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Enabling complete transcriptome sequencing

SENSE™

Making sense of RNA sequencing

mRNA-Seq Library Prep Kit V2

User Guide

Catalog Numbers:
001 (SENSE mRNA-Seq Library Prep Kit V2 for Illumina)
020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
024 (Automation Module for SENSE mRNA-Seq V2)
025 (SIRVs Spike-in RNA Variant Control Mixes)
047 (i5 Dual Indexing Add-on Kit for QuantSeq/SENSE for Illumina)

001UG004V0320

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ATTENTION: Barcodes rearranged!

001UI094V0200

The SENSE mRNA-Seq Library Prep Kit V2 has been updated!

Major changes of the update:

- **New arrangement and renaming of the barcode plate to improve the nucleotide balance → i7 Index Plate (7001-7096), unique set of barcodes – no overlap with Illumina-specific indices (BC05 removed).** An evaluation tool to check the color balance of index subsets is available on the Lexogen website.
- Barcode 00 (BC00) renamed to P7 Primer 7000.
- qPCR endpoint determination using only 1.7 µl template and set to 50 % of the maximum fluorescence. Subtract 3 cycles from determined cycle number for the endpoint PCR when using 10x as much template.
- Optional dual indexing for up to 384 unique barcode combinations introduced.

For more details please have a look at the Revision History at the end of this User Guide or contact us under support@lexogen.com or +43 1 3451212 41.

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When describing a procedure for publication using this product, please refer to it as Lexogen's SENSE™ mRNA-Seq Kit V2.

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1. Overview

This SENSE mRNA-Seq kit V2 is an all-in-one library preparation protocol designed to generate Illumina-compatible libraries from total RNA in less than 5 hours. The SENSE protocol maintains strand-specificity (>99.9 %) and allows the mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. SENSE includes an integrated poly(A) selection, hence prior rRNA depletion is not required. Insert size can be varied during the library preparation protocol itself, meaning that size selection with additional kits is not necessary. Multiplexing of libraries can be carried out using up to 96 i7 indices. An i5 Dual Indexing Add-on Kit (Cat- No. 047) is also available for multiplexing or unique indexing of up to 384 libraries. Libraries are compatible with both single-read and paired-end sequencing reagents.

The SENSE protocol comprises a highly specific bead-based poly(A) selection step which removes almost all traces of rRNA, tRNA, and non-polyadenylated RNA. Information regarding input RNA requirements can be found in Appendices A and B, p.19 and 21, respectively.

Library production is initiated by the random hybridization of starter/stopper heterodimers to the poly(A) RNA still bound to the magnetic beads. These starter/stopper heterodimers contain Illumina-compatible linker sequences. A single-tube reverse transcription and ligation reaction extends the starter to the next hybridized heterodimer, where the newly-synthesized cDNA insert is ligated to the stopper. As the insert size is determined by the distance between starter/stopper binding sites, RNA fragmentation is not required. Therefore, spurious second strand synthesis from the 5' ends of fragments is absent, providing the basis for the excellent strand-specificity of the SENSE protocol.

Second strand synthesis is performed to release the library from the oligodT beads, and the library is purified using magnetic beads, rendering the protocol highly suitable for automation. In a subsequent PCR amplification the complete sequences required for cluster generation are introduced (see Appendix G, p.29, for a schematic representation of the finished library). Library quantification can be performed with standard protocols and is further discussed in Appendix E, p.25. Libraries are compatible with single-read or paired-end sequencing. Up to 96 i7 indices are included in the kit and are introduced during the PCR amplification step (Appendix F, p.27). Data can be analyzed with a number of standard bioinformatic pipelines. Special considerations for the analysis of SENSE data, such as read orientation, are presented in Appendix H, p.30.

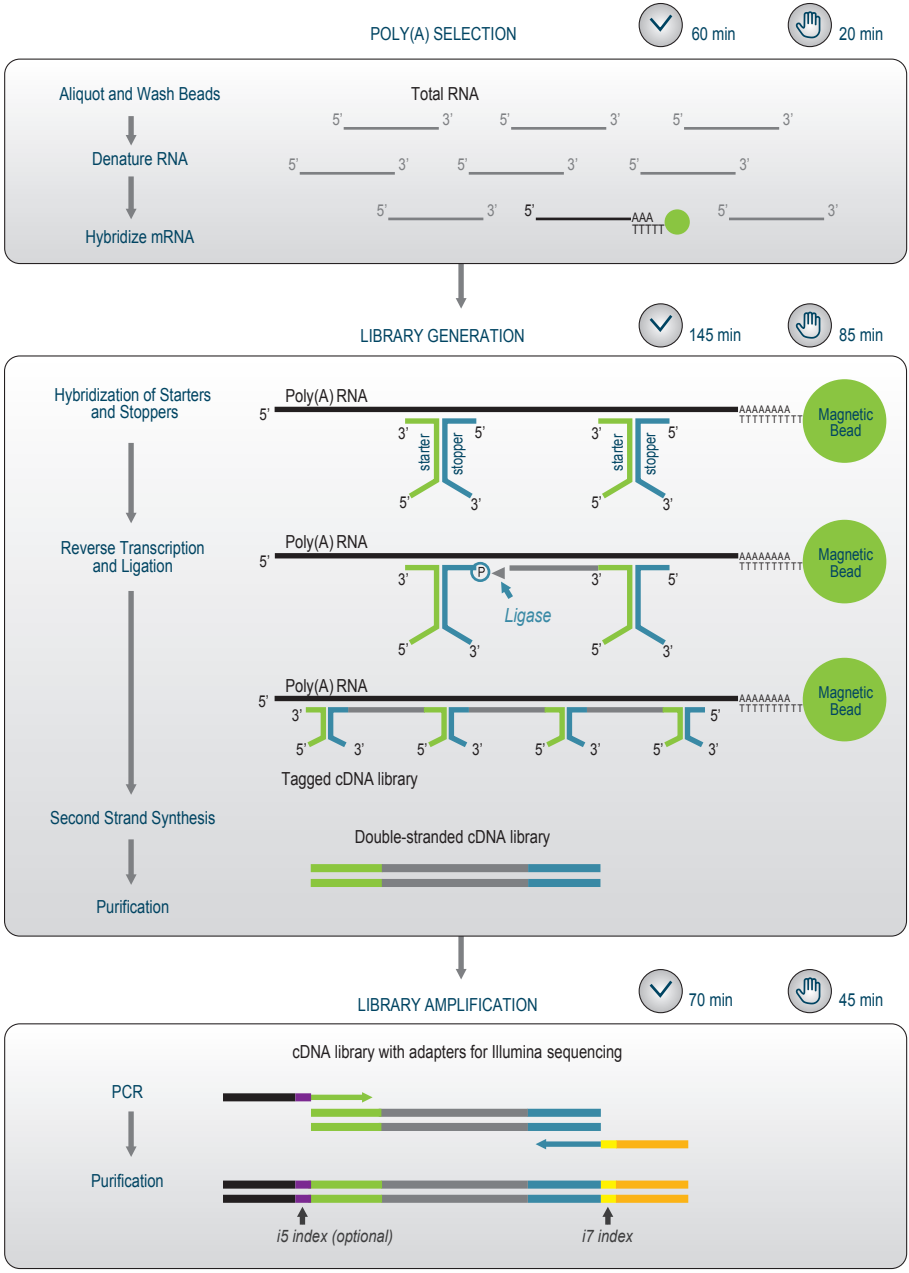

Figure 1. Schematic overview of the SENSE workflow.

2. Kit Components and Storage Conditions

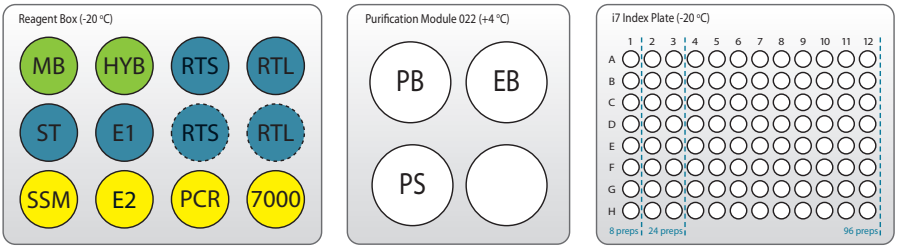

Figure 2. Location of kit contents in a 96 prep kit. For 8 prep and 24 prep kits the purification solutions (PB, PS, EB) and BW are provided in smaller containers and the dotted solutions of the reagent box are missing. i7 indices are provided in a 96-well plate. For 8 prep kits column 1 (up to first dotted line, Indices 7001-7008), for 24 prep kits columns 1-3 (up to the second dotted line, indices 7001-7024) and for 96 prep kits all columns are filled with indices 7001-7096, respectively. For index sequences see p.27.

Kit Component	Tube Label	Volume*			Storage
		8 preps	24 preps	96 preps	
Magnosphere MS150 / OligodT Beads	**MB**	88 µl	264 µl	1,056 µl	-20 °C
RNA Hybridization Buffer	**HYB**	88 µl	264 µl	1,056 µl	-20 °C
Reverse Transcription and Ligation Mix Short	**RTS**	132 µl	396 µl	1,584 µl	-20 °C
Reverse Transcription and Ligation Mix Long	**RTL**	132 µl	396 µl	1,584 µl	-20 °C
Starter/Stopper Mix	**ST**	18 µl	53 µl	212 µl	-20 °C
Enzyme Mix 1	**E1**	27 µl	80 µl	317 µl	-20 °C
Second Strand Synthesis Mix	**SSM**	150 µl	449 µl	1796 µl	-20 °C
Enzyme Mix 2**	**E2**	27 µl	62 µl	220 µl	-20 °C
PCR Mix**	**PCR**	124 µl	247 µl	801 µl	-20 °C
P7 Primer	**7000**	5 µl / reaction (for 8 qPCR rxns)			-20 °C
i7 Index Plate (96-well plate)		5 µl / reaction (included in 96-well plate)			-20 °C
Bead Wash Buffer	**BW**	7,040 µl	21,120 µl	84,480 µl	+4 °C / -20 °C
Purification Module (Cat. No. 022) included in the kit					
Purification Beads	**PB**	388 µl	1162 µl	4647 µl	+4 °C
Purification Solution	**PS**	1,080 µl	3,221 µl	12,884 µl	+4 °C
Elution Buffer**	**EB**	1,232 µl	3,344 µl	12,848 µl	+4 °C / -20 °C

**including additional volume for 8 qPCR reactions

*including 10 % surplus

Upon receiving the SENSE kit, store the Purification Module (Cat. No. 022.96), containing **PB**, **PS**, and **EB** at +4 °C and the rest of the kit in a -20 °C freezer. **REMARK**: **BW** and **EB** can be stored either at +4 °C or -20 °C. Before use, check the contents of **PS** and **BW** which may precipitate during shipping. If a precipitate is visible or the content appears milky, incubate at 37 °C until buffer components dissolve completely.

3. User-supplied Consumables and Equipment

Check to ensure that you have all of the necessary materials and equipment before beginning library preparation. All reagents, equipment, and labware must be free of nucleases and nucleic acid contamination.

Reagents

- 80 % fresh ethanol (washing of Purification Beads, **PB**).
- SYBR Green I (Sigma-Aldrich, Cat. No. S9430), 10,000x in DMSO for qPCR.

Equipment

- Magnetic rack / plate e.g., for 1.5 ml tubes: BcMag Magnetic separator-24, article# MS-03 from Bioclone; for 96-well plates: 96S Super Magnet Plate, article# A001322 from Alpaqua.
- Benchtop centrifuge (rotor compatible with 1.5 ml tubes or 96-well plates).
- Calibrated single-channel pipettes for handling 1 µl to 1,000 µl volumes.
- Thermomixer for 1.5 ml tubes or 96-well plates (dry bath incubator with shaking function).
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

Labware

- Suitable low binding pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml reaction tubes, low binding, certified ribonuclease-free.
- 200 µl PCR tubes or 96-well plates and caps or sealing foil.
- Vortex mixer.

Optional Equipment

- Automated microfluidic electrophoresis station (e.g., Agilent Technologies 2100 Bioanalyzer).
- qPCR machine and library standards (for library quantification).
- Benchtop fluorometer and appropriate assays (for RNA quality control and library quantification).
- Agarose gels, dyes, and electrophoresis rig (for RNA quality control).

The complete set of materials, reagents and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A (p.19) for more information on RNA quality.

Consult Appendix E, p.25 for information on library quantification methods.

4. Guidelines

RNA Handling

- RNases are ubiquitous, and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e., RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination. Well before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions. **ATTENTION:** Do not forget to rinse off any RNaseZap residue with RNase-free water after usage! Residues of RNaseZap may damage the RNA.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Bead Handling

- Beads are stored at +4 °C and must be resuspended before usage. Beads can be resuspended by pipetting up and down several times or by vortexing. When properly resuspended, the solution should have a uniform brown color with no visible clumping on the walls or on the bottom of the tube.
- Beads may stick to certain pipette tips, in which case removing the beads from the inside of the tip may be impossible. Avoid resuspending by repeated pipetting, and instead resuspend by vortexing if this occurs with your tips.
- Beads are superparamagnetic and are collected by placing the plate / tube in a magnetic 96-well plate or a magnetic stand. The time required for complete separation will vary depending on the strength of your magnets, tube thickness, viscosity of the solution, and the proximity of the tube to the magnet. Separation time may need to be adjusted accordingly. When fully separated, the supernatant should be completely clear, and the beads collected at one point or as a ring along the wall of the tube, depending on the magnet that was used.
- To remove the supernatant, the plate / tube containing the beads has to stay in close contact with the magnet. Do not remove the plate / tube from the magnetic stand when re-

moving the supernatant, as the absence of the magnet will cause the beads to go into suspension again.

- In general, beads should not be centrifuged during the protocol. However, should liquid condense (e.g., after step 16) or become entrapped in the cap or drops of fluid stay on the side of the reaction tube, centrifugation at 2,000 x g for 30 seconds should be carried out before placing the tube on the magnetic rack.
- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant but before adding the next reagent. Beads can be resuspended by vortexing, but make sure that beads are not deposited on the tube walls above the level of the liquid, where they can dry during incubation. If necessary, stuck beads can be collected by centrifuging the tube briefly with a benchtop centrifuge.

General

- Always spin down the microtubes (except **PB**) or plates before opening! This prevents cross-contamination and spillage.
- Unless explicitly mentioned, all steps should be carried out at room temperature (RT) between 20 °C and 25 °C. Results may be negatively impacted if the protocol is performed at temperatures outside of this range. While reaction set-up is often performed at RT, incubation temperatures are explicitly defined and must be strictly adhered to.
- To further increase reproducibility, centrifugation should be performed at 18 °C. If a refrigerated centrifuge is not available, centrifugation can be carried out at RT.
- Ensure that adequate volumes of all reagents and the necessary equipment are available and set to the proper temperatures before beginning the protocol.
- Make sure to pre-heat thermomixers (dry bath incubators) well in advance.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips. Pipette carefully to avoid foaming as some solutions contain detergents.
- Thaw all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of each step of the detailed protocol. Mix reagents well by vortexing or pipetting repeatedly, and centrifuge briefly with a benchtop centrifuge to collect contents before use.
- Keep enzyme mixes at -20 °C until immediately before use or store in a -20 °C benchtop cooler.
- Steps requiring a thermocycler have been tested with a maximum ramp speed of 5 °C/sec before denaturation and extension, and 2.5 °C/sec during primer annealing. While these ramp speeds are typical for most modern thermocyclers, some models can exceed these rates, and ramp speed may need to be decreased to ensure efficient annealing. Preheat lid to 105 °C, in case this has to be adjusted manually.

Pipetting and Handling of (Viscous) Solutions

- Enzyme mixes, **RTS** ●, **RTL** ●, and **PS** are viscous solutions which require care to pipette accurately. Quickly spin down the tubes to collect all liquid at the bottom of the tube. Be sure to pipette slowly and check the graduation marks on your pipette tips when removing an aliquot.
- When drawing up liquid, the tip should be dipped 3 to 5 mm below the surface of the liquid, always at a 90 ° degree angle. Do not dip the tip in any further, as viscous solutions tend to stick to the outside of the pipette tip.
- Any residual liquid adhering to the tip should be removed by sliding the tip up the wall or edge of the tube from which the liquid was taken. Spin down the tube afterwards again to ensure that all liquid is collected at the bottom of the tube for further storage.
- When dispensing, the pipette should be held at a 45 degree angle, and the tip placed against the side of the receiving vessel.
- When pipetting liquids from bottles, take special care that only the sterile pipette tip touches the bottle opening to prevent introducing RNases or other contaminants. Tips are sterile whereas the pipette itself is not. If necessary, tilt the bottle to bring the liquid closer to the opening and facilitate pipetting.

Preparation of Mastermixes and Pipetting with Multi-Channel Pipettes

In steps 19 and 35 of the SENSE mRNA-Seq protocol mastermixes of enzymes and reaction buffers can be prepared. When preparing mastermixes and when using multi-channel pipettes, always include a 10 % surplus per reaction in order to have enough solution available for all reactions.

EXAMPLE: Step 35 for 24 preps: use 184.8 µl **PCR** ● (= 7 µl x 24 preps x 1.1)
+ 26.4 µl **E2** ● (= 1 µl x 24 preps x 1.1)
resulting in a total of 211.2 µl, which is well enough for multi-channel pipetting.
All reagents of the SENSE mRNA-Seq V2 kit include a 10 % surplus.

Automation

SENSE mRNA-Seq V2 is compatible with automation. If you are interested in an automated protocol or need help automating the protocol on your NGS workstation, please contact Lexogen (info@lexogen.com).

5. Detailed Protocol

5.1 Poly(A) Selection

Preparation

Aliquot and Wash Beads	Denature RNA	Hybridize mRNA
MB ● – thawed at RT **HYB** ● – thawed at RT **BW** – stored at +4 °C	**Total RNA** – thawed on ice	**BW** – thawed at RT or stored at +4 °C
Magnetic rack / plate	Thermocycler 60 °C, 1 min 25 °C, ∞	Thermomixer set to 25 °C, 1,250 rpm

Aliquot and Wash Beads

SENSE uses Magnosphere MS150/oligodT beads from JSR Life Sciences. The magnetic beads must be washed before use. All steps are performed at room temperature.

1. Mix the beads (**MB** ●) well. Transfer 10 µl of the resuspended beads per library preparation into a new 1.5 ml tube. Beads can be washed as a batch if multiple library preparations are required.
2. Place the tube in a magnetic rack and let the beads collect for 5 minutes or until the supernatant is completely clear (depends on the magnet strength). Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
3. Remove the tube from the magnetic rack and add 200 µl Bead Wash Buffer (**BW**) per library preparation. Resuspend the beads and transfer the tube to the magnetic rack. Let the beads collect for 5 minutes; remove and discard the supernatant.
4. Repeat this washing step once (for a total of two washes).
5. Resuspend the beads in 10 µl RNA Hybridization Buffer (**HYB** ●) per library preparation. Pipette and mix carefully to avoid introducing air bubbles.

Denature RNA

RNA samples are briefly heated to resolve secondary structures and promote efficient hybridization. For information on appropriate amounts of total RNA input as well as RNA quantification and quality control see Appendix A, p.19 and Appendix B, p.21.

6. Dilute 500 ng to 2 µg of total RNA to a volume of 10 µl with RNase-free Water.
7. Denature RNA samples using a thermocycler at 60 °C for 1 minute and then hold at 25 °C. Do not cool samples excessively or place denatured RNA on ice.

Hybridize mRNA

The denatured total RNA is incubated with the washed beads, which specifically bind polyadenylated RNAs. RNAs lacking a poly(A) tail are then washed away, leaving only purified poly(A) RNA hybridized to the beads.

8	Add the 10 µl of denatured RNA to 10 µl of washed beads and incubate using a thermomixer at 25 °C for 20 minutes with 1,250 rpm agitation. **NOTE:** If no thermomixer is available, incubate in a thermocycler with mixing (vortex & spin down) every 10 minutes.
9	Transfer the tube onto a magnetic rack and let the beads collect for 5 minutes (or until the supernatant is clear). Remove and discard the supernatant.
10	Remove the tube from the magnetic rack and add 100 µl Bead Wash Buffer (**BW**). Resuspend the beads and mix well. Incubate using a thermomixer at 25 °C for 5 minutes with 1,250 rpm agitation. Collect the beads by placing the tube onto a magnetic stand for 5 minutes. Remove and discard the supernatant.
11	Repeat this washing step once (for a total of two washes).

5.2 Library Generation

Preparation

Reverse Transcription and Ligation	Second Strand Synthesis	Purification
RTS ● } **thawed on thermomixer,** **RTL** ● } **5 MIN 25 °C, 1,250 RPM** **ST** ● – thawed at RT **E1** ● – keep on ice or at -20 °C **BW** – thawed at RT or stored at +4 °C	**SSM** ● – thawed at RT **E2** ● – keep on ice or at -20 °C	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – thawed at RT or stored at +4 °C
Thermomixer set to 25 °C, 1,250 rpm Magnetic rack / plate	Thermocycler 98 °C, 90 sec 65 °C, 60 sec 72 °C, 5 min 25 °C, ∞	Magnetic rack / plate

Reverse Transcription and Ligation

The Starter/Stopper Heterodimer Mix is hybridized to the RNA, and reverse transcription and ligation is performed, generating short cDNA fragments with linker sequences at either end.

12 After removing the supernatant from the last wash, add 15 µl Reverse Transcription and Ligation Mix **RTS** ● or **RTL** ●. **ATTENTION: RTS** ● is used for sequencing runs of up to 100 nt single-read or 50 nt paired-end; **RTL** ● is used for sequencing runs of 100 nt paired-end and larger. Please also consult Appendix C: Adjusting Library Size, p.23.

13 Add 2 µl Starter/Stopper Mix (**ST** ●). Mix by vortexing.

14 Incubate at 25 °C for 5 minutes using a thermomixer with 1,250 rpm agitation. **REMARK:** For low input RNA (≤50 ng total RNA) extend this incubation to 20 min.

15 Add 3 µl of Enzyme Mix 1 (**E1** ●), mix by vortexing, and incubate at 25 °C for an additional 2 minutes at 1,250 rpm.

16 Raise the temperature on the thermomixer to 37 °C and incubate for one hour with 1,250 rpm agitation. **OPTIONAL:** This step can be extended to 2 hours to increase the yield, e.g., for low input RNA. **REMARK:** At this point we recommend placing the purification components (**PB, PS, EB**) for step 22 at room temperature to give them enough time to equilibrate.

17 Apply 100 µl Bead Wash Buffer (**BW**) to the RT/ligation reaction and mix thoroughly. Collect the beads with a magnetic rack for 5 minutes or until the supernatant is completely clear (depends on the magnet strength). Remove and discard the supernatant.

18 Apply 100 µl **BW** to the beads and resuspend by pipetting or vortexing gently. Collect the beads with a magnetic rack for 5 minutes. Remove and discard the supernatant.

Second Strand Synthesis

During this step the library is converted to dsDNA and is freed from the hybridized RNA by both the hydrolysis of the RNA and the second strand synthesis reaction itself.

19	After removing the supernatant from the second wash, resuspend the beads in 17 µl Second Strand Synthesis Mix (**SSM** ●). Transfer the resuspended beads to a PCR tube or plate. **OPTIONAL:** An **SSM** / **E2** mastermix can be prepared.
20	Add 1 µl Enzyme Mix 2 (**E2** ●) and mix well.
21	Conduct one cycle of thermocycling with the following program: 98 °C for 90 seconds, 65 °C for 60 seconds, 72 °C for 5 minutes, hold at 25 °C. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The double-stranded library is purified using magnetic beads to remove second strand synthesis reaction components. The purification components **PB** and **PS** should equilibrate for 30 minutes at room temperature before use. **PB** may have settled and must be properly resuspended before adding them to the reaction.
ATTENTION: By adding different amounts of the purification components **PB** and **PS** the desired library size can be defined. Please consult Appendix C: Adjusting Library Size, p.23.

22	Add **x** µl Purification Beads (**PB**) and **y** µl Purification Solution (**PS**). For obtaining libraries suitable for PE100 sequencing add 14 µl **PB** and 2 µl **PS**. For other read lengths please see Appendix C, p.23. Mix well by pipetting and vortexing. Incubate for 5 minutes at room temperature.
23	Place the plate onto a magnetic plate and let the beads collect for 5 minutes or until the supernatant is completely clear.
24	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
25	Add 50 µl of Elution Buffer (**EB**), remove the plate from the magnet and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
26	Add 70 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.
27	Place the plate onto a magnetic plate and let the beads collect for 5 minutes or until the supernatant is completely clear.
28	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.

29	Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
30	Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely, as traces of ethanol can inhibit the subsequent PCR reaction.
31	Leave the plate in contact with the magnet and let the beads dry for 5 -10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long (visible cracks appear), as this will negatively influence the elution and hence the resulting library yield.
32	Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
33	Place the plate onto a magnetic plate and let the beads collect for 5 minutes. Transfer 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.
34	**ATTENTION:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, add 2 µl RNase-free water or **EB** to the eluted library. The qPCR option is available for a total of 8 samples. For further details please refer to Appendix D, p.24.

5.3. Library Amplification - Single Indexing (i7 only)

This section describes single indexing PCR (i7 indices only) for multiplexing and unique indexing of up to 96 libraries using the i7 indices included in the kit. Lexogen also offers an i5 Dual Indexing Add-on Kit (Cat. No. 047) for unique indexing of up to 384 libraries. For details, please refer to the i5 Dual Indexing Add-on Kit Instruction Manual (047IM109).

Preparation

PCR		Purification	
PCR ●	– thawed at RT	**PB**	– stored at 4 °C
E2 ●	– keep on ice or at -20 °C	**80 % EtOH**	– provided by user **prepare fresh!**
i7 Index Plate	– thawed at RT; **spin down before opening!**	**EB**	– thawed at RT or stored at 4 °C
		PS	– stored at 4 °C
Thermocycler	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 11 -25x see Appendix C, p.23; for low input RNA see Appendix B, p.21 72 °C, 1 min 10 °C, ∞	Magnetic rack / plate	

PCR

The library is amplified to add the complete adapter sequences required for cluster generation, to add unique indices for multiplexing, and to generate sufficient material for quality control and sequencing.

NOTE: At this point we recommend placing the purification components (**PB, PS, EB**) for step 39 at room temperature to give them enough time to equilibrate.

35 Prepare a mastermix containing 7 µl of PCR Mix (**PCR** ●) and 1 µl Enzyme Mix 2 (**E2** ●) per reaction.

36 Add 8 µl of this **PCR / E2** mastermix to 17 µl of the eluted library.

37 Add 5 µl of the respective i7 index (**7001-7096**, in 96-well plate). Mix well by pipetting. Seal the PCR plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **ATTENTION:** Spin down the i7 index plate before opening! Visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired indices. Avoid cross contamination! Reseal opened wells of the i7 primer plate after usage to prevent cross contamination! **REMARK:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, use 5 µl of the P7 Primer (**7000** ●) per reaction. The qPCR option is available for a total of 8 samples. For further details please refer to Appendix D, p.24.

38 Conduct 11 to 25 cycles of PCR (depending on the amount of input RNA and size selection used) with the following program: Initial denaturation at 98 °C for 30 seconds, 11 to 25 cycles of 98 °C for 10 seconds, 65 °C for 20 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 1 minute, hold at 10 °C.

ATTENTION: Cycle numbers vary depending on the size selection performed in step 22 (see Appendix C, p.23), your input RNA amount (see Appendix B, p.21), and the tissue or organism your RNA was extracted from. Please refer to the tables in Appendix B, p.21 and Appendix C, p.23 and/or also take advantage of the qPCR assay as described in Appendix D, p.24.

☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The finished library is purified from PCR components that can interfere with quantification. The Purification Beads (**PB**) may have settled and must be properly resuspended before adding them to the reaction.

39	Add 30 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **REMARK:** For input RNA amounts ≤50 ng total RNA reduce the amount of **PB** to 27 µl for better removal of unwanted side-products.
40	Place the plate onto a magnetic plate and let the beads collect for 5 minutes or until the supernatant is completely clear.
41	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
42	Add 30 µl of Elution Buffer (**EB**), remove the plate from the magnet and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
43	Add 30 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.
44	Place the plate onto a magnetic plate and let the beads collect for 5 minutes or until the supernatant is completely clear..
45	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
46	Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.
47	Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.
48	Leave the plate in contact with the magnet and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Do not let the beads dry too long (visible cracks appear), as this will negatively influence the elution and hence the resulting library yield.
49	Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet and resuspend the beads properly in **EB**. Incubate for 2 minutes at room temperature.
50	Place the plate onto a magnetic plate and let the beads collect for 5 minutes or until the supernatant is completely clear.
51	Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.
52	At this point, the libraries are finished and ready for quality control (Appendix E, p.25), pooling (for multiplexing see Appendix F, p.27), and cluster generation.

6. Short Procedure

ATTENTION: Spin down solutions before opening tubes or plates!

60 min Poly(A) Selection

☐☐	Wash 10 µl beads (**MB** ●) twice with 200 µl **BW**.	Aliquot and Wash Beads
☐	Resuspend beads with 10 µl **HYB** ●.	
☐	Dilute 500 ng to 2 µg total RNA in a volume of 10 µl e.g., RNAse-free water.	Denature RNA
☐	Incubate for 1 min at 60 °C, hold at 25 °C.	
☐	Add RNA (10 µl) to beads (10 µl).	
☐	Incubate for 20 min at 25 °C / 1,250 rpm.	Hybridize mRNA
☐☐	Wash 2x for 5 min at 25 °C / 1,250 rpm with 100 µl **BW**.	
☐	Withdraw supernatant.	

145 min Library Generation

☐	Add 15 µl **RTS** ● or **RTL** ● (see p.23) and resuspend beads.	Reverse Transcription and Ligation
☐	Add 2 µl **ST** ● and incubate for 5 min at 25 °C / 1,250 rpm (20 min for ≤50 ng RNA).	
☐	Add 3 µl **E1** ● and incubate for 2 min at 25 °C / 1,250 rpm.	
☐	Raise temp. to 37 °C and incubate for 1 h / 1,250 rpm.	
☐☐	Wash twice with 100 µl **BW**.	
☐	Resuspend beads with 17 µl **SSM** ●.	Second Strand Synthesis
☐	Transfer **SSM**/beads mix into a PCR plate or tube and add 1 µl **E2** ●.	
☐	Incubate: 98 °C / 90 sec, 65 °C / 60 sec, 72 °C / 5 min. ☞ Safe stopping point.	
☐	Add .....µl **PB +** .....µl **PS** (see p.23) per rxn, mix well, incubate 5 min.	Purification
☐	Place on magnet for 5 min, remove and discard supernatant.	
☐	Add 50 µl **EB**, mix well, incubate 2 min at RT.	
☐	Add 70 µl **PS**, mix well, incubate 5 min at RT.	
☐	Place on magnet for 5 min, remove and discard supernatant.	
☐☐	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
☐	Air dry beads for 5 - 10 min. **ATTENTION:** Do not let the beads dry too long!	
☐	Add 20 µl **EB**, mix well, incubate 2 min at RT.	
☐	Place on magnet for 5 min, transfer 17 µl of the supernatant into a fresh PCR plate (see p.24 for qPCR). ☞ Safe stopping point.	

70 min Library Amplification

☐	Pre-mix 7 µl **PCR** ● and 1 µl **E2** ● per reaction, mix well.	PCR
☐	Add 8 µl **PCR** / **E2** premix to 17 µl purified cDNA library.	
☐	Add 5 µl i7 primer (**7001-7096**, from the 96-well plate), mix well. **ATTENTION:** Reseal opened index wells after usage!	
☐	PCR: 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 11 - 25x (see p.21 and p.23) 72 °C, 1 min 10 °C, ∞ ☞ Safe stopping point. **ATTENTION:** Increase cycle number for low input RNA and samples with low mRNA content! (see p.21)	
☐	Add 30 µl **PB** (or 27 µl **PB** for ≤50 ng input RNA) per reaction, mix well, incubate 5 min.	Purification
☐	Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐	Add 30 µl **EB**, mix well, incubate 2 min at RT.	
☐	Add 30 µl **PS**, mix well, incubate 5 min at RT.	
☐	Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐☐	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.	
☐	Air dry beads for 5 - 10 min. **ATTENTION:** Do not let the beads dry too long!	
☐	Add 20 µl **EB**, mix well, incubate 2 min at RT.	
☐	Place on magnet for 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate.	

7. Appendix A: General RNA Requirements

Potential Contaminants

RNA samples should be free of salts, metal ions, and organic solvents which can be carried over from RNA extraction. Several sources of contamination can be detected with an UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio should be approximately 2. Several common contaminants including proteins, chaotropic salts, and phenol absorb strongly between 220 and 230 nm and can often be identified as peaks in this region. Contamination with any of these generates a lower A260/230 ratio. Phenol also has an absorption maximum between 250 and 280 nm which overlaps that of nucleic acid, so high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts.

Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of gDNA, which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids more intensively than double-stranded, low to moderate amounts of gDNA may not be readily visible with an RNA-specific microfluidics assay. We highly recommend examining all RNA samples on a denaturing agarose gel or using a fluorometric assay with DNA- and RNA-specific dyes to check samples for DNA contamination. On an agarose gel, gDNA can appear as either a dark mass which remains in the slot if relatively intact, or as a high molecular weight smear if it has been sheared during extraction. SENSE libraries generated from samples containing gDNA may have an increased number of intergenic reads or lower strandedness.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA such as Lexogen's SPLIT RNA Extraction Kit (Cat. No. 008.48). However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. We do not recommend DNase treatment as the extended incubation with divalent cations can lead to RNA hydrolysis and decrease RNA integrity. If samples must be DNase treated, heat inactivation should be avoided, and the enzyme deactivated by other means such as phenol/chloroform extraction or silica column purification.

RNA Integrity

The integrity of an RNA sample can be assessed with a variety of methods. We recommend the use of a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN), and we recommend a RIN score of 8 or greater for optimal sequencing results. Typically such samples have easily detectable

rRNA peaks and a comparatively low abundance of short RNAs, which can arise from both intact short transcripts as well as from RNA degradation. Libraries can also be generated from lower quality RNA, but this may lead to 3'-bias in sequencing results.

Mitochondrial Ribosomal RNA

Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also bind to the oligodT beads and be reverse transcribed and converted into a cDNA library. Optional an rRNA depletion method, which also removes mt-rRNAs, such as Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037), can be used before starting the SENSE library preparation if it is essential to remove mt-rRNA transcripts.

RNA Storage

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen or submerged in RNAlater (Life Technologies Inc.) and stored at -80 °C. After extraction, RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Avoid frequent freeze/thaw cycles as RNA might be sheared.

SIRVs Spike-in RNA Variant Control Mixes

Lexogen offers a set of artificial spike in transcripts called SIRVs (Spike-In RNA Variant Control Mixes, Cat. No. 025.03), which provide for the first time a comprehensive set of transcript variants to validate the performance of isoform-specific RNA-Seq workflows, and to serve as a control and anchor set for the comparison of RNA-Seq experiments. The SIRV sequences enable bioinformatic algorithms to accurately map, assemble, and quantify isoforms, and provide the means for the validation of these algorithms in conjunction with the preceding RNA-Seq pipelines. The SIRVs consist of 69 artificial RNA transcripts with no identity to any known genomic sequences hence they can be spiked into any RNA. Each SIRVs box contains 3 tubes labeled E0, E1, or E2. Each mix contains all 69 SIRV transcripts, but at different molar concentrations to each other. SIRV Mixes can be used as single spike-ins, or as a combination of two or three different SIRV mixes for the assessment of differential gene expression.

ERCC RNA Spike-in Controls

To enable the hypothesis-neutral calculation of strandedness, we highly recommend the addition of artificial transcripts of known strand orientation and concentration such as the ERCC RNA spike-in controls (Ambion Inc.). These sets of RNAs, just as the SIRVs, have a known strand orientation and no (or in case of the SIRVs only known) antisense transcripts, so the calculation of strandedness based on Spike-in sequences is more accurate than calculations based on reads aligning to the genome. ERCC Spike-in transcripts are however monoexonic hence for SENSE we recommend using SIRVs.

8. Appendix B: RNA Input Amount and Quality

High quality mRNA-Seq data relies on high-quality input RNA. The amount of total RNA required for SENSE depends on the poly(A) RNA content of the sample in question. This protocol was tested extensively with various mouse tissues, fungi, plants, yeast, and human reference RNAs (Human Brain Reference RNA (HBRR) and Universal Human Reference RNA (UHRRR)).

Typical inputs of 500 ng total RNA for mRNA-rich tissues (such as kidney, liver, and brain) or 2 µg total RNA for tissues with lower mRNA content (such as lung and heart) generate high quality libraries for single-read 50 nt sequencing (SR50) with 11 cycles of library amplification. For other library sizes PCR cycles need to be adjusted as described in the table of Appendix C, p.23.

RNA inputs down to 1 ng total RNA from mRNA-rich samples such as Universal Human Reference RNA were used for successful library generation with SENSE mRNA-Seq V2. However, reducing the input RNA also requires increasing the number of PCR cycles during the PCR amplification step. Some reference cycle numbers for lower Universal Human Reference RNA inputs can be found on p.22.
ATTENTION: If using 50 ng total RNA input or less, we recommend increasing the **ST** ● hybridization time (step 14) from 5 minutes to 20 minutes and reducing the amount of PB in step 39 to 27 µl. For reduced RNA inputs we also strongly recommend performing a qPCR assay (see Appendix D, p.24) to determine the appropriate cycle number for the endpoint PCR.

The input requirements for your particular experiment may be different, and we have included extra reagents for library amplification and purification to assist with optimization. If RNA input is not sufficient, either due to naturally low poly(A) RNA content or degraded RNA, additional cycles of library amplification may be necessary. However, as additional cycles of library amplification may increase the proportion of PCR duplicates, it is more desirable to increase the amount of input RNA (if possible for your application) rather than to rely on extra PCR cycles to increase library yield.

As a starting point, we recommend performing the protocol initially with 500 ng or 2 µg of total RNA according to the expected poly(A) content. To determine the exact cycle number needed for your endpoint PCRs, you have the option to perform a qPCR for up to 8 samples. Therefore, we recommend diluting the samples you want to check by qPCR by adding 2 µl RNase-free water or **EB** to the 17 µl of your eluted library from step 33 and continue as described in Appendix D, p.24.
Lexogen also offers a PCR Add-on Kit for Illumina, which can be used for additional qPCR assays (Cat. No. 020.96), if you need to determine your exact endpoints for more than 8 samples.

For Universal Human Reference RNA the following table can be used as a reference. Keep in mind that for different RNA inputs the cycle numbers may need to be increased, hence we

strongly recommend taking advantage of the qPCR assay (see Appendix D, p.24).

Universal Human Reference RNA Input	Step 12	Step 14	µl PB and PS Used in Step 22		Step 39 (PB)	Library Yield		PCR Cycles for UHRRR
			PB x	PS y		ng/µl	nM	
500 ng UHRRR	RTS	5 min	14 µl	20 µl	30 µl	2.52	11.97	11
100 ng UHRRR						2.59	12.48	14
50 ng UHRRR		20 min			27 µl	1.58	6.93	16
10 ng UHRRR						1.24	5.60	18
1 ng UHRRR						1.28	3.29	22
500 ng UHRR	RTL	5 min	14 µl	2 µl	30 µl	2.89	10.06	12
100 ng UHRR						1.45	7.55	16
50 ng UHRR		20 min			27 µl	1.62	6.32	17
10 ng UHRR						1.11	4.15	20
1 ng UHRR						1.58	7.08	25

9. Appendix C: Adjusting Library Size

The size of SENSE libraries can be adjusted to the desired sequencing length. This is accomplished by modulating the insert range of the library generated during RT / ligation and by using different size selections during purification.

SENSE is offered with two different Reverse Transcription and Ligation Mixes to be used in step 12 of library generation. As shown in the table below, **RTS** ● will produce libraries with shorter mean insert sizes, while **RTL** ● generates libraries with longer inserts. Additionally, the desired library size can be further fine-tuned by modulating the magnetic bead-based purification in step 22.

Please refer to the table below to see which size selection (volume of **PB**/**PS** to be added) is appropriate for your desired read length.

The required volumes of **PB** and **PS** can be added directly to the sample after second strand synthesis. The µl listed refer to the volumes needed per sample to be purified.

Depending on your selected insert range the number of PCR cycles during library amplification varies slightly (from 11 to 13 cycles). Check the table to see which cycle number is required to obtain >3 nM of library for your selected read length. **ATTENTION:** All reference values shown here refer to 500 ng total RNA starting material (Universal Human Reference RNA, UHRRR). If using less input RNA or RNA with low mRNA content or quaity, further cycles need to be added. In this case we strongly recommend performing the qPCR assay as described in Appendix D, p.24.

Sequencing Length up to	Step 12	µl PB and PS in Step 22		Library*			Insert**				Library Yield		Recommended PCR Cycles
		PB x	PS y	Start [bp]	End [bp]	Mean Size*	Mean Size	>100 nt	>200 nt	>300 nt	ng/µl	nM	
≤PE50	RTS	14 µl	20 µl	150	1500	387	265	90 %	36 %	13 %	2.52	11.97	11
PE100	RTL	14 µl	2 µl	198	2000	535	413	99 %	77 %	44 %	2.89	10.06	12
≥PE150		12 µl	- µl	225	2000	607	485	100 %	87 %	67 %	1.31	3.91	13

*For multiplexed (i7 indexed) libraries. Libraries prepared without indexing (P7 Primer 7000) are 6 bp shorter.
** Mean insert size is the library size minus 122 bp (116 bp adaptor sequences + 6 bp barcode).
PE: Paired-End Sequencing

REMARK: Additional variations of the size selection are possible e.g., using **RTS** ● in step 12 and adding 2 µl **PS** in step 22 which would result in a smaller insert size for a PE100 sequencing run.

The combinations (**RTS**/**RTL**/**PS**) recommended for the different sequencing lengths in the table above were selected to provide a good balance between maximizing the total number of bases sequenced and an even coverage distribution. These settings are optimal for gene expression (counting) applications. If full-length transcript assembly or isoform detection (e.g., splice variants) is important, we recommend using buffer combinations for the next shorter library size. This should provide insert sizes slightly below the chosen read length of the run. While losing some sequencing space by reading into the 3' adapter sequence, the coverage along the transcripts will improve.

9. Appendix C: qPCR and Reamplification

qPCR to Determine the Exact Cycle Number of Your Endpoint PCRs

Dilute the samples you want to check by qPCR by adding 2 µl of Elution Buffer (**EB**) or RNase-free water to the 17 µl of your eluted library from step 33. For determining the cycle number of your endpoint PCR, please use 5 µl of the P7 primer without index (**7000** ● included in this kit for 8 reactions or **7000** ● from the PCR Add-on Kit for Illumina (Cat. No. 020.96)) in step 37 of the protocol. Insert 1.7 µl (of the diluted 19 µl double-stranded library, step 21) into a qPCR reaction. Simply add SYBR Green I (or an equivalent fluorophore) to the PCR reaction to a final concentration of 0.1x. For SYBR Green I use 1.2 µl of a 2.5x SYBR Green I solution (1:4000 SYBR Green I dilution, diluted in DMSO). Fill up the total PCR reaction volume to 30 µl with water or 10 mM Tris, pH8.0. Alternatively, if 8 or more qPCRs are run at the same time, best practice would be to prepare a mastermix with 0.15 µl of a 20x SYBR Green I solution (1:100 SYBR Green I dilution in DMSO) per reaction. SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually. Overcycle this initial qPCR (30 cycles or even more if little input material was used; include a no template control!). Determine the fluorescence value at which the fluorescence reaches the plateau. Calculate where **the fluorescence is at 50 % of the maximum** and determine at which cycle these 50 % of fluorescence are reached. As in the endpoint PCR 10x more cDNA will be used compared to the qPCR, three cycles can be subtracted from the determined cycle number. This is the cycle number you should use for the endpoint PCR using the remaining 17 µl of the template. There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer.

EXAMPLE: 500 ng input RNA was used for generating two libraries. The cDNA of library 1 was diluted by adding 2 µl of **EB.** When inserting 1.7 µl of the cDNA into a qPCR, the cycle number determined in the overcycled qPCR (50 % of the maximum fluorescence) was 14 cycles. 17 µl template (i.e.,10 times more cDNA than in the qPCR, hence -3 cycles) should be amplified with 11 cycles. The undiluted cDNA of library 2 can be amplified with 11 cycles as well.

ATTENTION: The qPCR approach described here is valid regardless if dual or single indexed endpoint PCRs are intended.

Reamplification of i7 Indexed Libraries (i7 only)

Lexogen's PCR Add-on Kit also contains a Reamplification Primer (**RE** ○) that can be used to reamplify single indexed (i7) libraries to get enough material for sequencing if they were undercycled. For details please refer to the PCR Add-on Kit (Cat. No. 020.96) Instruction Manual.

ATTENTION: Do not use **7000** ● for the reamplification of i7 indexed libraries! This will lead to a loss of indices and to a mixed and not assignable sequence pool in the NGS run.

ATTENTION: Do not use the Reamplification Primer (**RE** ○) for a qPCR assay on the cDNA-library as the cDNA lacks binding sites for the Reamplification Primer. **RE** ○ can be only used on i7 indexed, amplified PCR libraries. For reamplification of dual indexed libraries contact Lexogen at info@lexogen.com.

11. Appendix E: Library Quality Control

Quality control of finished SENSE libraries is highly recommended and can be carried out with various methods depending on available equipment. A thorough quality control procedure should include the analysis of both the concentration and the size distribution of libraries.

Quality Control Methods

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become the de facto standard for many NGS laboratories and generates information regarding library concentration and size distribution. Several electrophoresis platforms are available from various manufacturers. For low- to medium-throughput applications, we recommend the Agilent Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies Inc.). Typically, 1 µl of SENSE library produced according to the directions in this manual can be analyzed directly on a High Sensitivity DNA chip. However, samples may need to be diluted to prevent detector saturation if additional PCR cycles were used. For high throughput applications instruments such as the Fragment Analyzer (Advanced Analytical Technologies, Inc.), LabChip GX II (Perkin Elmer) or 2200 TapeStation (Agilent Technologies, Inc.) are recommended. Depending on the minimum sample loading requirements for each instrument, 1 µl of the finished library may be diluted to the required volume (e.g., 2 µl sample for TapeStation and 10 µl for LabChip GX II).

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished SENSE library is calculated by comparing Cq values to a set of known standards. While generating more accurate quantification, these assays do not supply the user with information regarding library size distribution. Unwanted side-products such as linker-linker artifacts are not discernible from the actual library in the qPCR assay as both will be amplified. Hence it is highly recommended to combine such an assay for quantification with microcapillary electrophoresis analysis for library size distribution.

If microcapillary electrophoresis platforms and qPCR machines are not available, very basic quality control can also be performed by separating a small aliquot of the library on a polyacrylamide or agarose gel. Library quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays, e.g., Qubit dsDNA HS assay. Most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify NGS libraries and should be avoided.

Typical Results

SENSE mRNA-Seq V2 kits are provided with 2 different reaction buffers **RTS** ● and **RTL** ● which generate libraries with different size ranges. Additionally, the library size can be varied depend-

ing on the amount of **PS** added in step 22. For a detailed overview regarding library size, insert range, and yield please refer to the table in Appendix C: Adjusting Library Size, p.23. Reducing the RNA input amount requires an increase in PCR cycle numbers to yield enough library for quality control. Please refer to input RNA amount table presented in Appendix B, p.21 for reference values for Universal Human Reference RNA. For other RNAs, cycle numbers and yield may differ hence performing a qPCR assay as described in Appendix D, p.24 is strongly recommended.

Typical concentrations are between 3.29 - 12.48 nM (1.3 - 2.6 ng/µl), which are well suited for cluster generation without further processing. A shorter side-product caused by the direct ligation of starter/stopper heterodimers to one another is sometimes visible at ~135 bp and should compose no more than 0 - 3 % of the total library. Higher proportions of this side-product can indicate problems during library preparation.

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. This might have an impact on library quantification and PCR duplication rate. Performing the qPCR reaction to determine the cycle number of your endpoint PCR as recommended in Appendix D, p.24 should prevent overcycling. Still, even overcycled PCR products can be used for subsequent sequencing reactions without significantly compromising your results. However, for further experiments using the same input RNA please adjust your cycle number accordingly or take advantage of the qPCR option.

Should your i7 indexed libraries be undercycled, you can reamplify them using Lexogen's PCR Add-on Kit for Illumina (Cat. No. 020.96). For more details please refer to Appendix D, p.24 as well as the PCR Add-on Kit Instruction Manual.

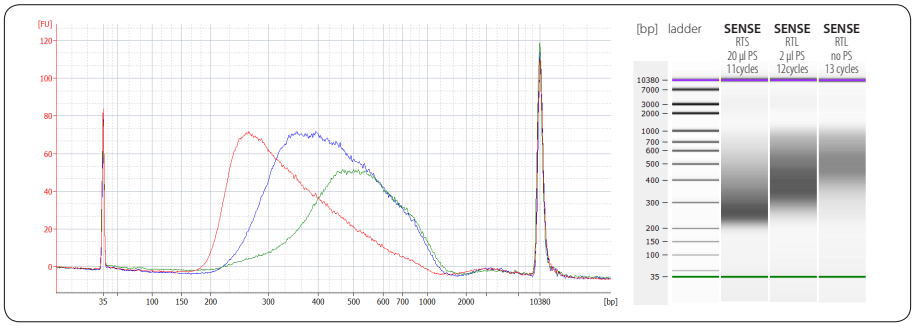

Figure 3. Bioanalyzer traces of RTS (red trace) and RTL (blue and green traces) synthesized SENSE libraries from 500 ng Universal Human Reference RNA (UHRRR) and purified with varying amounts of PS in step 22. Red trace: 14 µl PB and 20 µl PS, dark blue trace: 14 µl PB and 2 µl PS, and green trace: only 12 µl PB and no PS added in step 22.

12. Appendix F: Multiplexing

SENSE libraries can be multiplexed. i7 indices (included in the kit) are introduced during the PCR amplification step (step 37). The i5 Dual Indexing Add-on Kit (Cat. No. 047) allows for up to 384 different indexing combinations. For details on i5 indexing and sample sheet entry, please refer to the i5 Dual Indexing Add-on Kit Instruction Manual (047IM109).

The i5 Dual Indexing Add-on Kit (Cat. No. 047) enables dual indexing (also introduced during the PCR amplification step) and allows for up to 384 different indexing combinations. For details on i5 indexing and sample sheet entry please refer to the i5 Dual Indexing Add-on Kit Instruction Manual.

i7 Indices

i7 indices allowing up to 96 samples to be sequenced per lane on an Illumina flow cell are included in the kit (i7 Index Plate, 96-well plate). i7 indices are 6 nt long and require an additional index-specific sequencing reaction (Index 1 Read).

	1	2	3	4	5	6	7	8	9	10	11	12
A	7001: CAGCGT	7009: TCAGGA	7017: TCTTAA	7025: TTTATG	7033: AGATAG	7041: CTCTCG	7049: GTGCCA	7057: AGTACT	7065: AAGCTC	7073: GACATC	7081: GCAGCC	7089: CGCGGA
B	7002: GATCAC	7010: CGGTTA	7018: GTCAGG	7026: AACGCC	7034: TTGGTA	7042: TGACAC	7050: TCGAGG	7058: ATAAGA	7066: GACGAT	7074: CGATCT	7082: ACTCTT	7090: CCTGCT
C	7003: ACCAGT	7011: TTAACT	7019: ATACTG	7027: CAAGCA	7035: GTTACC	7043: AAGACA	7051: CACTAA	7059: GGTGAG	7067: TCGTTC	7075: CGTCGC	7083: TGCTAT	7091: GCGCTG
D	7004: TGCACG	7012: ATGAAC	7020: TATGTC	7028: GCTCGA	7036: CGCAAC	7044: ACAGAT	7052: GGTATA	7060: TTCCGC	7068: CCAATT	7076: ATGGCG	7084: AAGTGG	7092: GAACCT
E	7005: ACATTA	7013: CCTAAG	7021: GAGTCC	7029: GCGAAT	7037: TGGCGA	7045: TAGGCT	7053: CGCCTG	7061: GAAGTG	7069: AGTTGA	7077: ATTGGT	7085: CTCATA	7093: TTCGAG
F	7006: GTGTAG	7014: AATCCG	7022: GGAGGT	7030: TGGATT	7038: ACCGTG	7046: CTCCAT	7054: AATGAA	7062: CAATGC	7070: AACCGA	7078: GCCACA	7086: CCGACC	7094: AGAATC
G	7007: CTAGTC	7015: GGCTGC	7023: CACACT	7031: ACCTAC	7039: CAACAG	7047: GCATGG	7055: ACAACG	7063: ACGTCT	7071: CAGATG	7079: CATCTA	7087: GGCCAA	7095: AGGCAT
H	7008: TGTGCA	7016: TACCTT	7024: CCGCAA	7032: CGAAGG	7040: GATTGT	7048: AATAGC	7056: ATATCC	7064: CAGGAC	7072: GTAGAA	7080: AACAAG	7088: AGACCA	7096: ACACGC

i7 index sequences are available for download at www.lexogen.com.

In general, we recommend processing a minimum of 8 samples, and using a complete set of eight i7 indices for multiplexing (e.g., **7001-7008**). However, if fewer indices are required care should be taken to always use indices which give a well-balanced signal in both lasers (red and green channels) for each nucleotide position. All columns (1 - 12) and rows (A - H) fulfill these criteria. An evaluation tool to check the color balance of index subsets is available at www.lexogen.com. The individual libraries within a lane should be mixed at an equimolar ratio to ensure this balance.

REMARK: If an 8 nt i7 index (Index 1) needs to be entered into an Illumina sample sheet, e.g.,

if SENSE libraries are multiplexed with 8 nt indexed libraries from other NGS-kit suppliers, add two nucleotides from the Illumina adapter sequence to the 3' end of the index. **EXAMPLE: 7001** would become CAGCGT**AT**, **7002** would become GATCAC**AT** and so on. These additional nucleotides are identical for all indices as they are derived from the Illumina adapter.

Some examples for subsets of indices are listed below.

Two samples per lane: In step 37 use 5 µl of an equimolar mix of **7001-7004** for one sample and 5 µl of an equimolar mix of **7005-7008** for the second. Here four indices are applied to each sample in order to provide a perfect nucleotide balance of the index read-out. Alternatively, two indices can be applied per sample but check the color balance using the evaluation tool at www.lexogen.com. For instance, use 2.5 µl of **7006** and 2.5 µl of **7008** for one sample and 2.5 µl of **7023** and 2.5 µl of **7096** for the second. Here two indices are applied to each sample in order to balance the red and green laser signals.

Four samples per lane: In step 37 use 5 µl of an equimolar mix of **7001-7002** for one sample, 5 µl of an equimolar mix of **7003-7004** for the second, 5 µl of an equimolar mix of **7005-7006** for the third, and 5 µl of an equimolar mix of **7007-7008** for the fourth. Here two indices are applied to each sample in order to provide a perfect nucleotide balance of the index read-out. Alternatively, when using only 1 index per sample we recommend checking the color balance with the evaluation tool provided at www.lexogen.com. Indices **7006**, **7008**, **7023**, and **7096** are examples of four well-balanced indices.

Eight samples per lane: In step 37 use 5 µl of indices **7001-7008** (column 1 of the i7 Index Plate). Apply only one index to each sample.

Twelve samples per lane: In step 37 use indices **7001-7008** (column 1 of the i7 Index Plate) plus indices **7009-7012** (first 4 indices of column 2 of the i7 Index Plate). Apply only one index to each sample.

ATTENTION: Take care to avoid cross contamination! Spin down the i7 Index Plate before carefully removing the seal. Tightly reseal the plate for later use of the remaining indices.

13. Appendix G: Sequencing*

General

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. Each sequencing facility has slightly different preferences of how much to load. From our experience a good starting point is to load between 7 and 14 pM of a SENSE library onto the flow cell. All SENSE libraries can be sequenced using the standard Illumina Multiplexing Read 1, Index Read, and Multiplexing Read 2 Sequencing Primers.
Multiplexing indices (i7) are included in the SENSE mRNA-Seq V2 kits (Cat. No. 001.08, Cat. No. 001.24, and Cat. No. 001.96). A schematic representation of those libraries is shown below.

Libraries with i7 Indexing

i7 indices (6 nt) are introduced during PCR (step 37). Multiplexing indices are included in the SENSE mRNA-Seq V2 kits (Cat. No. 001.08, 001.24, 001.96) and are provided in a 96-well plate (see also Appendix F, p.27).

```
          5'-(Read 1 Sequencing Primer)-3'
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-(Insert…
3'TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA-(Insert…

                 5'-(Index 1 (i7) Sequencing Primer)-3'
   …Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG-i7-TAGAGCATACGGCAGAAGACGAAC 5'
             3'-(Read 2 Sequencing Primer)-5'
```

Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'

Index 1 Read (i7): Multiplexing Index 1 Sequencing Primer (not supplied):
5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'

Read 2: Multiplexing Read 2 Sequencing Primer (not supplied):
5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

ATTENTION: If P7 Primer **7000** ● supplied with the basic kit (Cat. No. 001.08, 001.24, 001.96, for 8 rxns only) or from the PCR Add-on Kit for Illumina (Cat. No. 020.96) is used in step 37 the libraries will not contain an index (i7), and hence cannot be demultiplexed.

*Note: Some nucleotide sequences shown in Appendix G may be copyrighted by Illumina, Inc.

14. Appendix H: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of SENSE NGS data, and is kept as general as possible for integration with your standard pipeline. In contrast to most other library preparation protocols, SENSE libraries generate reads in a strand orientation opposite to the genomic reference. More information about the principal data analysis can be found at www.lexogen.com/sense-data-analysis/.

Processing Raw Reads

We recommend the use of a general fastq quality control tool such as FastQC or NGS QC Toolkit to examine the quality of the sequencing run. These tools can also identify over-represented sequences, which may optionally be removed from the dataset.

In order to reduce bias introduced by the reverse transcriptase and hence to achieve better cluster identification on Illumina platforms, SENSE starters are random nonamer starters.

Demultiplexing

i5 and i7 indices: Demultiplexing can be carried out by the standard Illumina pipeline. 7 index sequences are available for download at www.lexogen.com.

Trimming

As SENSE is based on random priming, there may be a higher proportion of errors at the first nucleotides of the insert due to non-specific hybridization of the starter/stopper heterodimer to the RNA. These mismatches can lead to a lower percentage of mappable reads when using a stringent aligner, in which case it may be beneficial to trim these nucleotides. Trimming can be done with the same work-flow for both reads in a paired-end dataset. Please ensure that the selected tool preserves the read-pair information. The first nine nucleotides need to be removed from Read 1 (starter side), while on the stopper side it is only six nucleotides (Read 2).
While trimming the first nucleotides introduced by the starter/stopper can decrease the number of reads of suitable length, the absolute number of mapping reads usually increases due to the improved read quality. Reads which are too short or have generally low quality scores should be removed from the set.

Alignment

At this point the filtered and trimmed reads can be aligned with a short read aligner to the reference genome or assembled de novo.

Transcriptome Modeling

The resulting alignment files are used to model the transcriptome and assess transcript abundance. Further analyses are experiment-specific and can include differential expression, differential splicing, and promoter usage.

15. Appendix I: Revision History

Revision date / Publication No.	Change	Page
001UG004V0320	Referral to i5 Dual Indexing Add-on Kit (Cat. No. 047) for up to 384 unique indexing options.	4-5, 27
	Update of Figures (Optional Dual Indexing). Kit Contents: i7 Index Plate, BC00 renamed to 7000.	6
	Barcode Plate (BC) was rearranged for improved balance and renamed to i7 Index Plate (7001-7096). Previous BC05: TAATCG replaced by 7025: TTTATG to avoid overlap with Illumina-specific indices.	6, 15, 18, 24, 27
	Rinse with RNAse-free water after RNaseZap usage!	8
	Restructuring of Appendices.	19-31
	qPCR endpoint determination using only 1.7 µl template and set to 50 % FU (previously 33 %). Subtract 3 cycles from determined endpoint (EP) when using 10x as much template (17 µl in EP, 1.7 µl in qPCR).	24
	Evaluation tool to check the color balance of index subsets.	27-28
001UG004V0313	Indication of safe stopping points.	14, 15, 16, 18
	Fixed Typo in Figure 3 legend.	25
001UG004V0312	Pierce or cut open new barcode sealing.	16
001UG004V0311	Typo in short protocol fixed.	18
001UG004V0310	RNAse-free water removed from kit components. New Figure 2.	6
	Increased ST hybridization for lower RNA input at step 14.	13
	Increased RT/Lig time at step 16 for higher yield.	13
	New SSM formulation. Beads resuspension directly in SSM, one step less.	14
	Reduced PB amount in step 39 for RNA inputs lower than 50 ng.	16
	Minimum input RNA amount reduced to 1 ng.	19
	Table for lower UHRR RNA input amounts and required cycle numbers.	20
	Endpoint PCR set at 33 % of the maximum qPCR fluorescence.	20
	Reamplification primer in PCR Add-on Kit.	20, 25
	Spike-in RNA Variant Control Mixes, Cat. No. 025.03.	22
	Section on lower input RNA amounts included. New Figure 3.	25
001UG004V0302	Changes in Figure 1 for easier understanding.	5
	SYBRGreen I recommendation.	7
001UG004V0301	Consistency changes.	
	Increased ST hybridization for lower RNA input. PCR Add-on Kit for more qPCR assays.	19
001UG004V0300	Initial Release SENSE mRNA-Seq V2.	
2012-10-01 / 3	Initial Release SENSE mRNA-Seq.	

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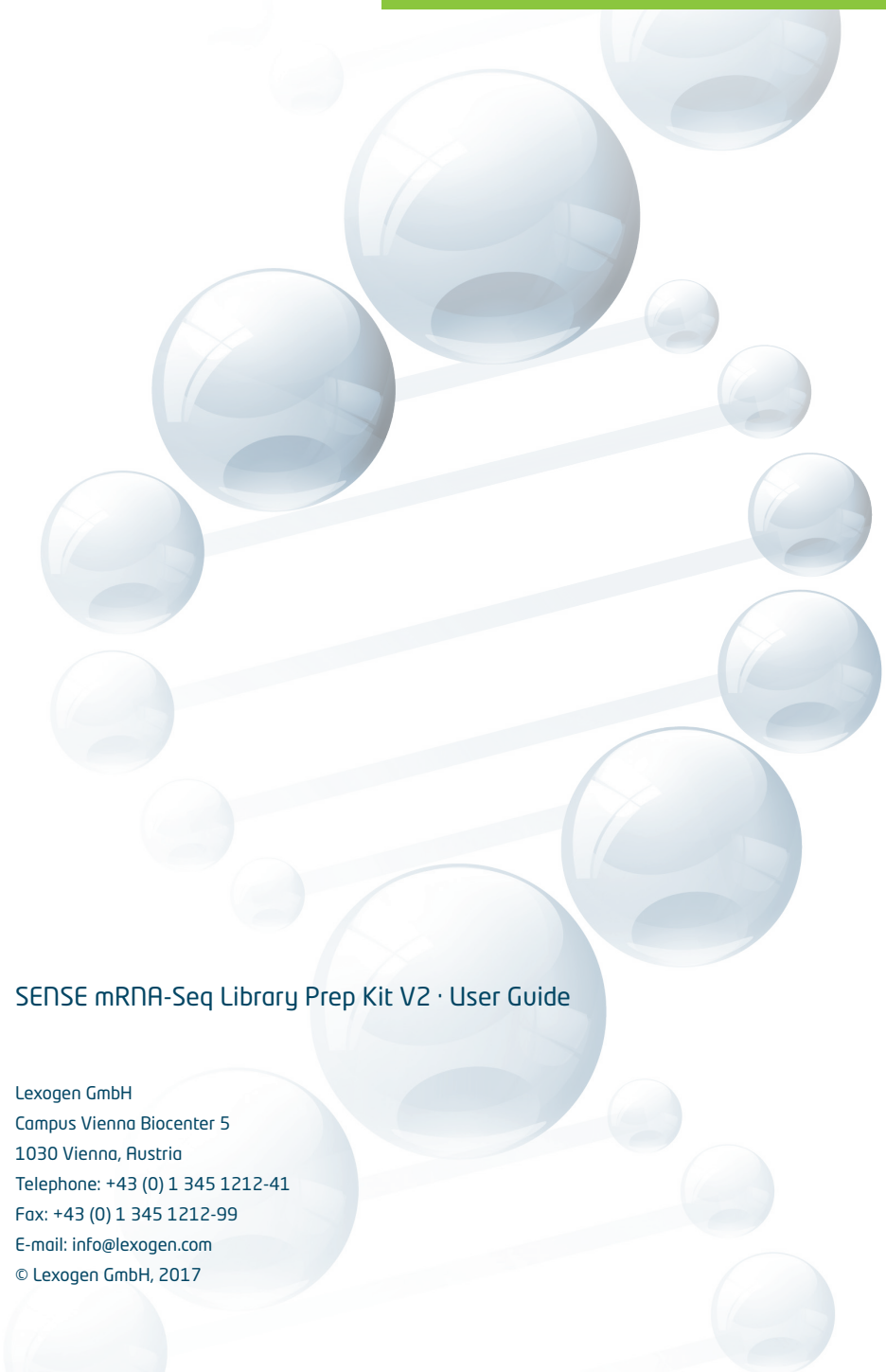

SENSE mRNA-Seq Library Prep Kit V2 · User Guide

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