LACEseq ribosome profiling kit for Illumina

Product	Catalog no	Rxns.
LACEseq	#LS-001	9

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 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 2000/2500, NextSeq550).

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Reagents provided

Product (label)	Cap Color	Catalog no.	Store Conditions	Quantity
Buffer BPK	Red	#LS001-1	-20°C	100 µL
PK enzyme (PK)	Red	#LS001-2	-20°C	10 µL
ATP	Red	#LS001-3	-20°C	100 µL
Buffer BA	Blue	#LS001-4	-20°C	50 µL
Enzyme Mix A (Mix A)	Blue	#LS001-5	-20°C	10 µL
$MnCl_2$	Blue	#LS001-6	-20°C	30 µL
GTP	Blue	#LS001-7	-20°C	30 µL
Linker MC (1 µM)	Blue	#LS001-8	**-80°C**	20 µL
Buffer BLB	Yellow	#LS001-9	-20°C	30 µL
Enzyme Mix B (Mix B)	Yellow	#LS001-10	-20°C	10 µL
PEG 8000	Yellow	#LS001-11	-20°C	200 µL
RT_T Primer (RT_T)	Green	#LS001-12	-20°C	10 µL
Buffer BRT	Green	#LS001-13	-20°C	50 µL
RT enzyme (RT)	Green	#LS001-14	-20°C	10 µL
dNTPs	Green	#LS001-15	-20°C	10 µL
DTT	Green	#LS001-16	-20°C	15 µL
Amplification mix (AM)	Clear	#LS001-17	-20°C	1.2 mL
Fw PCR1 (F1)	Clear	#LS001-18	-20°C	10 µL
Rev PCR1 (R1)	Clear	#LS001-19	-20°C	10 µL
Control (CTRL)	Clear	#LS001-20	**-80°C**	10 µL

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Reagents and equipment to be supplied by user:

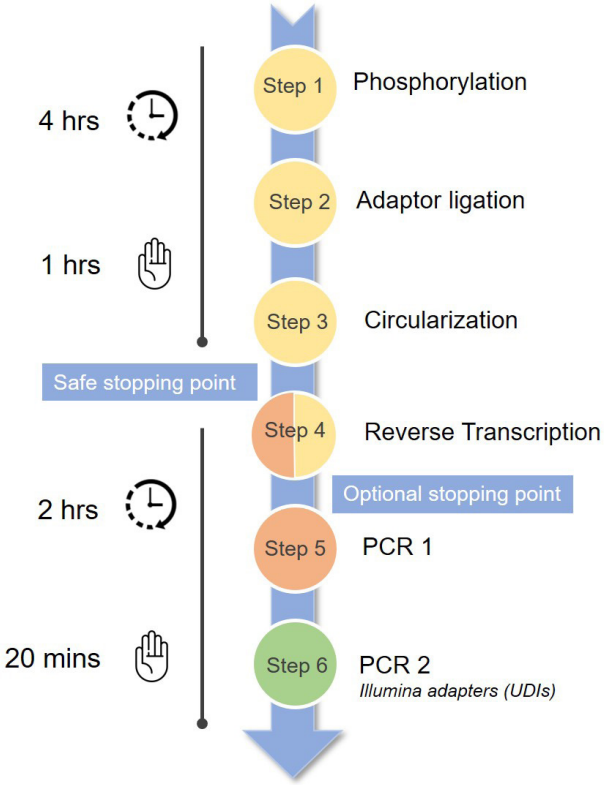
- RNA Clean & Concentrator™-5 (Zymo catalog. no. R1015 & R1016)
- Nuclease-free water
- GlycoBlue (Ambion catalog no. AM9515)
- Isopropanol (Sigma catalog no. 278475)
- Microcentrifuge and nonstick RNase-free microfuge tubes (0.2 mL and 1.5 mL)
- Magnetic stand for 1.5mL tube
- iUDIs plate LACEseq (IMMAGINA catalog no. #LS-UDI-001-16)
- AMPure XP for PCR Purification (Beckman Coulter catalog no. A63881)
- NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel catalog no 740609.10/.50/.250)
- Agilent 2100 Bioanalyzer
- High-Sensitivity DNA chip (Agilent Tech. catalog no. 5067-4626)
- Qubit Fluorometer
- Qubit miRNA Qubit (Thermo, catalog no.Q32880)

Optional:

- PAGExt Gel extraction kit (IMMAGINA catalog no. #KGE-002

Work always in an RNase-free environment.

Workflow



Sample Recommendations

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

If you are using this kit for the first time, we recommend performing a positive control reaction. The positive control (Control, clear cap) 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 fragment.

Step 1
5' phosphorylation

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

Buffer BPK	5µL
ATP	5 µL
PK	1 µL
RPFs	5ng – 40 ng
H_2O	Up to 50 µL

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

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

	RPFs amount			
	5 ng	**10 ng**	**20 ng**	**40 ng**
RNA (from Step 1)	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 1 µM*	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).

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

- Prepare a 1 mM ATP solution by diluting the ATP stock in nuclease-free water (e.g. 1 uL ATP + 9 uL 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)	**8 µL**
Buffer BLB	2 µL
ATP (1mM)	1 µL
PEG8000	8 µL
Enzyme Mix B	1 µL

- Incubate the reaction for 2h at 25 °C in a thermal cycler.
- 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 Transcription

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

Circular RNA (from step 3)	10 µL
dNTPs	1 µL
RT_T Primer	1 µL
H_2O	Up to 14 µL

- Incubate the circular RNA-primer mix at 70°C for 5 minutes and then transfer to ice for at least 1minute.
- Add the following reagents to the annealed RNA:

Buffer BRT	4 µL
DTT	1 µL
RT enzyme	1 µL

- 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

- Combine the following reagents (per reaction) in final volume of 100 µL:

cDNA (from step 4)	20 µL
Amplification Mix	50 µL
F1	0.8 µL
R1	0.8 µL
H_2O	28.4 µL

- 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.

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

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

Step 6
PCR amplification - PCR 2

- Combine the following reagents per reaction (final volume 100 µL):

PCR1	24 µL
Amplification Mix 2x	50 µL
LACEseq UDIs (10 µM)	1 µL
H_2O	25 µL

- 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.

- Use Agencourt XP beads (1.6x ratio) or NucleoSpin Gel and PCR Clean-Up 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 user manual. Elute each sample in 20 µl of nuclease-free water.

Please note: size selection performed using IMMAGINA PAGExt allows for more stringent selection of libraries than bead-based selection. Please refer to Appendix 2 for detailed instructions.

Appendix 1
smRNA Column purification

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.

1. 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.
2. Add 2 volumes of the adjusted buffer to the sample and mix. Example: Mix 100 µl adjusted buffer and 50 µl sample.
3. Transfer the mixture to the Zymo-Spin™ Column and centrifuge. Save the flow-through!
4. 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.
5. Add 400 µl RNA Prep Buffer to the column and centrifuge. Discard the flow-through.
6. Add 700 µl RNA Wash Buffer to the column and centrifuge. Discard the flow-through.
7. Add 400 µl RNA Wash Buffer to the column and centrifuge for 1 minute ensure complete removal of the wash buffer. Carefully, transfer the column into a RNase-free tube (not provided).
8. Add up to 12 µl DNase/RNase-Free Water directly to the column matrix and centrifuge

Appendix 2
PAGE purification

Library PAGE purification with PAGExt kit

To perform library purification, we recommending PAGE purification.
If you are using PAGExt kit IMMAGINA (#KGE-002) follow the protocol below:

1. Prepare samples: add 4 µL of 6x DNA loading dye to 20 µL of cleaned-up PCR;
2. Prepare M2 marker: mix 1 µL M2, 9 µL nuclease-free water and 2 µL of 6x DNA loading dye;
3. Load the samples and marker on a 10% TBE polyacrylamide gel (split the sample total volume into 3 adjacent lanes) and run the gel at 200 V until the bromophenol blue band reaches the bottom of the gel;
4. Size select the fragments at ~ 200 nt according to M2;
5. Place each gel slice in a pierced tube (provided) and nest the pierced small tube inside a 1.5-ml RNase-free Microcentrifuge tube (provided). Spin at maximum speed for 3 min at 4°C. Transfer carefully any remaining gel debris from the pierced tube discard the pierced tube and keep the 1.5 ml microcentrifuge tube;
6. Add 400 µL of DEB (DNA Extraction Buffer), close the vial with the provided cup, incubate the tubes for 1 hour at - 80°C, thaw them at RT and then place the samples on a wheel in slow motion at RT overnight;
7. With a 1 mL cut-tip, transfer the liquid and gel slurry into a spin filter (provided) and spin at 650g for 3 min at 4°C to remove the gel debris. Transfer the eluted solution to a new 1.5 ml tube (not provided);
8. Add 700 µL of Isopropanol and 1.5 µL GlycoBlue to the eluted sample;
9. Store at - 80°C for 2h (fast procedure) or overnight (safe procedure);
10. Thaw the samples on ice, pellet the library by centrifugation (20000g) for 30 min at 4°C and air-dry the pellet;
11. Resuspend the pellet in 11-15 µL TR buffer.
12. Proceed with Library validation and quantification.

Appendix 3
Library quality check

Library quality check and pre-sequencing quantification

Validation is performed to determine if library production was successful.

1. Evaluate the size distribution of each library by running samples on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit.
2. Use the library profile results to determine whether each sample is suitable for further processing. Successful library production should yield a major peak at ~200 bp for Control RNA (figure 1). Small peaks observed in the ~170 bp size range are the result of adapter dimers (figure 1). When calculating expected library molecule sizes for a particular RNA input, use the following formula: 176 + input RNA size (nt) = expected size of library molecules (bp).
3. Perform a qPCR analysis using P5 and P7 primers and Illumina standard for a high accurate library quantification.

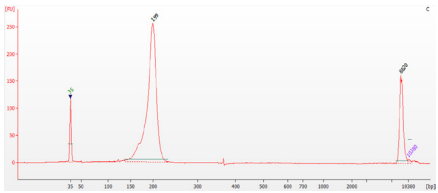


Figure 1. Agilent High Sensitivity DNA Kit. Libraries were generated using LACEseq kit starting from 7 ng of Ribosome Protected Fragment (RPFs).

Related products

Product	Catalog no	Rxns.
RiboLace_Mod.1	#RL001_Mod1	9
PAGExt	#KGE-002	18
iUDIs plate LACEseq	#LS-UDI-001-16	16

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Notes: