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 small RNA bearing the 3'-Phosphate signature. This product is particularly selected for performing libraries from Ribosome Protected Fragments (RPFs) isolated using RiboLace kits (#RL001_XL, #RL001_Mod.1_12) and or other protocols.

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
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™ microRNA 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

Please start the LACEseq library only if you have at least 5 ng of RNAs bearing a 3'-Phospate signature (e.g., at least 5 ng of Ribosomes protected fragments (RPFs) after PAGExt extraction). For quantification of RPFs, you could utilize a Qubit™ microRNA Assay Kit after gel extraction.

If you do not have at least 5 ng of RNA, please contact out specialists at techsupport@immaginabiotech.com before starting the library preparation. The RNA amount you utilize for library preparation is important to tailor specifically the amount of linker to add in Step 3 and the number of PCR cycles to run for Step 5 and Step 6 respectively.

In case you are dealing with RPFs and retrieved less than 5 ng, combine multiple ribosomes' pulldowns for the same sample. 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.

Using the Positive Controls

The kit contains a positive control reaction for library preparation (CTRL, Cat. Nr. #LS001-22) which 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) starting from Step 1 – phosphorylation until Step 6, then please run the obtained library on a gel for visualization, or run it on a bioanalyzer. Two bands of similar quantity should be visible as depicted in the image (Fig.2 page 13).

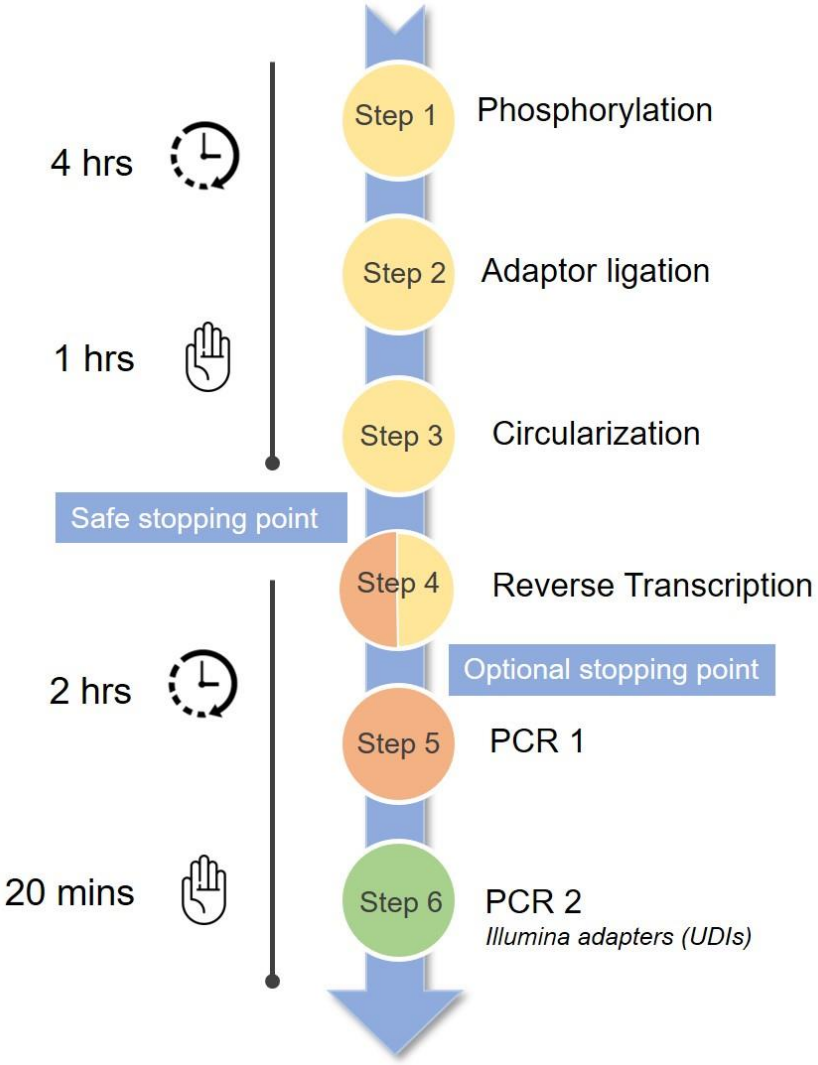

Figure 1. ***Overview of the LACEseq workflow.***

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	#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	1.25 mL	-20°C	Vial	Clear
Fw PCR1 (F1)	#LS001-18	12 µL	-20°C	Vial	Clear
Rev PCR1 (R1)	#LS001-19	12 µL	-20°C	Vial	Clear
Control (CTRL)	#LS001-22	10 µL	-80°C	Vial	Clear

Note:

Input RPFs amount: ≥ 5 ng (quantified by Qubit™ microRNA Assay Kit after gel extraction). In case of less RPFs amount, combine multiple ribosomes' pulldowns for the same sample. The RPFs amount you utilize for library preparation is important to tailor specifically the amount of linker to add in Step 3 and the number of PCR cycles to run for Step 5 and Step 6 respectively. 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.

If you are using this kit for the first time, we recommend performing the positive control reaction. The positive control (CTRL, 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 control (10 ng).

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
RNA with 3'P (e.g., RPF)	5ng – 40 ng
H_2O	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.** Perform all steps at room temperature and centrifugation at 10,000-16,000 g for 30 seconds, unless otherwise specified.

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

☐ **1.5** Add 100 µL adjusted RNA Binding Buffer (from step 1.4) to the sample and mix.

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

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

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

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

☐ **1.10** 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.

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

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

STEP 2. 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.

☐ **2.1** 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 2.1)	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 1µM	0.25 µL	0.5 µL	1 µL	2 µL
H_2O	0.75 µL	0.5 µL	-	-

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

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

☐ **2.4** 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.

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

☐ **2.6** Add the 100 µL adjusted RNA Binding Buffer (from step 2.5) to the sample and mix.

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

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

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

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

☐ **2.11** 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.

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

STEP 3. CIRCULARIZATION

☐ 3.1 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 2.12)	**8 µL**
Buffer BLB	2 µL
ATP (1mM)	1 µL
PEG8000*	8 µL
Enzyme Mix B	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.

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

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

☐ 3.4 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.

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

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

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

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

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

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

☐ 3.11 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.

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

⚠ **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 3.12)	**12 µL**
dNTPs	1 µL
RT_T Primer	1 µL

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

☐ **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

☐ **Note:** the cycles of Step 11 – PCR 1 and Step 12 – PCR 2 depend on the starting RPFs that you used in Step 7. The number of cycles is important for avoiding overamplification, please refer to the following table for the correct number of cycles to utilize.

Table 1 *Number of cycles of PCR to use in Step 5 (PCR1) and 6 (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

☐ **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
H_2O	28.4 µL

☐ **5.2** Place the tube(s) in a thermal cycler with a heated lid and run the following program:

Step	**Temperatu**	**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 1 for correct number of cycles.

☐ **5.3** 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.

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

☐ **5.5** 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.

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

- ☐ **5.7** 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.
- ☐ **5.8** Repeat washing step with 75% ethanol once, keeping the beads on the magnet.
- ☐ **5.9** Let dry the beads pellet 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.**
- ☐ **5.10** 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.
- ☐ **5.11** Place the sample tubes on the magnetic rack for 2 minutes or longer, until the solution is completely clear.
- ☐ **5.12** Transfer the supernatant (about 50 µL) from each tube to a clean tube and proceed with the next step.

STEP 6. PCR AMPLIFICATION – PCR 2

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

PCR1 **(from Step 5.12)**	49 µL
Amplification Mix	50 µL
LACEseq UDIs (10 µM)*	1 µL

*LACEseq UDIs (10 µM) are sold separately. Please ask for them while ordering the LACEseq kit.

☐ **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	∞

*Please refers to Table 1 for correct number of cycles.

☐ **6.3** Purify the PCR reaction by using NucleoSpin Gel and PCR CleanUp kit (or equivalent) follow the PCR clean-up protocol of the manufacture manual. Elute each sample in 20 µl of nuclease-free water.

Please note: the libraries originated contains adaptor dimers together with the library originated from the RPFs. We suggest extract 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. #KGE-002_12) which allows for more stringent selection of libraries than bead-based selection. We recommended to Evaluate each size selected library by Agilent 2100 Bioanalyzer (Step 7).

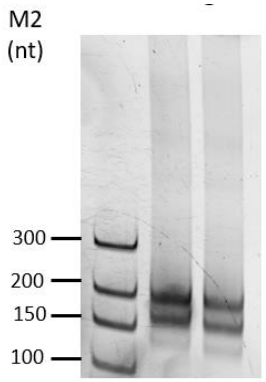

Fig.2 The Library is 200 nt length, while the adaptor dimer is 170 nt.

STEP 7. LIBRARY QUALITY CHECK

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

☐ **7.2** 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 40% of the principal 200 bp peak, you need to purify again the libraries from gel before proceeding with sequencing.

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

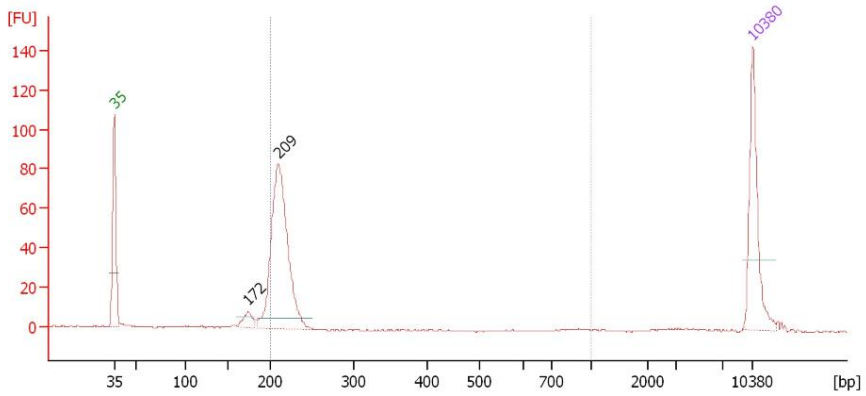

Fig. 3 Example electropherogram results for LaceSeq Set libraries after gel extraction using IMMAGINA PAGE Gel Extraction Kit (cat no. #KGE-002_12). Library was analysed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. The peak at 209 bp corresponds to the size of RPFs, while the peaks at 172 bp correspond with the size of adaptor dimers.

STEP 15. 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 between 100 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 disomes and trisomes, longer reads are required, thus in this case we suggest sequencing 150-200 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 470 pM, while for standard protocol 700 pM. In general, we prefer adding 3.5% quantity of PhiX.

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

For NextSeq 1000/2000 we suggest loading the library at 500 pM, with a 10% spike in of PhiX.

Finally, it is possible to sequence our libraries PE 150 and using only the FW reads as input for data analysis. For this reason, we suggest sequencing deep to retrieve 100 to 120 M forward reads/sample.

Contacts

Info

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