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, including Barcodes)
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)

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Table of Contents

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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. Optional multiplexing of libraries can be carried out using up to 96 external barcodes. Libraries are compatible with both single-end 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 Appendix A (p.19).

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 E, p.28, for a schematic representation of the finished library). Library quantification can be performed with standard protocols and is further discussed in Appendix C (p.24). Libraries are compatible with single-end or paired-end sequencing. External barcodes are included in the kit and are also introduced during the PCR amplification step (Appendix D, p.26). 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 F (p.30).

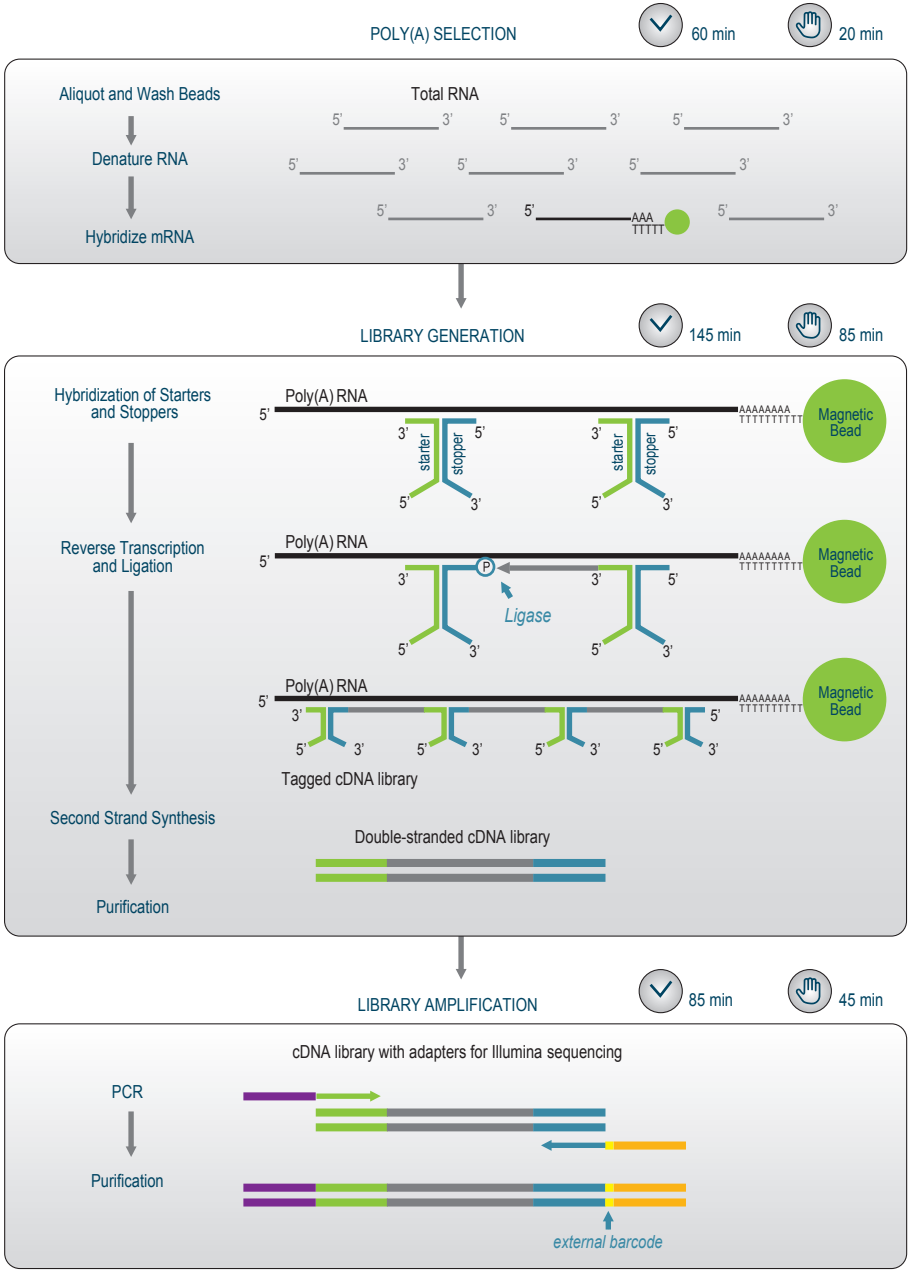

Figure 1. Schematic overview of the SENSE workflow.

2. Kit Components and Storage Conditions

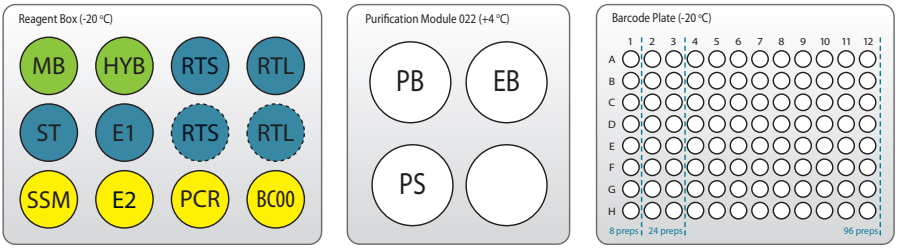

Figure 2. Location of kit contents in a 96 prep kit. For 8 prep and 24 prep kits the purification solutions (PB, PS, EB) and BW are provided in smaller containers and the dotted solutions of the reagent box are missing. External barcodes are provided in a 96-well plate. For 8 prep kits column 1 (up to first dotted line), for 24 prep kits columns 1-3 (up to the second dotted line) and for 96 prep kits all columns are filled with barcodes, respectively. For barcode sequences see p.26.

Kit Component	Tube Label	Volume* provided			Storage
		8 preps	24 preps	96 preps	
Magnosphere MS150 / OligodT Beads	MB	88 µl	264 µl	1056 µl	-20 °C
RNA Hybridization Buffer	HYB	88 µl	264 µl	1056 µl	-20 °C
Reverse Transcription and Ligation Mix Short	RTS	132 µl	396 µl	1584 µl	-20 °C
Reverse Transcription and Ligation Mix Long	RTL	132 µl	396 µl	1584 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
BC00	BC00	44 µl	44 µl	44 µl	-20 °C
Bead Wash Buffer	BW	7040 µl	21120 µl	84480 µl	+4 °C
Barcode Plate (96-well plate)	BC	Set 1	Set 1-3	Set 1-12	-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	1080 µl	3221 µl	12884 µl	+4 °C
Elution Buffer**	EB	1232 µl	3344 µl	12848 µl	-20 °C/+4 °C

**including additional volume for 8 qPCR reactions

*including a 10 % surplus

Upon receiving the SENSE kit, store **PB** and **PS** at +4 °C. The rest of the kit should be stored in a -20 °C freezer. **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), 10,000 x 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 1000 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).

Optional Equipment

- Automated microfluidic electrophoresis station (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).

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.

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 C (p.24) 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. 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.
- 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 removing 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.

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** – 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).

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.
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** – 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-end or 50 nt paired-end; **RTL** ● is used for sequencing runs of 100 nt paired-end and larger. Please also consult Appendix B: 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 (1 ng - 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 Beads (**PB**) 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. 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.

Purification

The double-stranded library is purified using magnetic beads to remove second strand synthesis reaction components. The Purification Beads (**PB**) 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 B: 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 B, 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.
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 re-precipitate 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.
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 wash 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. **ATTENTION:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, add 17 µl RNase-free water or 10 mM Tris, pH 8.0 to the eluted library. The qPCR option is available for a total of 8 samples. For further details please refer to Appendix A (p.19).

34 After elution, libraries can be stored at -20 °C for later amplification.

5.3 Library Amplification

Preparation

PCR	Purification
PCR – thawed at RT **BC** – thawed at RT; **spin down before opening!** **E2** – keep on ice or at -20 °C	**PB** – stored at 4 °C **80 % EtOH** – provided by user **prepare fresh!** **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 -25 x see Appendix B, p.23 for low input RNA see Appendix A, p.19 72 °C, 1 min 10 °C, ∞	Magnetic rack / plate

PCR

The library is amplified to add the complete adaptor sequences required for cluster generation and to generate sufficient material for quality control and sequencing.

NOTE: At this point we recommend placing the Purification Beads (**PB**) 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 external Barcode Primer (**BC01-96**, in 96-well plate, **ATTENTION:** Spin down before opening! Pierce or cut open the sealing foil of the wells containing the desired barcodes. Avoid cross contamination!). Mix well by pipetting. Seal the plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **REMARK:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, use 5 µl of Barcode 00 (**BC00** ●) per reaction. The qPCR option is available for a total of 8 samples. For further details please refer to Appendix A (p.19).

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 B, p.23), your input RNA amount (see Appendix A, p.19), and the tissue or organism your RNA was extracted from. Please refer to the tables in Appendix A (p.19) and Appendix B (p.23) and /or also take advantage of the qPCR assay as described in Appendix A (p.19).

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 lower than 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 re-precipitate 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.
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 wash 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 C, p.24), pooling (for multiplexing see Appendix D, p.26), 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 2 x 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.	
☐	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** ●.	2nd 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	
☐	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.19 for qPCR).	

85 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 **BC** (from the 96-well plate) for each reaction, mix well.	
☐	PCR: 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 11 - 25 x (see p.20 and p.23) 72 °C, 1 min 10 °C, ∞ **ATTENTION:** Increase cycle number for low input RNA and samples with low mRNA content! (see p.20)	
☐	Add 30 µl **PB** (or 27 µl **PB** for low 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: RNA Requirements - PCR Cycles

RNA Input Amount

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 (HBR) and Universal Human Reference RNA (UHRR)). 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-end 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 B (p.23).

RNA inputs down to 1 ng total RNA from mRNA-rich samples such as Universal Human Reference RNA (UHRR) 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 page 20. **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. For reduced RNA inputs we also strongly recommend performing a qPCR assay (see below) 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 17 µl RNase-free water or 10 mM Tris, pH 8.0 to the 17 µl of your eluted library from step 33.
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.

qPCR to Determine the Exact Cycle Number of Your Endpoint PCRs

For determining the cycle number of your endpoint PCR please use 5 µl of Barcode 00 (**BC00** ●) in step 37 of the protocol. Insert 17 µl (of the diluted 34 µl double stranded library, step 33) 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 0.1x SYBR Green I add 1.2 µl 2.5x SYBR Green I solution (1:4,000 SYBR Green I dilution, diluted in DMSO). The total PCR reaction volume will be 31.2 µl. Alternatively, if 8 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 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) and then determine **the maximum fluorescence value** at which the fluorescence reaches a plateau. Calculate where the fluorescence is 33 % of the maximum, and use the corresponding cycle number for the endpoint PCR with the remaining 17 µl of the template. There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer.
For similar samples that have not been diluted for the qPCR assay the cycle number determined in the overcycled qPCR can be reduced by 1 cycle.
EXAMPLE: 500 ng input RNA was used for generating two libraries. The cDNA of library 1 was diluted and inserted into the qPCR assay. The cycle number determined in the overcycled qPCR (33 % of the maximum fluorescence) was 12 cycles. The remaining half of the template should be amplified with 12 cycles, whereas the undiluted cDNA of library 2 can be amplified with 11 cycles, as here double the amount of template is inserted into the PCR.

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.

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 UHRR
			PB x	PS y		ng/µl	nM	
500 ng UHR	RTS	5 min	14 µl	20 µl	30 µl	2.52	11.97	11
100 ng UHR						2.59	12.48	14
50 ng UHR		20 min			27 µl	1.58	6.93	16
10 ng UHR						1.24	5.60	18
1 ng UHR						1.28	3.29	22
500 ng UHR	RTL	5 min	14 µl	2 µl	30 µl	2.89	10.06	12
100 ng UHR						1.45	7.55	16
50 ng UHR		20 min			27 µl	1.62	6.32	17
10 ng UHR						1.11	4.15	20
1 ng UHR						1.58	7.08	25

Reamplification of Barcoded Libraries with the PCR Add-on Kit

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

ATTENTION: Do not use the **BC00** ● for the reamplification of already barcoded libraries! This will lead to a loss of barcodes and to a mixed and not assignable sequence pool in an 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. The Reamplification Primer can only be used on already amplified PCR libraries.

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.

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 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 have a known strand orientation and no antisense transcripts, so the calculation of strandedness based on ERCC sequences is more accurate than calculations based on reads aligning to the genome.

8. Appendix B: 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, UHRR). If using less input RNA or RNA with low mRNA content, further cycles need to be added. In this case we strongly recommend performing the qPCR assay as described in Appendix A, p.19.

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	RTL	12 µl	- µl	225	2000	607	485	100 %	87 %	67 %	1.31	3.91	13

*For multiplexed (externally barcoded) libraries. Libraries prepared without external barcodes (BC00) 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: 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. 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 B: 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 A: RNA Requirements - PCR cycles (p.20) 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 A, p.19 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 1000 - 9000 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 on page 19 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 barcoded 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 A, p.20 as well as the PCR Add-on Kit Instruction Manual.

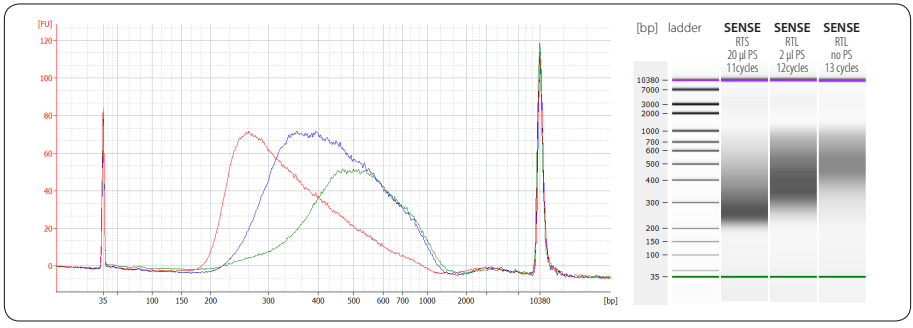

Figure 3. Bioanalyzer traces of RTS (red trace) and RTL (blue and green traces) synthesized SENSE libraries from 500 ng Universal Human Reference RNA (UHRR) and purified with varying amounts of PS in step 22. Red trace: 14 µl PB and 20 µl PS, dark blue trace: 14 ml PB and 2 µl PS, and green trace: only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

10. Appendix D: Multiplexing

SENSE libraries can be multiplexed. Barcodes can be introduced as standard external barcodes during the PCR amplification step (step 37).

External Barcodes

External barcodes allowing up to 96 samples to be sequenced per lane on an Illumina flow cell are included in the kit in the Barcode Plate (**BC**). External barcodes require an additional index-specific sequencing reaction and are 6 nt long.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC01: ACATTA	BC02: GGTGAG	BC03: CGAAGG	BC04: AAGACA	BC05: TAATCG	BC06: CGCAAC	BC07: AATAGC	BC08: TTAACT	BC09: AATGAA	BC10: GATTGT	BC11: ATAAGA	BC12: GCCACA
B	BC13: GAACCT	BC14: CGGTTA	BC15: AACGCC	BC16: CAGATG	BC17: GATCAC	BC18: CGCGGA	BC19: CCTAAG	BC20: GGCTGC	BC21: ACCAGT	BC22: GTGCCA	BC23: AGATAG	BC24: TCGAGG
C	BC25: ACAACG	BC26: GCGCTG	BC27: CAAGCA	BC28: GTTACC	BC29: CTCTCG	BC30: CCAATT	BC31: TTCGAG	BC32: CGTCGC	BC33: TGTGCA	BC34: ACCGTG	BC35: ATACTG	BC36: ATGAAC
D	BC37: AGTTGA	BC38: GACGAT	BC39: CACACT	BC40: CAGCGT	BC41: TGCTAT	BC42: TCTTAA	BC43: CCGCAA	BC44: CTCCAT	BC45: GTCAGG	BC46: ACGTCT	BC47: GAGTCC	BC48: GACATC
E	BC49: AGGCAT	BC50: ACCTAC	BC51: TGGATT	BC52: GCAGCC	BC53: CGCCTG	BC54: CCGACC	BC55: TATGTC	BC56: TGACAC	BC57: ACAGAT	BC58: AGACCA	BC59: GCTCGA	BC60: ATGGCG
F	BC61: GAAGTG	BC62: AGAATC	BC63: GCGAAT	BC64: CGATCT	BC65: CATCTA	BC66: AAGTGG	BC67: TGCACG	BC68: TCGTTC	BC69: ACACGC	BC70: GTAGAA	BC71: AGTACT	BC72: GCATGG
G	BC73: AACAAG	BC74: AACCGA	BC75: TGGCGA	BC76: CACTAA	BC77: AAGCTC	BC78: TACCTT	BC79: CTAGTC	BC80: AATCCG	BC81: GTGTAG	BC82: ACTCTT	BC83: TCAGGA	BC84: ATTGGT
H	BC85: TTGGTA	BC86: CAACAG	BC87: CAATGC	BC88: GGAGGT	BC89: CAGGAC	BC90: GGCCAA	BC91: CTCATA	BC92: CCTGCT	BC93: GGTATA	BC94: TTCCGC	BC95: TAGGCT	BC96: ATATCC

External barcode sequences are available for download at www.lexogen.com.

When choosing subsets of barcodes, it is important to make sure that both color channels used by Illumina platforms (red laser: A/C and green laser: G/T) register a signal at each nucleotide position.
Barcodes can also be combined across rows or columns.
A schematic representation of the provided barcodes is shown in Figure 2, p.6.
The 8 reaction SENSE mRNA-Seq V2 kit (Cat. No. 001.08) includes Barcode Set 1 (BC01/BC13/BC25/BC37/BC49/BC61/BC73, and BC85).
The 24 reaction SENSE mRNA-Seq V2 kit (Cat. No. 001.24) includes Barcode Set 1 (BC01/BC13/BC25/BC37/BC49/BC61/BC73, and BC85), Barcode Set 2 (BC02/BC14/BC26/BC38/BC50/BC62/BC74, and BC86), and Barcode Set 3 (BC03/BC15/BC27/BC39/BC51/BC63/BC75, and BC87).
The 96 reaction kit (Cat. No. 001.96) includes all Barcode Sets (Set 1 - 12) and, as mentioned previously, here barcodes can be combined across rows (Set A: BC01 - 12, Set B: BC13 - 24, and so on) or columns (Set 1 - 12).

In general we recommend using a complete set of 8 or 12 barcodes for multiplexing (e.g., Set 1

or Set A if the 96 reaction kit is used, respectively). However, if fewer barcodes are required also subsets of each set can be chosen. Some examples for subsets of barcodes are listed below.

Two samples per lane: In step 37 use 2.5 µl of BC01 and 2.5 µl BC13 for one sample and 2.5 µl BC25 and 2.5 µl BC37 for the second. Here two barcodes 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 BC01 for one sample, 5 µl BC13 for the second, 5 µl BC25 for the third, and 5 µl BC37 for the fourth. Apply only one barcode to each sample.

Eight samples per lane: In step 37 use all the barcodes from Set 1 (BC01/BC13/BC25/BC37/BC49/BC61/BC73, and BC85). Apply only one barcode to each sample.

Twelve samples per lane: In step 37 use all the barcodes from Set 1 (BC01/BC13/BC25/BC37/BC49/BC61/BC73, and BC85) plus 4 barcodes from Set 2 (BC02/BC14/BC26, and BC38) if you have the 24 reaction kit (Cat. No. 001.24). Alternatively, if using the 96 reaction kit (Cat. No. 001.96) barcodes BC01 - 12 from row A can be used. Apply only one barcode to each sample.

ATTENTION: If using subset of barcodes, take care to avoid cross contamination! Spin down the barcode plate before carefully removing the seal. Tightly re-seal the plate for later use of the remaining barcodes.

Various multiplexing options are available to suit your experimental design. However, care should be taken to always use sets of barcodes that give a signal in both color channels for each nucleotide position. In detail, at least one of the two bases A or C (red channel) **AND** one of the two bases G or T (green channel) should be present at a given nucleotide position for all Illumina sequencers except NextSeq machines which uses a different color coding system.
All barcode sets included in this kit fulfill those criteria.

The individual libraries within a lane should be mixed in an equimolar ratio to ensure this balance and to ensure equal read depth per sample.

11. Appendix E: Sequencing*

General

The amount of library loaded onto the flowcell 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 flowcell. All SENSE libraries can be sequenced using the standard Illumina Multiplexing Read 1, Index read, and Multiplexing Read 2 Sequencing Primers.
External barcodes 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 without Barcodes (BC00●)

Here **BC00** ● (for 8 rxns only) supplied with the basic kit (Cat. No. 001.08, 001.24, 001.96) is used in step 37 .

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

   …Insert)- AGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert)- TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTAGAGCATACGGCAGAAGACGAAC 5'
               3'-(Read 2 Sequencing Primer)-5'
```

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

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

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

Libraries with External Barcodes

External barcodes (6 nt) are introduced during PCR (step 37). External barcodes 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 D, p.26).

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

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

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

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

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

12. Appendix F: 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 under www.lexogen.com.

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.

De-Multiplexing

SENSE External Barcodes: The barcode is contained in the Index Read, and demultiplexing can be carried out by the standard Illumina pipeline.

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.

13. Appendix G: Revision History

Revision date / Publication No.	Change	Page
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 UHR RNA input amounts and required cycle numbers.	20
	Endpoint PCR set at 33 % of the maximum qPCR fluorescence.	20
	Re-Amplification 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.	
October 01st 2012 / 3	Initial Release SENSE mRNA-Seq.	

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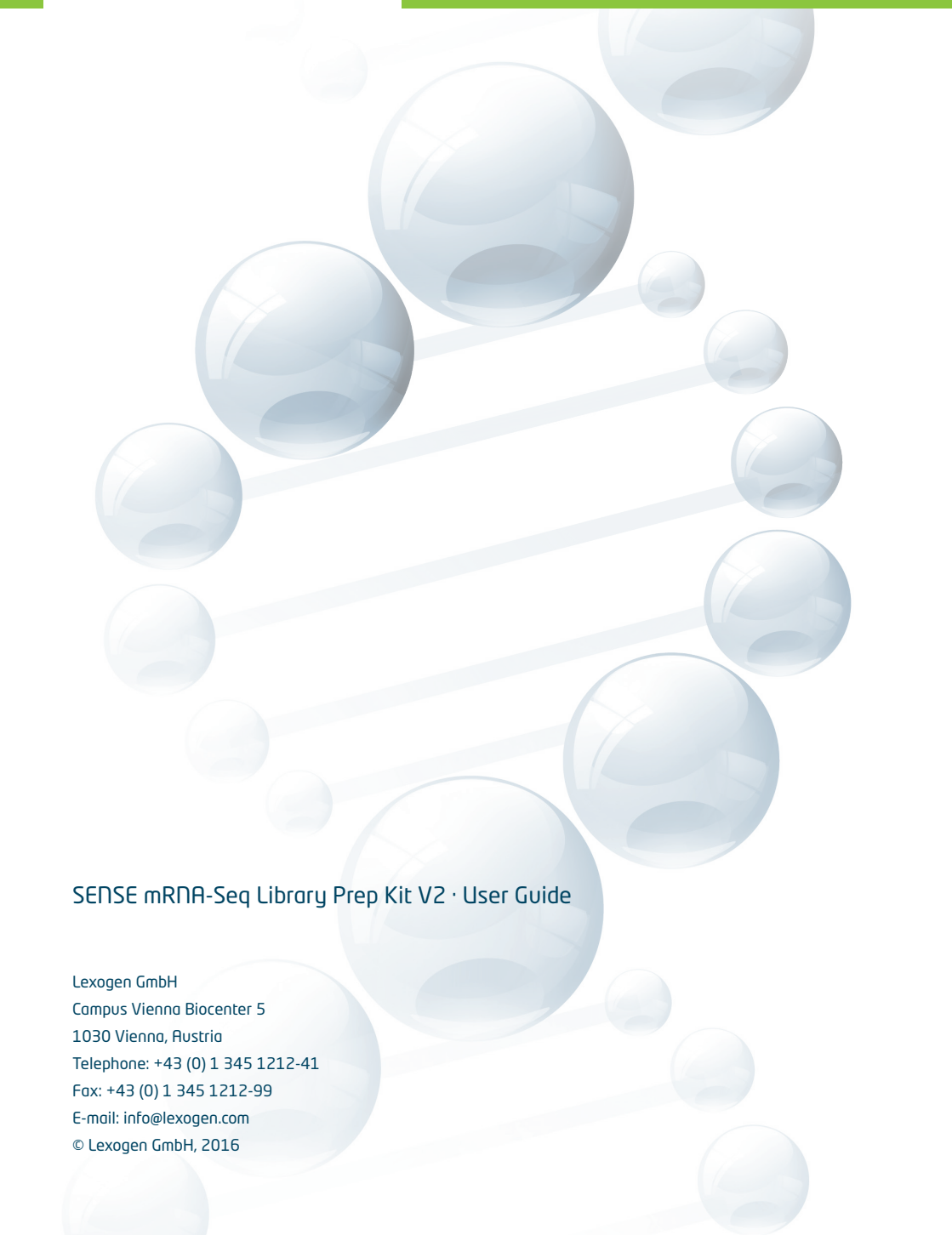

SENSE mRNA-Seq Library Prep Kit V2 · User Guide

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