

The background of the entire page is decorated with a network of light blue lines and numerous translucent blue spheres of varying sizes, creating a molecular or network-like aesthetic.

LEXOGEN

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)

001UG004V0330



Important changes to the SENSE mRNA-Seq Library Prep Kit V2 protocol and User Guide!

- Steps **19** - **20**: Prepare a mastermix of **SSM** and **E2**, and resuspend the beads in the **SSM / E2** mastermix. OPTIONAL: **SSM** and **E2** can be added separately.
- Step **21**: Hold temperature was changed from 25 °C to 4 °C.
- The Short Procedure was updated and reformatted to include low input protocol modifications (p.19 - 20).
- Appendices text and tables were updated, including:
 - Appendix D: qPCR (p.25) - Attention notes added, minor protocol rewording.
 - Appendix G: Multiplexing (p.29 - 31) - New protocols added for lane mix preparation and repurification to remove linker linker artefacts.

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Table of Contents

1. Overview	4
2. Kit Components and Storage Conditions	6
3. User-Supplied Consumables and Equipment	7
4. Guidelines	8
5. Detailed Protocol	11
5.1 Poly(A) Selection	11
5.2 Library Generation	13
5.3 Library Amplification.	16
6. Short Procedure	19
7. Appendix A: General RNA Requirements.	21
8. Appendix B: RNA Input Amount and Quality.	23
9. Appendix C: Adjusting Library Size	24
10. Appendix D: qPCR	25
11. Appendix E: Library Reamplification	26
12. Appendix F: Library Quality Control.	27
13. Appendix G: Multiplexing	29
14. Appendix H: Sequencing	32
15. Appendix I: Data Analysis.	33
16. Appendix J: Revision History	34
17. Notes	35

1. Overview

The 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 mRNA 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.

The SENSE mRNA protocol includes a highly specific bead-based poly(A) selection step which removes almost all traces of rRNA, tRNA, and non-polyadenylated RNA, meaning prior rRNA depletion is not required. Information regarding input RNA requirements can be found in Appendix A, p.21 and Appendix B, p.23, respectively.

Library generation is initiated by the random hybridization of starter/stopper heterodimers to the poly(A) RNA, while it is 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 mRNA protocol. Additionally, insert size can be selectively adjusted during the library preparation protocol itself, meaning that size selection with additional kits is not necessary.

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 i7 and (optional) i5 indices, and complete adapter sequences required for cluster generation are introduced (see also Appendix H, p.32). Library quantification can be performed with standard protocols and is further discussed in Appendix F, p.27.

Multiplexing of libraries can be carried out using up to 96 i7 indices (included in the kit). Additional i5 indices can also be included using the i5 Dual Indexing Add-on Kits (Cat. No. 047). Used together, Lexogen's 96 i7 and 96 i5 indices enable up to 9,216 unique indexing possibilities for sequencing (Appendix G, p.29). SENSE mRNA libraries are compatible with both single-read and paired-end sequencing on all Illumina instruments (Appendix H, p.32). Data can be analyzed with a number of standard bioinformatic pipelines. Special considerations for the analysis of SENSE mRNA data, such as read orientation, are provided in Appendix I, p.33).

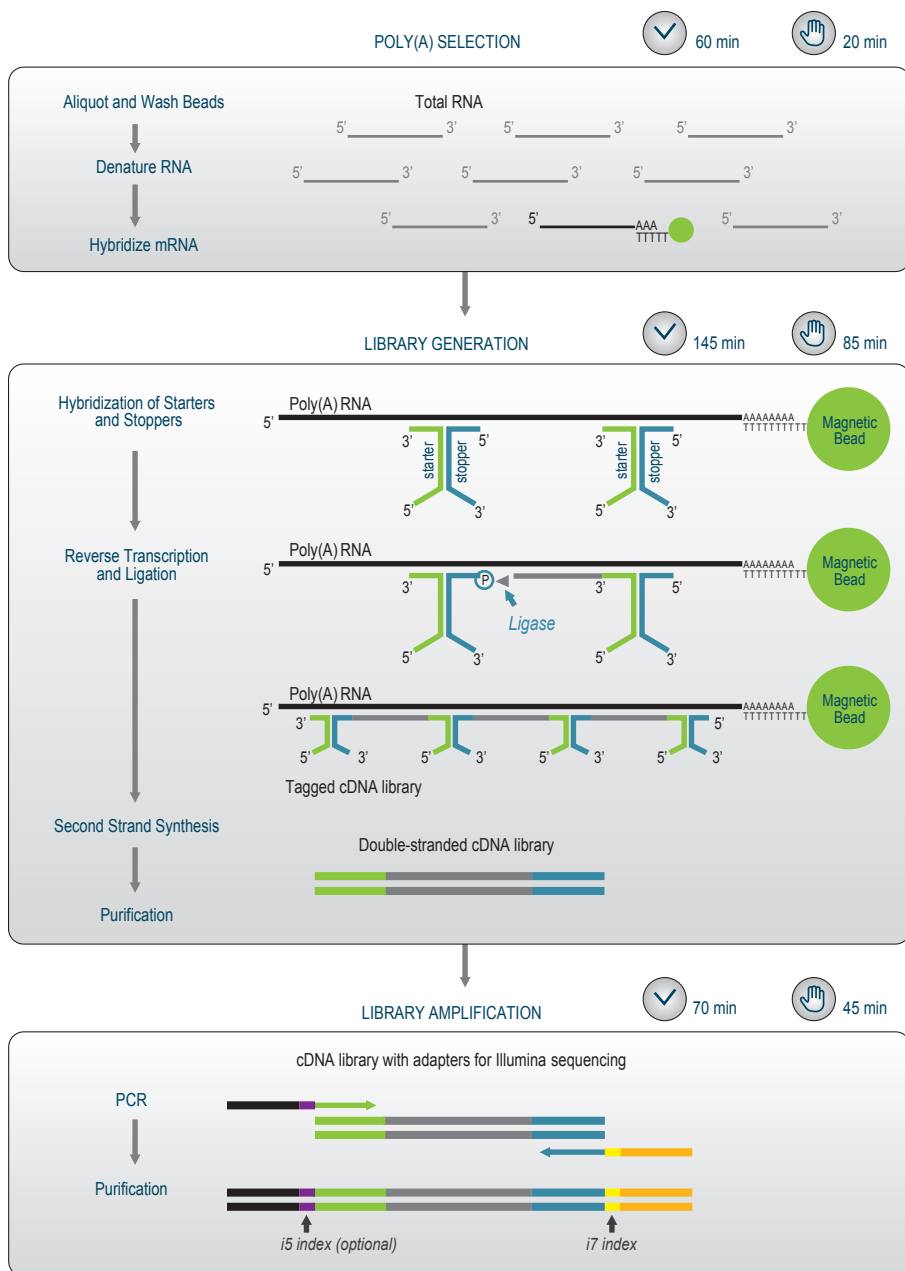


Figure 1. Schematic overview of the SENSE mRNA V2 workflow.

2. Kit Components and Storage Conditions

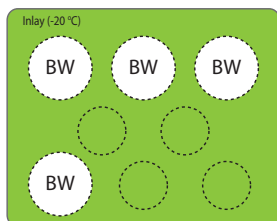
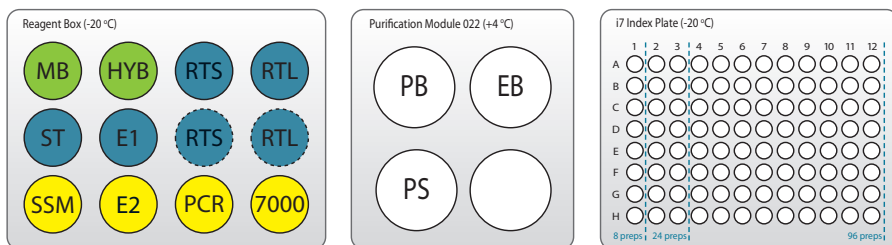


Figure 2. Location of kit contents in a 96 prep kit. For 8 prep and 24 prep kits the purification solutions (PB, PS, EB) and BW are provided in smaller boxes and the dotted solutions of the reagent box are missing. i7 indices are provided in a 96-well plate. For 8 prep kits column 1 (up to first dotted 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.29.

Kit Component	Tube Label	Volume*			Storage
		8 preps	24 preps	96 preps	
Magnosphere MS150 / OligodT Beads	MB ●	88 µl	264 µl	1,056 µl	+4 °C / -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 mRNA-Seq Kit V2, 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:** Store **BW** and **EB** at either +4 °C or -20 °C. Store **MB** at +4 °C after first use. Before use, check the contents of **PS** and **BW** for precipitate. 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 (for washing of Purification Beads, **PB**).
- SYBR Green I nucleic acid stain (Sigma Aldrich, S9430; ThermoFisher S7563), 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 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. See Appendix A, p.21 for more information on RNA quality.

See Appendix F, p.27 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

- Purification Beads **PB** must be stored at +4 °C and must be equilibrated to room temperature and 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.
- Magnosphere MS150 OligodT beads (**MB**) are shipped at -20 °C and can be stored further at -20 °C until use. Thaw at room temperature and vortex well to resuspend in solution. After thawing for first use, store the beads at +4 °C and avoid freeze-thaw cycles.
- 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 removing the supernatant, as the absence of the magnet will cause the beads to go into

suspension again.

- When performing ethanol washes, avoid pipetting ethanol directly over the beads and keep the plate in contact with the magnet to avoid bead loss during washing steps.
- Ensure that all the liquid supernatant is removed and discarded after adding PB and after the second ethanol wash steps for both the pre- and post-PCR Purifications. Use a p10 pipette to remove additional liquid from the bottom of the well / tube.
- 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 on the side of the reaction tube, centrifugation at 2,000x 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, or hydrated, 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 Purification Beads (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. Pre-heat 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 ° 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 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 Kit V2 include a 10 % surplus.

Automation

The SENSE mRNA-Seq Kit 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 (support@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 RNAse-Free Water - user-provided	BW – thawed at RT or stored at +4 °C
	Thermocycler 60 °C, 1 min 25 °C, ∞	Thermomixer set to 25 °C, 1,250 rpm

Aliquot and Wash Beads

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

ATTENTION: Do not let the beads dry out between washing steps. After removing the supernatant, immediately add the next volume of Bead Wash Buffer (**BW**).

NOTE: Beads can be washed as a batch in a single tube if multiple library preparations are required. We recommend using a maximum of 50 µl beads per 1.5 ml tube, to ensure Bead Wash Buffer volumes do not exceed the tube capacity.

- 1 Mix the beads (**MB** ●) well. Transfer 10 µl of the resuspended beads per library preparation into a new 1.5 ml tube.
- 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.21 and Appendix B, p.23.

- 6 In a PCR plate or tubes, prepare 500 ng - 2 µg of total RNA in a total volume of 10 µl using RNase-free Water.

- 7 Denature the RNA in a thermocycler, for 1 minute at 60 °C, moved dthen hold at 25 °C.
ATTENTION: Do not place denatured RNA on ice or cool samples below room temperature.

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.

ATTENTION: Do not let the beads dry out between washing steps. After removing the supernatant, immediately add the next volume of Bead Wash Buffer (**BW**), or Reverse Transcription and Ligation Mix (**RTS** ● or **RTL** ●) after step 11.

- 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. **REMARK:** 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: 25 °C, 1,250 rpm; 37 °C, 1,250 rpm;	Thermocycler 98 °C, 90 sec 65 °C, 60 sec 72 °C, 5 min 4 °C, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use.

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.

ATTENTION: Two Reverse Transcription and Ligation Mixes are provided: **RTS ●** is used for libraries intended for SR100 or PE50 sequencing; **RTL ●** is intended for ≥PE100 sequencing. See Appendix C, p.24 for further details.

12 After removing the supernatant from the last wash, add 15 µl Reverse Transcription and Ligation Mix **RTS ●** or **RTL ●**.

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

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

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

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**, and **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 and oligodT beads, by both the hydrolysis of the RNA and the second strand synthesis reaction itself.

- 19 Prepare a mastermix of 17 μ l Second Strand Synthesis Mix (**SSM** ●) and 1 μ l Enzyme Mix 2 (**E2** ●) per sample (include 10 % excess).

- 20 After removing the supernatant from the second wash, resuspend the beads in 18 μ l of **SSM/E2** mastermix, then transfer the resuspended beads to a PCR tube or plate.
OPTIONAL: Resuspend the beads in 17 μ l Second Strand Synthesis Mix (**SSM** ●), then add 1 μ l Enzyme Mix 2 (**E2** ●) and mix well.

- 21 Conduct one cycle in a thermocycler with the following program: 98 °C for 90 seconds, 65 °C for 60 seconds, 72 °C for 5 minutes, shortly hold at 4 °C or store at -20 °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. By adding different amounts of the purification components **PB** and **PS** the desired library size can be defined. Please consult Appendix C, p.24.

ATTENTION: Ensure the samples from step 21 are equilibrated to room temperature before beginning the purification. The purification components **PB** and **PS** should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB**) must be fully resuspended before use.

- 22 Add **x** μ l Purification Beads (**PB**) and **y** μ l Purification Solution (**PS**). Mix well by pipetting and vortexing. Incubate for 5 minutes at room temperature. **REMARK:** For obtaining libraries suitable for PE100 sequencing add 14 μ l **PB** and 2 μ l **PS**. For other read lengths see Appendix C, p.24.

- 23 Place the plate onto a magnet and let the beads collect for 2 - 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. Do not disturb the beads.

- 25 Add 50 μ l 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 μ l 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 magnet, and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

- 28 Remove and discard the clear supernatant without removing the PCR plate from the magnet. Do not disturb the beads.

- 29 Add 120 μ l 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. Remove the supernatant completely.
-
- 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:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
-
- 32 Add 20 μ l of Elution Buffer (**EB**) per well, remove the plate from the magnet and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
-
- 33 Place the plate onto a magnet and let the beads collect for 2 - 5 minutes.
-
- 34 Transfer 17 μ l of the supernatant into a fresh PCR plate.  Safe stopping point. Libraries can be stored at -20 °C at this point. **ATTENTION:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, add 2 μ l RNase-free water or **EB** to the eluted library. The qPCR option is available for a total of 8 samples. For further details see Appendix D, p.25.
-

5.3. Library Amplification - Single Indexing (i7 only)

This section describes single indexing PCR (i7 indices only) using the i7 indices included in the kit. Alternatively the Lexogen i5 6 nt Dual Indexing Add-on Kits (Cat. No. 047) can be used for preparing dual-indexed 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	PS – stored at 4 °C
i7 6 nt Index Set	– thawed at RT; spin down before opening!	80 % EtOH – provided by user prepare fresh!
		EB – thawed at RT or stored at 4 °C
Thermocycler	98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use.
	} 11 -25x see Appendix D, p.25	

PCR


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

Cycle numbers vary depending on the size selection performed in step 22 (see Appendix C, p.24), input RNA amount, and the tissue or organism your RNA was extracted from. Before proceeding to library amplification, **we strongly recommend performing a qPCR Assay to determine the optimal number of PCR cycles to use for the Endpoint PCR**. For details please see Appendix D, p.25.

ATTENTION: Important notes for Library Amplification.

- Avoid cross contamination when using the Lexogen i7 6 nt Index Set. Spin down the plate before opening and visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired indices only. Reseal opened wells after use.
- Do not add the P7 Primer (7000 ●) to the Endpoint PCR. Primer 7000 ● should only be used for the qPCR Assay to determine the exact cycle number for the Endpoint PCR (see Appendix D, p.25).

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

- 35 Prepare a mastermix containing 7 μ l of PCR Mix (**PCR** ●) and 1 μ l Enzyme Mix 2 (**E2** ●) per reaction.
-
- 36 Add 8 μ l of the **PCR / E2** mastermix to 17 μ l of the eluted library.
-
- 37 Add 5 μ l 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:** Reseal opened wells after usage to prevent cross contamination!
-
- 38 Conduct 11 to 25 cycles of PCR (as determined by qPCR, see Appendix D, p.25) 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.  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**) must be fully resuspended before use.

ATTENTION: If the libraries were stored at -20 °C, ensure that they are thawed and equilibrated to room temperature before restarting the protocol.

- 39 Add 30 µl of Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature. **REMARK:** For ≤50 ng total RNA input add 27 µl of **PB**.
- 40 Place the plate onto a magnet and let the beads collect for 2 - 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 fully 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 magnet and let the beads collect for 2 - 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. Do not disturb the beads.
- 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. 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:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and the resulting library yield.
- 49 Add 20 µl of Elution Buffer (**EB**) per well, remove the plate from the magnet and resuspend the beads fully 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, pooling, and sequencing.  Safe stopping point. Libraries can be stored at -20 °C at this point.

6. Short Procedure

1 hr Poly(A) Selection

Aliquot and Wash Beads	
<input type="checkbox"/>	Wash 10 µl beads (MB ●) 2x with 200 µl BW .
<input type="checkbox"/>	Resuspend beads with 10 µl HYB ●.
Denature RNA	
<input type="checkbox"/>	Prepare 500 ng - 2 µg total RNA in a volume of 10 µl.
<input type="checkbox"/>	Incubate for 1 min at 60 °C, hold at 25 °C.
<input type="checkbox"/>	Add RNA (10 µl) to beads (10 µl).
Hybridize mRNA	
<input type="checkbox"/>	Incubate for 20 min at 25 °C at 1,250 rpm.
<input type="checkbox"/>	Wash 2x with 100 µl BW for 5 min at 25 °C / 1,250 rpm.
<input type="checkbox"/>	Withdraw supernatant.

2.4 hr Library Generation

Standard Input	Low Input (≤50 ng)
Reverse Transcription and Ligation	
<input type="checkbox"/>	Add 15 µl RTS ● or RTL ● (see p.24) and resuspend beads.
<input type="checkbox"/>	Add 2 µl ST ● and incubate for 5 min at 25 °C / 1,250 rpm.
<input type="checkbox"/>	Add 3 µl E1 ● and incubate for 2 min at 25 °C / 1,250 rpm.
<input type="checkbox"/>	Raise temp. to 37 °C and incubate for 1 h / 1,250 rpm.
<input type="checkbox"/>	<input type="checkbox"/> OPTIONAL: Raise temp. to 37 °C and incubate for 2 h / 1,250 rpm.
<input type="checkbox"/>	Wash 2x with 100 µl BW for 5 min at 25 °C.
Second Strand Synthesis	
<input type="checkbox"/>	Prepare mastermix of 17 µl SSM ● and 1 µl E2 ● per sample, mix well.
<input type="checkbox"/>	Resuspend beads in 18 µl SSM / E2 mastermix.
<input type="checkbox"/>	Incubate: 98 °C / 90 sec, 65 °C / 60 sec, 72 °C / 5 min, 4 °C / ∞ . ⚡ Safe stopping point.
Purification	
<input type="checkbox"/>	Addµl PB +µl PS (see p.24) per rxn, mix well, incubate 5 min at RT.
<input type="checkbox"/>	Place on magnet for 5 min, discard supernatant.
<input type="checkbox"/>	Add 50 µl EB , mix well, incubate 2 min at RT.
<input type="checkbox"/>	Add 70 µl PS , mix well, incubate 5 min at RT.
<input type="checkbox"/>	Place on magnet for 5 min, discard supernatant.
<input type="checkbox"/>	Rinse the beads twice with 120 µl 80 % EtOH, 30 sec.
<input type="checkbox"/>	Air dry beads for 5 - 10 min. ATTENTION: Do not let the beads dry too long!
<input type="checkbox"/>	Add 20 µl EB , mix well, incubate 2 min at RT.
<input type="checkbox"/>	Place on magnet for 5 min, transfer 17 µl of supernatant to a fresh PCR plate. ⚡ Safe stopping point.

ATTENTION: Spin down solutions before opening tubes or plates!

qPCR [Strongly Recommended! Requires PCR Add-on Kit (Cat. No. 020.96)]

- ☐ Add 2 µl of **EB** to the 17 µl of eluted cDNA.
- ☐ Prepare a 2.5x stock of SYBR Green I nucleic acid stain (i.e., 1:4,000 dilution in DMSO; use Sigma-Aldrich, Cat. No. S9430; or ThermoFisher S7563).
- ☐ Combine 1.7 µl of cDNA with: 7 µl **PCR** ●, 5 µl Primer **7000** ●, 1 µl **E** ● (from PCR Add-on Kit), 1.2 µl of 2.5x SYBR Green I nucleic acid stain, and 14.1 µl of **EB**, per reaction. Mix well.
- ☐ PCR: 98 °C, 30 sec

98 °C, 10 sec	}	35x (see p.25)
65 °C, 20 sec		
72 °C, 30 sec		
72 °C, 1 min		
10 °C, ∞	Calculate the optimal cycle number for Endpoint PCR (see Appendix D, p.25)	

1.2 hr Library Amplification

PCR

- ☐ Prepare a mastermix with 7 µl **PCR** ● and 1 µl **E2** ● per reaction, mix well.
- ☐ Add 8 µl **PCR** / **E2** mastermix to 17 µl purified cDNA library.
- ☐ Add 5 µl i7 primer (**7001-7096**, from the 96-well plate) to each reaction, mix well.
ATTENTION: Reseal opened index wells after use!
- ☐ PCR: 98 °C, 30 sec

98 °C, 10 sec	}	11 - 25x (see p.25)	ATTENTION: Higher cycle numbers may be needed for low input / low mRNA content samples.
65 °C, 20 sec			
72 °C, 30 sec			
72 °C, 1 min			
10 °C, ∞	Safe stopping point.		

Standard Input

Purification

- ☐ Add 30 µl **PB** per reaction, mix well, incubate 5 min at RT.
- ☐ Place on magnet for 2 - 5 min, 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, 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 supernatant to a fresh PCR plate. ⚡ Safe stopping point.

Low Input (≤50 ng)

- ☐ Add 27 µl **PB** per reaction, mix well, incubate 5 min at RT.

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 a 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 has an additional 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 genomic DNA (gDNA), which is indistinguishable from RNA on a spectrophotometer. Please note that 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. In general the poly(A) selection step of SENSE mRNA will also remove residual gDNA, as this is unlikely to bind to oligodT beads and is therefore eliminated during the bead washing steps.

To avoid gDNA contamination at all, use an RNA extraction protocol that does not co-isolate gDNA (e.g., Lexogen's SPLIT RNA Extraction Kit, Cat. No. 008). However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase digestion. In general 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 should be deactivated by other means such as phenol / chloroform extraction or silica column purification.

RNA Integrity

We recommend evaluating RNA integrity using a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies, Inc.). 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). For SENSE mRNA we recommend using high quality RNA input (RIN \geq 8).

Mitochondrial Ribosomal RNA

Mitochondrial ribosomal RNAs (mt-rRNAs) are polyadenylated and hence will also be captured during poly(A) selection. Typically, mt-rRNAs can make up 1 - 2 % of the reads when an mRNA-Seq protocol is used. Optionally, 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 mRNA library preparation protocol, if it is essential that all mt-rRNA transcripts are removed.

RNA Storage

After extraction, RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Addition of RNasin or an equivalent RNase inhibitor is recommended. Avoid frequent freeze / thaw cycles as RNA might be sheared.

SIRVs Spike-in RNA Variant Control Mixes

The Lexogen SIRV™ (Spike-In RNA Variant) controls are artificial spike in transcripts that serve as a control and anchor set for the comparison of RNA-Seq experiments. The SIRVs consist of 69 artificial RNA transcripts with no identity to any known genomic sequences, hence they can be spiked into any RNA. SIRVs are available in three sets, SIRV-Set 1 (Cat. No 025) contains the Isoform Mixes E0, E1, and E2 of the isoform module, SIRV-Set 2 (Cat. No. 050) provides the Isoform Mix E0 only, whereas SIRV-Set 3 (Cat. No. 051) has the SIRV Isoform Mix E0 in a mixture with the ERCC RNA Spike-in controls (Thermo Fisher Scientific Inc., see below). The SIRVs are polyadenylated mRNAs and therefore are efficiently captured for SENSE mRNA library generation. SIRV-Set 1 contains 3 tubes labeled E0, E1, or E2, each of which contain the same 69 SIRVs but at different molar concentrations to each other. These SIRV Mixes can be used as single spike-ins, or by spiking different mixes into different sample types, 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.). ERCCs 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 mRNA we recommend using SIRVs, or SIRV-Set 3 (Cat. No. 051), which contains ERCCs together with the SIRV isoform controls.

8. Appendix B: RNA Input Amount and Quality

High quality mRNA-Seq data relies on high-quality input RNA. We recommend using only high quality total RNA for SENSE mRNA (RIN ≥ 8). Lower quality RNA can be used as input but will likely require adjustment of PCR cycles for endpoint, and can result in 3' biased sequencing data.

The recommended input range is 1 ng - 2 μ g of total RNA. However, the ideal input amount 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 (UHRR)).

We recommend where possible to use a starting input amount of 500 ng total RNA. When using lower input amounts ≤ 50 ng total RNA, protocol modifications are advised (see below).

ATTENTION: Input amounts > 10 ng are advised for preparation of libraries with the longest size selection option (i.e., \geq PE150) using Reverse Transcription and Ligation Mix **RTL** (see Appendix C, p.24). Inputs ≤ 10 ng generate lead to excess linker-linker formation, which can affect optimal library amplification. Linker-linker peaks however, can be removed via additional purification if required (see Appendix G, p.29).

Low Input Protocol Modifications

For total RNA input amounts of 50 ng or less, we recommend the following protocol modifications:

- Step 14: Increase the **ST** hybridization time from 5 minutes to 20 minutes
- Step 16: **OPTIONAL:** Extend the incubation time for reverse transcription to 2 hours.
- Step 34: Perform a qPCR Assay to determine optimal cycle numbers, before performing Endpoint PCR (see Appendix D, p.25).
- Step 39: Add 27 μ l of **PB** (instead of 30 μ l) for final library purification.

NOTE: PCR cycles need to be adjusted depending on the input amount and sample type, as mRNA content varies for different species, tissue, and cell types. The PCR cycle number can also vary depending on the size selection option chosen (see Appendix C, p.24). Typically, longer libraries require an additional 1 - 2 PCR cycles. Optimal PCR cycle numbers can be evaluated by qPCR Assay, as described in Appendix D, p.25.

9. Appendix C: Adjusting Library Size

SENSE mRNA library prep includes three size selection options. Size selection is accomplished by modulating the insert range of the library generated during reverse transcription / ligation and by using different purification conditions.

For the reverse transcription / ligation at step 12, two different Reverse Transcription and Ligation Mixes are provided: **RTS** will produce libraries with shorter mean insert sizes, while **RTL** generates libraries with longer inserts. Additionally, the desired library size can be further modified during magnetic bead-based purification.

For purification, size selection is modified by altering the volume ratio of **PB:PS** added at step 22. The required volumes of **PB** and **PS** per sample are listed in the table below and should be added directly to the sample after second strand synthesis.

ATTENTION: All reference values shown here refer to 500 ng total RNA starting material of UHRR. RNA from different tissues and species, using less input RNA, or samples with low mRNA content or quality may alter the length profile. Libraries prepared with **RTL** (i.e., for longer size selection) typically require 1 -2 additional PCR cycles for final amplification. **We strongly recommend performing a qPCR assay** to determine the optimal number of cycles for Endpoint PCR (see Appendix D, p.25).

Sequencing Length up to	Step 12	Vol. PB and PS in Step 22 (µl)		Library*			Insert**			
		PB x	PS y	Start [bp]	End [bp]	Mean Size*	Mean Size	>100 nt	>200 nt	>300 nt
≤PE50	RTS	14 µl	20 µl	150	1500	387	265	90 %	36 %	13 %
PE100	RTL	14 µl	2 µl	198	2000	535	413	99 %	77 %	44 %
≥PE150		12 µl	-	225	2000	607	485	100 %	87 %	67 %

* 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 (the total length of added adapter sequences).

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, 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 and reads will overlap better for more complete coverage.

10. Appendix D: qPCR

The qPCR Assay is recommended to determine the exact number of cycles for the endpoint PCR in order to prevent under- or overcycling of the library.

qPCR Assay Protocol

Each SENSE mRNA-Seq Kit contains additional **PCR** ●, **E2** ●, and P7 Primer (**7000** ●) for 8 qPCRs. If more qPCRs are required, the PCR Add-in Kit for Illumina (Cat. No. 020.96) can be used. Enzyme Mix (**E** ●) from the PCR Add-on Kit is interchangeable with **E2** ● from the SENSE mRNA V2 Kit.

ATTENTION: Important notes for the qPCR Assay.

- SYBR Green I nucleic acid dye (Sigma Aldrich, S9430; ThermoFisher S7563) is required for the qPCR assay and must be supplied by the user.
- The use of SYBR Green I-containing qPCR mastermixes for the qPCR Assay is explicitly not recommended.

NOTE: SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines need to be adjusted manually.

1 Dilute the double-stranded library from step 34 to 19 µl by adding 2 µl Elution Buffer (**EB**) or molecular biology-grade water (H₂O).

2 Prepare a 1:4,000 dilution of SYBR Green I dye in DMSO, for a 2.5x working stock concentration. **ATTENTION:** The final concentration in the reaction should be 0.1x. Higher concentrations of SYBR Green I will inhibit the amplification.

3 For each reaction combine: 1.7 µl of the diluted cDNA library, 7 µl of PCR Mix (**PCR** ●), 5 µl of P7 Primer (**7000** ●), 1 µl of Enzyme Mix (**E** ●), and 1.2 µl of 2.5x SYBR Green I nucleic acid dye. Add 14.1 µl of Elution Buffer (**EB**) or molecular biology-grade water (H₂O) to bring the total volume to 30 µl. **ATTENTION:** Include a no template control!

4 Perform 35 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 35 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 **REMARK:** There is no need to purify or analyze the overcycled PCR product.

5 Using the amplification curves in linear scale, determine the value at which the fluorescence reaches the plateau. Calculate 50 % of this maximum fluorescence value and determine at which cycle this value is reached. As the endpoint PCR will contain 10x more cDNA compared to the qPCR, subtract three from this cycle number. This is then the final cycle number you should use for the endpoint PCR with the remaining 17 µl of the template.

Example for Endpoint Calculation

500 ng total RNA input was used for generating libraries. Using 1.7 μ l of cDNA for a qPCR, the cycle number corresponding to 50 % MF was 15 cycles. The remaining 17 μ l of the template should therefore be amplified with 12 cycles (15 - 3 cycles = 12 cycles).

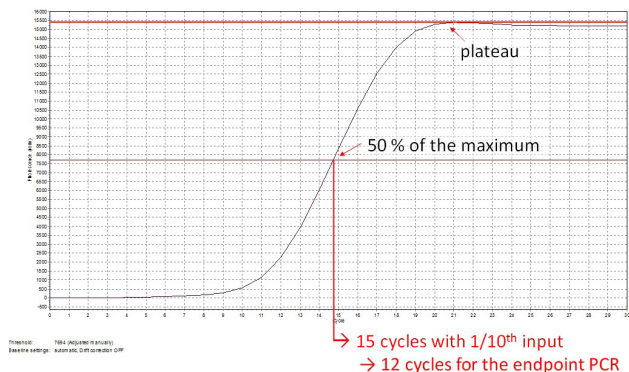


Figure 3. Calculation of the number of cycles for the endpoint PCR.

The optimal cycle number can also be determined using the amplification data from the qPCR software to calculate the 50 % MF value. Take the cycle number that is the next closest fluorescence value above the 50 % MF and subtract 3 to get the endpoint cycle number.

NOTE: Once the number of cycles for the Endpoint PCR is established for one type of sample (same input amount, tissue, and RNA quality), there is no need for further qPCRs. The entire cDNA can be used directly for the Endpoint PCR.

11. Appendix E: Library Reamplification

Reamplification of Single-Indexed Libraries (i7 only)

Lexogen's PCR Add-on Kit (Cat. No. 020) contains a i7 Reamplification Primer (**i7-RE** ○) that can be used to reamplify single-indexed (i7) libraries that are undercycled. For details please refer to the PCR Add-on Kit (Cat. No. 020.96) Instruction Manual.

Reamplification of Dual-Indexed Libraries (i5 and i7)

For reamplification of dual-indexed libraries the Reamplification Add-on Kit for Illumina (Cat. No. 080) is available on request. Please contact info@lexogen.com for further information.

12. Appendix F: Library Quality Control

Quality control of finished SENSE mRNA 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

Analyzing a small volume of the amplified library with microcapillary electrophoresis has become standard practice 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 Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies, Inc.). For high throughput applications instruments such as the Fragment Analyzer or 2200 TapeStation (Agilent Technologies, Inc.), or LabChip GX II (Perkin Elmer) are recommended. Typically, 1 μ l of SENSE mRNA library produced according to the directions in this manual can be analyzed directly on a High Sensitivity DNA chip. Depending on the minimum sample loading requirements for each instrument, 1 μ l of the finished library may be diluted to the required volume (e.g., 2 μ l sample for TapeStation and 10 μ l 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 mRNA 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

Library size profiles will depend on whether **RTS** or **RTL** was used for library generation, and which purification conditions were selected at step 22 (see Appendix C, p.24).

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 Assay to determine the optimal cycle number for Endpoint PCR should prevent overcycling. Overcycled PCR products can still be sequenced. However, for further experiments using the same input RNA please adjust your cycle number accordingly or take advantage of the qPCR option.

Should the libraries be undercycled and amounts insufficient for pooling and sequencing, these can be reamplified using Lexogen's PCR Add-on Kit for Illumina (Cat. No. 020). For more details please refer to the PCR Add-on Kit Instruction Manual.

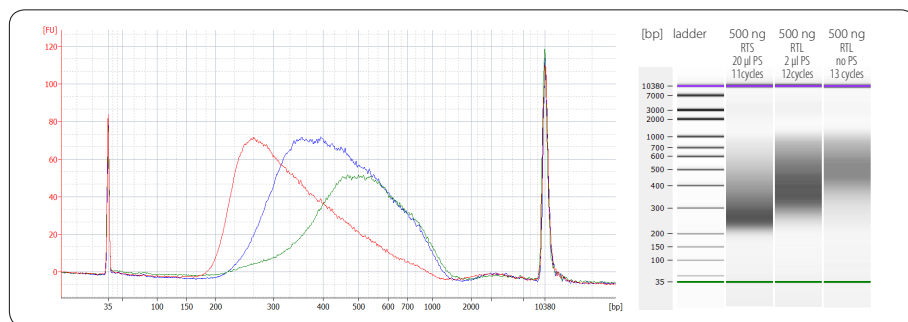


Figure 4. Bioanalyzer traces of SENSE mRNA libraries prepared with RTS (red trace) and RTL (blue and green traces). Input was 500 ng Universal Human Reference RNA (UHRR). Purification reagent volumes used at step 22 were: 14 µl PB and 20 µl PS (red trace), 14 µl PB and 2 µl PS (dark blue trace), and 12 µl PB only (no PS, green trace).

13. Appendix G: Multiplexing

SENSE mRNA libraries can be indexed using the Lexogen i7 6 nt indices included in the kit, to enable multiplexing for sequencing. The indices are introduced during the PCR amplification step (step [37](#)).

i7 Indices

The Lexogen i7 6 nt indices allow up to 96 samples to be sequenced per lane on an Illumina flow cell and are included in the kit (Lexogen i7 6 nt Index Set, 96-well plate). The 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: AAGGCC	7034: TTGGTA	7042: TGACAC	7050: TCGAGG	7058: ATAAGA	7066: GACGAT	7074: CGATCT	7082: ACTCTT	7090: CCTGCT
C	7003: ACCACT	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: GCCAAT	7037: TGCGCA	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

The Lexogen i7 6 nt index sequences are available for download at www.lexogen.com.

In general, we recommend processing a minimum of 8 samples, 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 select indices that 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 when individual libraries are mixed in an equimolar ratio. Use the online Index Balance Checker tool available at <https://www.lexogen.com/support-tools/index-balance-checker/>, to select the ideal combination of indices for optimal color and nucleotide balance.

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

Dual Indexing - i5 and i7 Indices

Two Lexogen i5 6 nt Dual Indexing Add-on Kits are available that enable dual indexing of SENSE mRNA libraries, for enhanced multiplexing capacity and improved control of index identification accuracy.

The Lexogen i5 6 nt Unique Dual Indexing Add-on Kit (5001–5096) provides a 96-well plate containing 96 unique i5 indices (Cat. No. 047.96). This kit is designed for unique dual indexing in combination with Lexogen's 96 i7 indices (included in all SENSE mRNA V2 Library Prep Kits). Up to 96 uniquely dual-indexed libraries can be prepared for sequencing in a single lane or run. Alternatively, used together with the 96 i7 indices, up to 9,216 dual-indexed libraries with different i5 / i7 index combinations can be prepared.

The Lexogen i5 6 nt Dual Indexing Add-on Kit (5001–5004, Cat. No. 047.4) provides four different i5 index primers (5001–5004). Each tube contains sufficient volume for preparing 24 (Cat. No. 047.4x24), or 96 (Cat. No. 047.4x96) libraries. In combination with 96 i7 indices, a maximum of 384 (4 i5 x 96 i7) dual-indexed libraries with different i5 / i7 index combinations can be prepared. For further information please consult the Lexogen i5 6 nt Dual Indexing Add-on Kit Instruction Manual (047IM109).

Lane Mix Preparation

Libraries should ideally be pooled in an equimolar ratio for multiplexed sequencing. It is important to ensure accurate quantification of the individual libraries prior to pooling, as well as for the library pool (lane mix). To quantify your libraries:

- 1 Measure the concentration of each library, using either qPCR or fluorescence-based assays (e.g., QuBit, Thermo Fisher Scientific Inc.).
- 2 Determine the average library size, using microcapillary electrophoresis analysis (e.g., Bioanalyzer, Agilent Technologies, Inc.). Set the range to 160 - 1,000 bp to include the whole library size distribution, and to exclude any linker-linker artifacts (~150 bp), or overcycling bumps (>1,000 bp).

Molarity is then calculated from the average library size and the concentration (ng/μl) using the following equation:

$$\text{Molarity} = (\text{library concentration (ng/}\mu\text{l)} \times 10^6) / (660 \times \text{average library size (bp)})$$


A template for molarity calculation is also available for download from www.lexogen.com.

After pooling the libraries, the prepared lane mix and any dilutions made for denaturing (e.g., 2 nM), should be reanalyzed to ensure the accuracy of the concentration. This can be performed according to steps 1 and 2 as above.

Lane Mix Repurification to Remove Linker-Linker Artifacts

A shorter side-product representing linker-linker artifacts is sometimes visible at ~150 bp, and should not compose more than 0 - 3 % of the total lane mix for sequencing. If the fraction of linker-linker, or other small fragments (≤ 150 bp) are too prominent, repurification of the lane mix prior to sequencing is advised.

Libraries or lane mixes can be repurified using the Purification Module with Magnetic Beads (Cat. No. 022) using the following protocol.

- 1 Measure the volume of the library or lane mix. If the volume is less than 20 μ l, adjust the total volume to 20 μ l using Elution Buffer (**EB**) or molecular biology-grade water (H_2O).
 - 2 Add 0.9 volumes (0.9x) of Purification Beads (**PB**). Mix thoroughly and incubate for 5 minutes at room temperature. **EXAMPLE:** For 50 μ l of lane mix, add 45 μ l **PB**.
-
-  Follow the detailed protocol from step 40 onwards (p.18).
-

14. Appendix H: Sequencing*

General

We do not recommend multiplexing Lexogen libraries with libraries from other vendors in the same sequencing lane.

Though this is possible in principle, specific optimization of index combinations, library pooling conditions, and loading amounts may be required, even for advanced users. Sequencing complex pools that include different library types at different lane shares may have unpredictable effects on sequencing run metrics, read quality, read outputs, and/or demultiplexing performance. Lexogen assumes no responsibility for the altered performance of Lexogen libraries sequenced in combination with external library types in the same lane (or run).

Due to size differences, libraries prepared with the Lexogen Small RNA-Seq Library Prep Kit (or any other small RNA library prep kit) should not be sequenced together with SENSE mRNA libraries.

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. In general SENSE mRNA libraries may be loaded according to standard Illumina loading recommendations. Additional guidelines may be found in the online Frequently Asked Questions. SENSE mRNA libraries can be sequenced using the standard Illumina Multiplexing Read 1, Index Read, and Multiplexing Read 2 Sequencing Primers.

Libraries with i7 Indexing

i7 indices (6 nt) are introduced during PCR (step 37). Multiplexing indices are included in the SENSE mRNA-Seq Kits V2 (Cat. No. 001) and are provided in a 96-well plate (see also Appendix G, p.29).

```
5'-(Read 1 Sequencing Primer)-3'
5' AATGATACGGCGACCAACGAGATCTACACTCTTCCCTACACGACGCTCTCCGATCT- (Insert...
3' TTAATATGCGCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA- (Insert...

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

Read 1: Multiplexing Read 1 Sequencing Primer (not supplied):

5'ACACTCTTCCCTACACGACGCTCTCCGATCT 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'

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

15. Appendix I: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of SENSE mRNA data, and is kept as general as possible for integration with your standard pipeline. SENSE mRNA 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 adapter contamination, which should be removed from the dataset.

The SENSE technology is based on random priming, which can be observed in the reads. The starter/stopper sequences may cause a higher proportion of errors at the first nucleotides, which is visible in the FastQC report.

Demultiplexing

Demultiplexing can be carried out by the standard Illumina pipeline. Lexogen i7 (and i5) 6 nt index sequences are available for download at www.lexogen.com.

Trimming

Due to the use of 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 sensitivity of assembly tools, in which case it may be beneficial to trim these nucleotides. 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). If adapter contamination is detected, adapter removal should be performed. Please ensure that the selected tool preserves the read pair information, or restores it afterwards.

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*. We recommend using STAR aligner with the ENCODE settings, increasing the mismatch rate to counter the starter/stopper errors.

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.

16. Appendix J: Revision History

The revision history table below shows user guide versions and kit changes made from 2017 onwards. The full revision history is available from www.lexogen.com.

Publication No. / Revision Date	Change	Page
001UG004V0330 Jul. 15, 2019	Attention notes added and reformatted for detailed protocol steps.	11 - 18
	Steps 19 -20: Prepare SSM/E2 mastermix and add to beads is now preferred handling, with option to add reagents separately.	14
	Step 21 hold temperature changed to 4 °C.	14
	Short Procedure was reformatted and updated to include low input protocol modifications.	19 - 20
	Text and tales updated for Appendices A - I.	21 - 33
	Appendix G: New protocols for lane mix preparation and repurification to remove linker-linker added.	30 - 31
	Revision History shortened to 2017 onwards.	34
001UG004V0322 Nov. 5, 2018	Temperature to hold the second strand synthesis is lowered to 4 °C.	14
	Restructuring of Appendix D (qPCR) and new Appendix E (Library Re-amplification)	25, 26
	Shortened Revision History (only 2016 onwards)	33
001UG004V0321 Dec. 4, 2017	Edited remark text for step 37 regarding use of P7 Primer for qPCR.	16
	Safe stopping points added.	17, 18
	Updated text for Appendices C and D: PCR cycle numbers and qPCR.	24, 25
	Updated text for Appendix H: Data Analysis.	31
001UG004V0320 Feb. 7, 2017	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
001UG004V0100 Oct. 1, 2012	Initial Release.	

17. Notes

Associated Products:

020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
024 (Automation Module for SENSE mRNA-Seq V2)
025, 050, 051 (SIRVs Spike-in RNA Variant Control Mixes)
044 (Lexogen i7 6 nt Index Set (7001-7096))
047 (Lexogen i5 6 nt Dual Indexing Add-on Kits (5001-5096))
080 (Reamplification Add-on Kit for Illumina)

SENSE mRNA-Seq Library Prep Kit V2 · User Guide

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