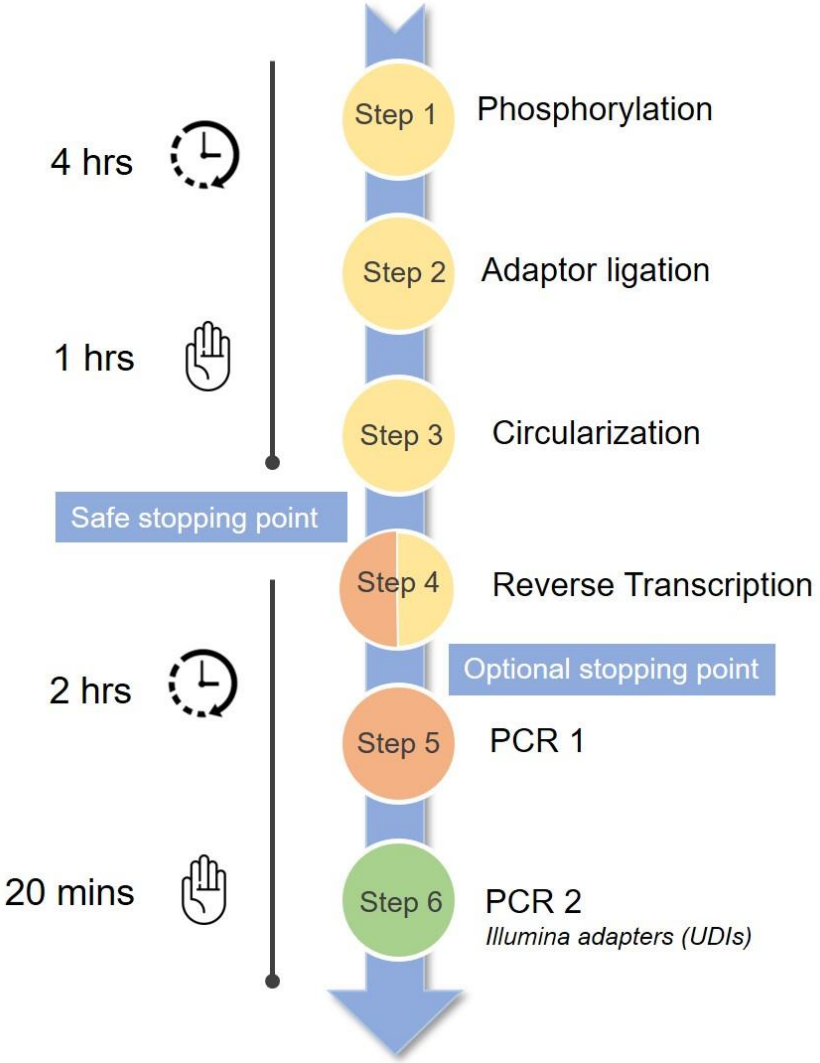


Figure 1. ***Overview of the LACEseq workflow.***

LIBRARY PREPARATION OF RIBOSOME PROTECTED FRAGMENTS

LACEseq components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial
Buffer BPK	#LS001-1	80 µL	-20°C	Vial	Red
PK enzyme (PK)	#LS001-2	13 µL	-20°C	Vial	Red
ATP (10 nM)	#LS001-3	80 µL	-20°C	Vial	Red
Buffer BA	#LS001-4	20 µL	-20°C	Vial	Blue
Enzyme Mix A (Mix A)	#LS001-5	13 µL	-20°C	Vial	Blue
MnCl2	#LS001-6	10 µL	-20°C	Vial	Blue
GTP	#LS001-7	10 µL	-20°C	Vial	Blue
Linker MC (1 µM)	#LS001-8	26 µL	-80°C	Vial	Blue
Buffer BLB	#LS001-9	30 µL	-20°C	Vial	Yellow
Enzyme Mix B (Mix B)	#LS001-10	13 µL	-20°C	Vial	Yellow
PEG 8000	#LS001-11	200 µL	-20°C	Vial	Yellow
RT_T Primer (RT_T)	#LS001-12	13 µL	-20°C	Vial	Green
Buffer BRT	#LS001-13	60 µL	-20°C	Vial	Green
RT enzyme (RT)	#LS001-14	13 µL	-20°C	Vial	Green
dNTPs	#LS001-15	13 µL	-20°C	Vial	Green
DTT	#LS001-16	15 µL	-20°C	Vial	Green
Amplification mix (AM)	#LS001-17	1.3 mL	-20°C	Vial	Clear
Fw PCR1 (F1)	#LS001-18	13 µL	-20°C	Vial	Clear
Rev PCR1 (R1)	#LS001-19	13 µL	-20°C	Vial	Clear
Control (CTRL)	#LS001-22	10 µL	-80°C	Vial	Clear

Note:

Input RPFs amount: ≥ 5 ng (quantified by Qubit after gel extraction). In case of less RPFs amount, combine multiple ribosomes' pulldowns for the same sample.

STEP 1. 5' PHOSPHORYLATION

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

Buffer BPK	5µL
ATP (10 mM)	5 µL
PK	1 µL
RPFs	5ng – 40 ng
H2O	Up to 50 µL

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

☐ **1.3** Purify the reaction through the **RNA Clean & Concentrator™-5 kit**, following the protocol for small RNAs and performing the final elution in a volume of 6 µL of **Nuclease-free water**. For more information, see Appendix 1.

STEP 2. LIGATION

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

	RPFs amount (25 – 35 nt)			
	5 ng	**10 ng**	**20 ng**	**40 ng**
RNA (from Step 1.3)	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	-	-

** use always the following ratio between RPFs and linker: 10 ng RPFs (quantified by miRNA Qubit), 0.5 µL of linker_MC. If it is necessary, you can dilute the Linker_MC in nuclease-free water (e.g instead of pipeting 0.25 µL, you can diluite the linker_MC 1:2 and add 0.5 µL of diluited Linker_MC into the reaction)*

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

☐ **2.3** Add **Nuclease-free water** up to 50 µL final volume, then purify the reaction through the **RNA Clean & Concentrator™-5 kit**, following the protocol for small RNAs and performing the final elution in a volume of 8 µL of **Nuclease-free water**. For more information, see Appendix 1.

STEP 3. CIRCULARIZATION

☐ 3.1 Prepare a 1 mM **ATP** solution by diluting the ATP stock (10 nM) 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 2.3)	**8 µL**
Buffer BLB	2 µL
ATP (1mM)	1 µL
PEG8000	8 µL
Enzyme Mix B	1 µL

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

☐ 3.3 Add **Nuclease free water** up to 50 µL final volume, then purify the reaction through **RNA Clean & Concentrator™-5 kit**, following the protocol for small RNAs and performing the final elution in a volume of 10 µL of **Nuclease-free water**.

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

STEP 4. REVERSE TRASCRIPTION

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

Circular RNA (from step 3.3)	**10 µL**
dNTPs	1 µL
RT_T Primer	1 µL
H2O	Up to 14 µL

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

☐ 4.3 Add the following reagents to the annealed RNA:

Buffer BRT	4 µL
DTT	1 µL
RT enzyme	1 µL

☐ 4.4 Incubate 40 min at 50°C, then heat-inactivate for 5 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 5. PCR AMPLIFICATION – PCR 1

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

cDNA (from Step 4.4)	**20 µL**
Amplification Mix	50 µL
F1	0.8 µL
R1	0.8 µL
H2O	28.4 µL

☐ **5.2** 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	∞

** we recommend performing a pilot experiment and reducing or increasing the number of PCR cycles, as necessary, to achieve adequate library yields.*

☐ **5.3** Purify PCR samples by using 1.6x volume **Agencourt AMPure XP beads** following manufacturer's instructions. Elute the sample in 40 µL of **Nuclease-free water**. The volume of Agencourt AMPure XP beads for a given reaction can be derived from the following equation:

Volume of Agencourt AMPure XP per reaction = 1.6 x Reaction Volume

Avoid over dried of the beads (pellet cracked) as this will significantly decrease elution efficiency

STEP 6. PCR AMPLIFICATION – PCR 2

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

PCR1 **(from Step 5.3)**	40 µL
Amplification Mix	50 µL
LACEseq UDIs (10 µM)	1 µL
H2O	9 µL

☐ **6.2** 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	∞

** we recommend performing a pilot experiment and reducing or increasing the number of PCR cycles, as necessary, to achieve adequate library yields.*

☐ **6.3** Use Agencourt XP beads (1.6x ratio) or NucleoSpin Gel and PCR CleanUp kit to purify the entire 100-µl PCR reaction. *Agencourt XP beads: follow manufacturer's instructions and elute the sample in 40 µL of nuclease-free water. Nucleospin Gel columns: follow the standard protocol in Section 5.1 of the manufacture manual. Elute each sample in 20 µl of nuclease-free water.*

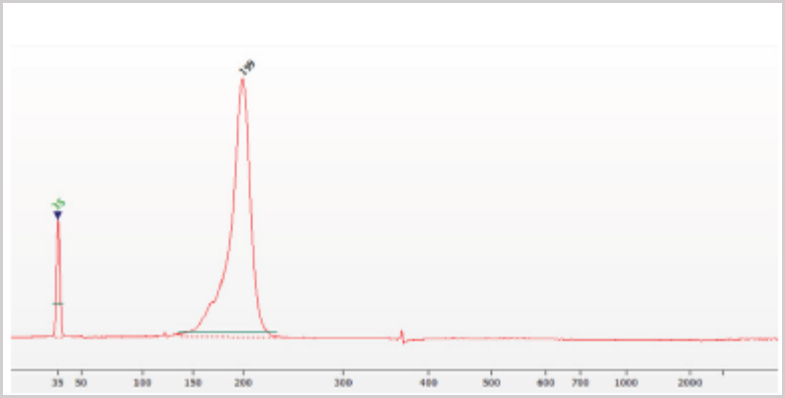
Please note: size selection performed using IMMAGINA PAGExt (catalog no. KGE002/KGE002_12) allows for more stringent selection of libraries than bead-based selection. We recommended to Evaluate each size selected library by Agilent 2100 Bioanalyzer (for more information, see Appendix 2).

APPENDIX 1: Zymo column purification for smRNA (RNA Clean & Concentrator™-5)

- ☐ Perform all steps at room temperature and centrifugation at 10,000-16,000 x g for 30 seconds, unless otherwise specified.
- ☐ Prepare adjusted RNA Binding Buffer (as needed). Mix an equal volume of buffer and ethanol (95-100%). Example: Mix 50 µl buffer and 50 µl ethanol.
- ☐ Add 2 volumes of the adjusted buffer to the sample and mix. Example: Mix 100 µl adjusted buffer and 50 µl sample.
- ☐ Transfer the mixture to the Zymo-Spin™ Column and centrifuge. Save the flow-through: Small RNAs (17-200 nt) are in the flow-through!
- ☐ Add 1 volume ethanol and mix. Example: Add 150 µl ethanol to 150 µl sample. Transfer the mixture to a new column and centrifuge. Discard the flow-through.
- ☐ Add 400 µl RNA Prep Buffer to the column and centrifuge. Discard the flow-through.
- ☐ Add 700 µl RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
- ☐ 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 (not provided).
- ☐ Add DNase/RNase-Free Water (for the elution volume see the specification at each Step) directly to the column matrix and centrifuge.

APPENDIX 2: Library quality check

- ☐ **1.1** Evaluate each size selected library by **Agilent 2100 Bioanalyzer** using the **Agilent High Sensitivity DNA Kit**.
- ☐ **1.2** 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. below). The peaks observed at ~170 and 189 bp comes from adapter dimers (up to 50% is acceptable for sequencing).
- ☐ **1.3** Perform a qPCR analysis using **P5 and P7 primers** on each libraries for high accurate library quantification.



Example electropherogram results LACEseq libraries after PAGE size selection.

Contacts

Info

info@immaginabiotech.com

Sales support (quoting, ordering and order status update)

orders@immaginabiotech.com

Technical service (technical enquiries and quality complaints)

techsupport@immaginabiotech.com

Via Sommarive 18, 38123, TRENTO, ITALY

www.immaginabiotech.com

+39 0461312018

Notes: