Lexogen
Enabling complete transcriptome sequencing

SENSE™

Making sense of RNA sequencing

Total RNA-Seq Library Prep Kit
User Guide

Catalog Numbers:
009 (SENSE Total RNA-Seq Library Prep Kit for Illumina)
020 (PCR Add-on Kit for Illumina)
022 (Purification Module with Magnetic Beads)
025 (SIRVs Spike-in RNA Variant Control Mixes)
037 (RiboCop rRNA Depletion Kit)
039 (Poly(A) RNA Selection Kit)
042 (SENSE Total RNA-Seq Library Prep Kit for Illumina with RiboCop)

009UG013V0122

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

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Enabling complete transcriptome sequencing

1. Overview

This SENSE Total RNA-Seq kit is a library preparation protocol designed to generate Illumina-compatible libraries from RNA within 3.5 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. Insert size can be varied during the library purification step, 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 input RNA can be either poly(A) RNA or rRNA-depleted samples including low quality RNA. Information regarding input RNA requirements can be found in Appendix A (p.18). We recommend using Lexogen's RiboCop rRNA Depletion Kit (Cat. No. 037.24 and 037.96). SENSE Total RNA-Seq is also available as a bundle with RiboCop (Cat. No. 042.08, 042.24, and 042.96). Alternatively, Lexogen also offers a Poly(A) RNA Selection Kit (Cat. No. 039.100), but if mRNA sequencing is of interest, we would recommend using the SENSE mRNA-Seq Library Prep Kit (Cat. No. 001.08, 001.24, and 001.96) which already includes poly(A) selection.

SENSE library production is initiated by the random hybridization of starter / stopper heterodimers to the RNA template. 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. The insert size is determined by the distance between starter / stopper binding sites. Lexogen's proprietary strand-displacement stop technology prevents spurious second strand synthesis, providing the basis for the excellent strand-specificity of the SENSE protocol.

Second strand synthesis is performed to degrade the RNA, and the library is then amplified, introducing the sequences required for cluster generation (see Appendix F, p.29, for a schematic representation of the finished library). Library quantification can be performed with standard protocols and is further discussed in Appendix D, p.25. Libraries are compatible with single-end or paired-end sequencing. Barcodes can be introduced during the PCR amplification step as standard external barcodes (Appendix E, p.27). Data can be analyzed with a number of standard bioinformatic pipelines. Special considerations for the analysis of SENSE data, such as read orientation, are presented in Appendix G, p.30.

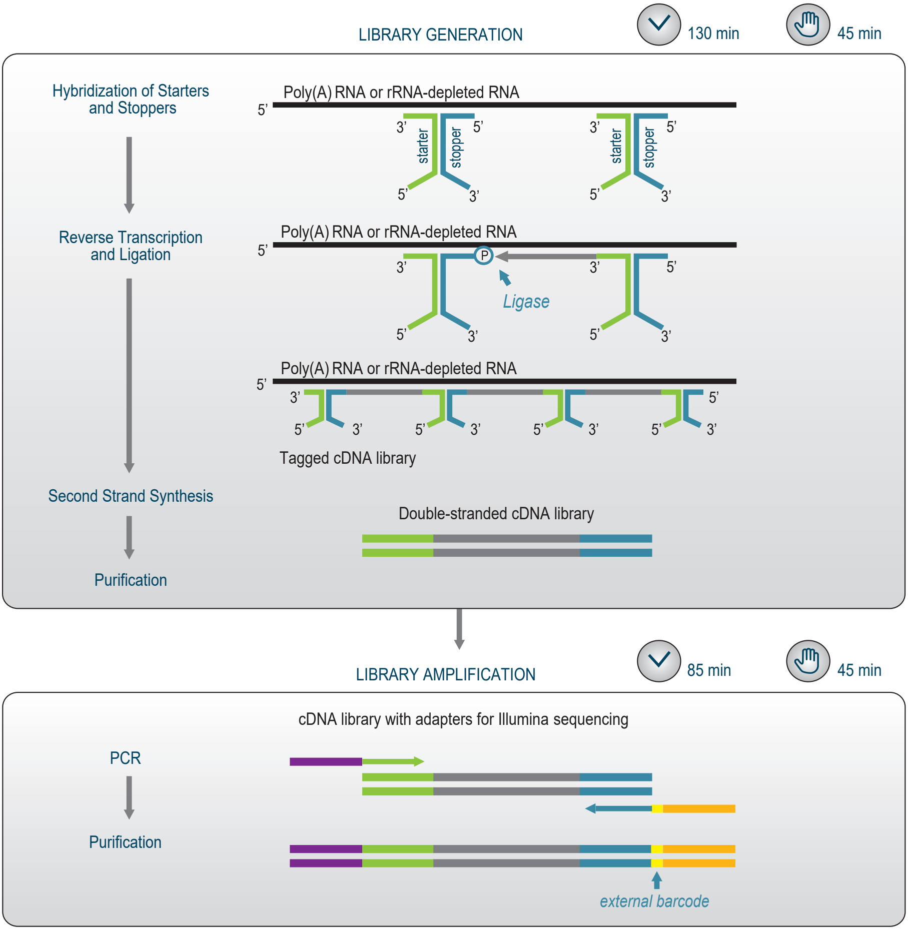

Figure 1. Schematic overview of the SENSE workflow.

2. Kit Components and Storage Conditions

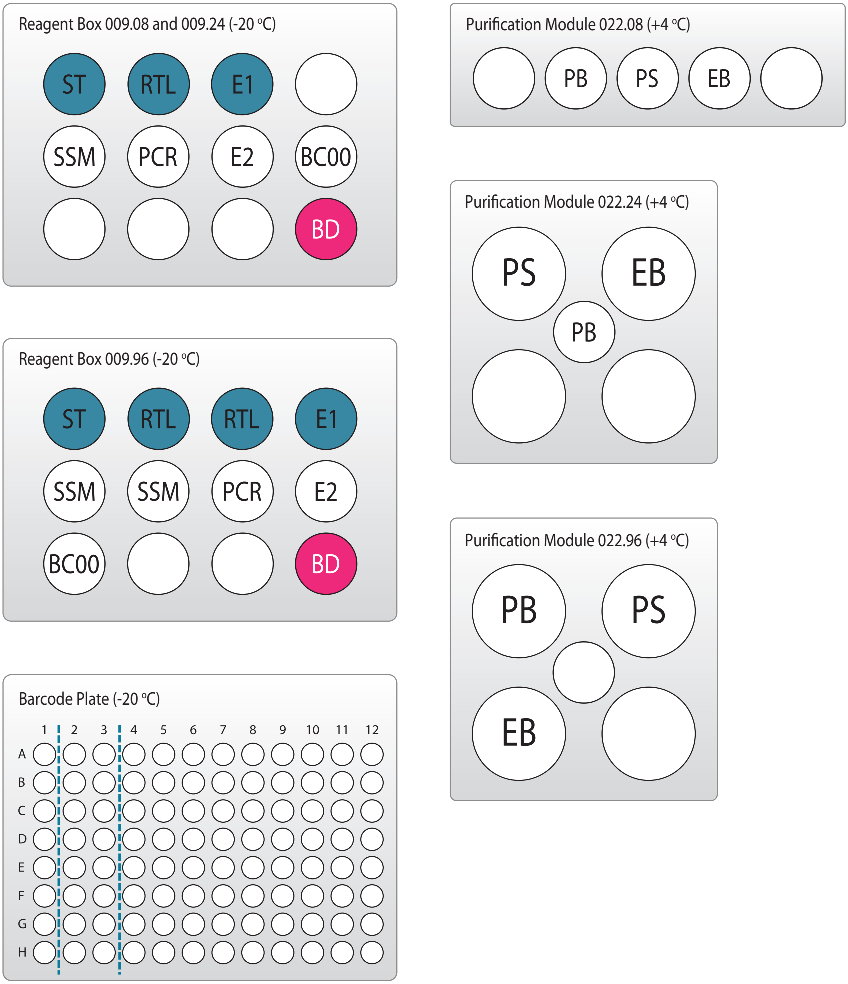

Figure 2. Location of kit contents. Each kit consists of a Reagent Box (to be stored at -20 °C) and a corresponding Purification Module (to be stored at +4 °C). For Cat. No. 009.08, 8 preps and Cat. No. 009.24, 24 preps, the included barcode plate is only filled in Column 1 and Column 1-3, respectively. For Cat. No. 009.96, 96 preps all 96 barcodes are included (to be stored at -20 °C).

Kit Component	Tube Label	Volume* for			Storage
		8 preps	24 preps	96 preps	
Starter / Stopper Mix	**ST** ●	44 µl	132 µl	528 µl	-20 °C
Reverse Transcription and Ligation Mix	**RTL** ●	124 µl	370 µl	1480 µl	-20 °C
Enzyme Mix 1	**E1** ●	27 µl	80 µl	317 µl	-20 °C
Second Strand Synthesis Mix	**SSM** ○	159 µl	476 µl	1901 µl	-20 °C
Enzyme Mix 2**	**E2** ○	36 µl	88 µl	326 µl	-20 °C
PCR Mix**	**PCR** ○	124 µl	247 µl	801 µl	-20 °C
Barcode 00	**BC00** ○	5 µl / reaction (for 8 qPCR rxns)			-20 °C
Barcode Plate (96-well plate)	**BC**	5 µl / reaction (included in 96-well plate)			-20 °C
Bead Diluent	**BD** ●	115 µl	344 µl	1373 µl	-20 °C
Purification Module (Cat. No. 022) included in the kit					
Purification Beads	**PB**	396 µl	1188 µl	4752 µl	+4 °C
Purification Solution	**PS**	1118 µl	3353 µl	13412 µl	+4 °C
Elution Buffer**	**EB**	1056 µl	3168 µl	12936 µl	-20 °C/+4 °C

**including additional volume for 8 qPCR reactions *including 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 **BD** ● and **PS** 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 (for washing of Purification Beads, **PB**).
- Optional: SYBR Green I (Sigma-Aldrich, Cat. No. S9430), 10,000 x in DMSO for qPCR.

Equipment

- Magnetic rack or 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 (12,000 x g, rotor compatible with 1.5 ml tubes or 3,000 x g, rotor compatible with 96-well plates).
- Calibrated single-channel and multi-channel pipettes for handling 1 µl to 1000 µl volumes.
- 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.18) for more information on RNA quality.

Consult Appendix D (p.25) for information on library quantification methods.

4. Guidelines

RNA Handling

- RNases are ubiquitous, and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e. RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination. 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 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.

- Always store beads in an upright position to ensure that beads are covered by liquid.
- In general, beads should not be centrifuged during the protocol. However, should drops of fluid stay on the side of the reaction tube (e.g., after mixing by vortexing), 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 plate / tube briefly with an appropriate centrifuge.

General

- The protocol is primarily designed to be performed in PCR plates or PCR strips. However, optionally SENSE Total RNA-Seq can also be carried out in 1.5 ml tubes and thermoblocks or thermomixers.
- Unless explicitly mentioned, all steps should be carried out at a 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, **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 7 and 23 of the SENSE Total RNA-Seq protocol mastermixes of enzymes and reaction buffers should 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 7 for 24 preps: use 475.2 µl **SSM** ○ (= 18 µl x 24 rxns x 1.1)
+ 52.8 µl **E2** ○ (= 2 µl x 24 rxns x 1.1)
resulting in a total of 528 µl, which is well enough for multi-channel pipetting.

All reagents of the SENSE Total RNA-Seq kit include 10 % surplus.

Automation

SENSE Total RNA-Seq is compatible with automation. If using an automated protocol, we recommend using only a volume of up to 5 µl of RNA in step 1. In step 4 please prepare a mastermix of 3 µl Elution Buffer (**EB**) and 3 µl Enzyme Mix 1 (**E1** ●) and then add 6 µl of the **EB** / **E1** mix to the reaction. Remember to include a 10 % surplus when preparing the mastermix. Please contact support@lexogen.com for more information.

5. Detailed Protocol

5.1 Library Generation

Preparation

Reverse Transcription and Ligation	Second Strand Synthesis	Purification
ST – thawed at RT **RTL** – THAWED FOR 5 MIN, 25 °C, MIX WELL BEFORE USE! **E1** – keep on ice or at -20 °C	**SSM** – thawed at RT **E2** – keep on ice or at -20 °C	**PB** – stored at +4 °C **BD** – thawed at RT **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – thawed at RT or stored at +4 °C
Thermocycler 94 °C, 3 min 25 °C, 15 min 25 °C, 2 min 37 °C, 1 h 70 °C, 5 min 25 °C, ∞ Magnetic plate / rack	Thermocycler 98 °C, 90 sec 65 °C, 60 sec 72 °C, 5 min 25 °C, ∞	96-well magnetic plate / rack 96-well PCR plate PCR sealing films Plate centrifuge

Reverse Transcription and Ligation

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

1 Mix up to 8 µl of your RNA with 5 µl **ST** ● and 14 µl Reverse Transcription and Ligation Mix **RTL** ● in a PCR plate or 8-well strip. **REMARK:** For degraded and FFPE RNA we recommend diluting **ST** ● 1:10 with 10 mM Tris, pH 8.0 before usage to prevent extensive linker-linker formation (see Appendix C, p.24). If a smaller volume of RNA is used, add RNAse-free water to a total volume of 27 µl. Make sure that **RTL** ● is properly thawed and mixed and mix RNA / **ST** / **RTL** well by pipetting and / or vortexing. Quickly spin down the tubes or PCR plate to ensure all liquid is collected at the bottom. **ATTENTION:** Proper mixing at this step is essential for high yield and excellent reproducibility.

2 Denature the RNA / **ST** / **RTL** mixture for 3 minutes at 94 °C in a thermocycler and then hold at 25 °C. **ATTENTION:** Modify this step for fragmented / degraded RNA such as FFPE RNA (see Appendix C, p.24). For heavily degraded RNA this step may be skipped entirely. Alternatively mix 8 µl RNA and 5 µl **ST** ●, denature for 1 minute at 60 °C before adding **RTL** ● at 25 °C. Mix properly, then continue with step 3.

3 Incubate for 15 minutes at 25 °C using a thermocycler.

4	Add 3 µl of Enzyme Mix 1 (**E1** ●), mix by vortexing. Spin down the liquid and incubate at 25 °C for an additional 2 minutes.
5	Raise the temperature on the thermocycler to 37 °C and incubate for one hour. **OPTIONAL:** Extending the incubation to two hours can increase the yield. **REMARK:** At this point we recommend placing the Purification Solutions (**PB**, **PS**, **EB**) for step 10 at room temperature to give them enough time to equilibrate.
6	Incubate for 5 minutes at 70 °C to inactivate the enzymes. Cool the reaction to 25 °C **OPTIONAL:** Libraries can be stored at -20 °C at this point.

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.

7	Prepare a mastermix containing 18 µl Second Strand Synthesis Mix (**SSM** ○) and 2 µl Enzyme Mix 2 (**E2** ○) per reaction. Mix well.
8	Add 20 µl of the **SSM** / **E2** mastermix directly to the reverse transcription and ligation reaction. Mix well.
9	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. **OPTIONAL:** Libraries can be stored at -20 °C at this point..

Purification

The double-stranded library is purified using magnetic beads to remove second strand synthesis reaction components. The Purification Beads (**PB**) should equilibrate for 30 min 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 Purification Solution (**PS**) the desired library size can be defined. Please consult Appendix B: Adjusting Library Size, p.22.

10	Add 15 µl Purification Beads (**PB**), **x** µl Bead Diluent (**BD** ●), and **y** µl of Purification Solution (**PS**) to the completed second strand synthesis reaction. For obtaining libraries suitable for PE100 sequencing add 13 µl **BD** ● and 27 µl **PS**. For other read lengths please see Appendix B, p.22. Mix well by pipetting and vortexing. Incubate for 5 minutes at room temperature. **ATTENTION:** For degraded and FFPE RNA use 13 µl **BD** ● and 27 µl **PS** (see Appendix C, p.24). Do not use the PE50 cut-off as this may increase the amount of unwanted side products.
11	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

12	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
13	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.
14	Add 42 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.
15	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
16	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
17	Add 120 µl of freshly prepared 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.
18	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.
19	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.
20	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.
21	Place the plate onto a magnetic plate and let the beads collect for 2 - 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 additional 17 µl Elution Buffer (**EB**) 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).
22	After elution, libraries can be stored at -20 °C for later amplification.

5.2 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 } 17 - 21 x see Appendix B, p.22 and Appendix C, p.24 72 °C, 1 min 10 °C, ∞	96-well magnetic plate 96-well PCR plates PCR sealing films Plate centrifuge

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 Solutions (**PB**, **PS**, **EB**) for step 27 at room temperature to give them enough time to equilibrate.

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

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

25 Add 5 µl of the respective external Barcode Primer (**BC01-96**, in 96-well plate). **ATTENTION:** Spin down barcode plate before opening! Pierce or cut open the sealing foil of the wells containing the desired barcodes. Avoid cross contamination! Reseal opened wells after usage to prevent cross contamination! Mix the PCR reaction 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).

26 Conduct 17 to 21 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 17 to 21 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 10 and your input RNA amount. Please refer to the tables in Appendix B, p.22 for reference values generated with Universal Human Reference RNA and Appendix C, p.24 for FFPE RNA. Other RNA inputs may require different cycle numbers, hence we recommend taking 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 may have settled and must be properly resuspended before adding them to the reaction.

27	Add 27 µl of properly resuspended Purification Beads (**PB**) to each reaction, mix well, and incubate for 5 minutes at room temperature.
28	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
29	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
30	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.
31	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.
32	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
33	Remove and discard the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed.
34	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.
35	Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.
36	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.
37	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.
38	Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
39	Transfer 15 -17 µl of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.
40	At this point, the libraries are finished and ready for quality control (Appendix D, p.25), pooling (for multiplexing see Appendix E, p.27), and cluster generation.

6. Short Procedure

ATTENTION: Spin down solutions before opening tubes or plates!

130 min Library Generation

☐	Mix 8 µl RNA, 5 µl **ST** ● and 14 µl **RTL** ●. **ATTENTION:** Dilute **ST** ● 1:10 for FFPE / degraded RNA.	Reverse Transcription and Ligation
☐	Heat to 94 °C for 3 min, then cool to 25 °C. **ATTENTION:** Modify this step for FFPE / degraded RNA.	
☐	Incubate for 15 min at 25 °C.	
☐	Add 3 µl **E1** ●, mix well and incubate for 2 min at 25 °C.	
☐	Raise temp. to 37 °C and incubate for 1 h (**OPTIONAL:** 2 h).	
☐	Heat to 70 °C for 5 min to inactivate the enzymes. =>Save stopping point.	
☐	Pre-mix 18 µl **SSM** ○ and 2 µl **E2** ○ per reaction, mix well.	2^{nd} Strand Synthesis
☐	Add 20 µl **SSM / E2** mix directly to the RT / lig reaction, mix well.	
☐	Incubate: 98 °C / 90 sec, 65 °C / 60 sec, 72 °C / 5 min , 25 °C / ∞. =>Save stopping point.	
☐	Add 15 µl **PB**+ .....µl **BD** ● + ...µl **PS**(see p.22), mix well, incubate 5 min at RT. **ATTENTION:** For FFPE / degraded RNA use 15 µl **PB** + 13 µl **BD** ● + 27 µl **PS**.	Purification
☐	Place on magnet for 2 - 5 min, remove and discard supernatant.	
☐	Add 30 µl **EB**, mix well, incubate 2 min at RT.	
☐	Add 42 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.	
☐	Add 20 µl **EB**, mix well, incubate 2 min at RT.	
☐	Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate (see p.19 for qPCR). =>Save stopping point.	

85 min Library Amplification

☐	Pre-mix 7 µl **PCR** ○ and 1 µl **E2** ○ per reaction, mix well.	PCR
☐	Add 8 µl **PCR** / **E2**mix to 17 µl of each purified library.	
☐	Add 5 µl **BC** for each reaction, mix well. **ATTENTION:** Reseal opened BC wells after usage!	
☐	PCR: 98 °C, 30 sec 98 °C, 10 sec / 65 °C, 20 sec / 72 °C, 30 sec } 17 - 21 x (see p.22 and p.24) 72 °C, 1 min 10 °C, ∞ =>Save stopping point.	
☐	Add 27 µl **PB**, 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 minutes.	
☐	Add 20 µl **EB**, mix well, incubate 2 min at RT.	
☐	Place on magnet for 2 - 5 min, transfer 15 - 17 µl of the supernatant into a fresh PCR plate.	

7. Appendix A: RNA Requirements - PCR Cycles

rRNA Removal

High quality RNA-Seq data relies on high quality input RNA. Ribosomal RNAs (rRNAs) should be removed before starting a SENSE Total RNA-Seq sample preparation as otherwise they will consume the majority of the sequencing space. rRNA removal can either be achieved by rRNA depletion or by poly(A) selection. There are several kits available on the market for both strategies. Lexogen also offers a Poly(A) RNA Selection Kit (Cat. No. 039.100) as well as the SENSE mRNA-Seq Library Preparation Kit (Cat. No. 001.08, 001.24, and 001.96) which already includes a poly(A) bead selection step. SENSE mRNA-Seq library generation is performed directly on the oligodT beads, hence even 1 ng total RNA can be used as staring material.

For rRNA depletion we recommend using an rRNA depletion kit that also the removes the mitochondrial rRNA (mt-rRNA), which would otherwise make up more than 40 % of the reads. For rat / human / mouse samples we recommend using Lexogen's RiboCop (Cat. No. 037.24, 037.96). A bundle version of SENSE Total RNA-Seq and RiboCop is also available (Cat. No. 042.08, 042.24, 042.96). Alternatively, Ribo-Zero Gold Kit from Epicentre (an Illumina company), GeneRead rRNA Depletion Kit (Qiagen), or NEBNext rRNA Depletion Kit may be used as well. For very limited amounts of RNA RiboGone from Clontech may be used. SENSE is compatible with all available rRNA depletion kits.

RNA Input Amount

200 ng total RNA inserted into a RiboCop rRNA depletion (~2 ng rRNA depleted RNA) can be channeled directly into a SENSE Total RNA-Seq sample prep and amplified with 18 cycles. The amount of non-ribosomal RNA recovered depends on the depletion kit. For Universal Human Reference RNA the non-ribosomal RNA fraction usually is between 1 % and 5 %. Higher recovery rates usually indicate rRNA contamination.

This protocol was tested extensively with poly(A)-selected and rRNA-depleted RNAs from various mouse tissues and Universal Human Reference RNA (UHR). Typical inputs between 0.6 ng and 8.8 ng rRNA-depleted or poly(A) RNA (which would correspond to 100 ng - 1 µg of total RNA) generate high quality libraries for single-end 50 nt sequencing (SR50) with 17 - 19 cycles of library amplification. For other library sizes PCR cycles may need to be adjusted to result in the desired library yield. Some reference values are shown in a table in Appendix B (p.22).

The input requirements for your particular experiment may be different, and we have included extra reagents (**PCR** ○, **E2** ○, and **BC00** ○) for 8 qPCR assays to help with optimization. If RNA input is not sufficient, 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 1 - 2 ng of rRNA-depleted RNA or mRNA. Dilute the samples you want to check by qPCR by adding 17 µl of Elution

Buffer (**EB**) or RNAse-free water to the 17 µl of your eluted library from step 21 and continue as described in the qPCR section (p.19) .
Lexogen also offers a PCR Add-on Kit for Illumina (Cat. No. 020.96), which can be used for additional qPCR assays, should 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 the primer without barcode referred to as Barcode 00 (**BC00** ○) in step 25 of the protocol. Insert 17 µl (of the diluted 34 µl double stranded library, step 21) into a qPCR reaction. Simply add SYBR Green I (or an equivalent fluorophore) to the PCR reaction to a final concentration of 0.1x. For SYBR Green I use 1.2 µl of a 2.5x SYBR Green I solution (1:4000 SYBR Green I dilution, diluted in DMSO). 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 (=1:100 SYBR Green I dilution) 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 or any equivalent devise.
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: 0.6 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 maximum fluorescence) was 20 cycles. The remaining half of the template should be amplified with 20 cycles, whereas the undiluted cDNA of library 2 can be amplified with 19 cycles, as here double the amount of template is inserted into the PCR.

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 but undercycled libraries 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 be used only 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. Best practice is to check the RNA before mRNA selection or rRNA depletion as well as afterwards. After poly(A) selection or rRNA depletion rRNA peaks should not be detectable any more. rRNA depleted samples will show a high abundance of short RNAs (especially tRNAs) which are not removed by the depletion methods. Using an RNA extraction method that avoids co-purifying those small RNA, such as Lexogen's SPLIT RNA Extraction Kit (Cat. No. 008.48, following the User Guide for extracting large RNA fractions) can be used if small RNAs are not of interest in a particular experiment. Libraries generated from lower quality input RNA may show a 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 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 also 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 agents generates a lower A260/230 ratio. Phenol also has an absorption maximum between 250 and 280 nm which overlaps that of nucleic acid, hence 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. These contaminants however should be removed by subsequent poly(A) selection or rRNA depletion.

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 much 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 (e.g., 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

SENSE Total RNA-Seq libraries can be adjusted to the desired sequencing length. This is accomplished during step 10 by modulating the amount of **BD** / **PS** addition. Please refer to the table below to see how much **BD** / **PS** is to be added for your desired read length. 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 from 17 to 19 cycles. Check the table to see which cycle number is required to obtain enough library for your selected read length. If a higher yield is required, adjust your cycle number accordingly. Each additional cycle roughly doubles your yield (depending on the amplification efficiency of the PCR). We recommend taking advantage of the qPCR assay described in Appendix A (p.19) to determine the exact cycle number necessary for your RNA input.
All reference values shown in the table below refer to 2 ng RiboCop rRNA depleted Universal Human Reference RNA (UHR) as starting material. 200 ng total RNA were inserted into a RiboCop depletion and eluted with 16 µl. 8 µl of the eluted RiboCop rRNA depleted RNA were used for the SENSE Total RNA-Seq library preparation.
SENSE Total RNA-Seq libraries are suitable for single-read (SR) and paired-end (PE) sequencing, e.g., libraries suited for ≤ PE50 are also suitable for SR50 and SR100 sequencing runs.

Sequencing length up to	Input RNA amount Universal Human Reference RNA – RiboCop depletion			µl added in step 10		Library*			Insert				Library yield		PCR cycles (2 ng depleted UHR)**
	Total RNA	rRNA depleted RNA µl	rRNA depleted RNA ng	BD	PS	Start [bp]	End [bp]	Mean size*	Mean size	> 100 nt	> 200 nt	> 300 nt	ng/µl	nM	
≤ PE50	200 ng	8 µl	2 ng	-	35 µl	150	1500	370	248	89 %	32 %	12 %	2.2	10.8	18
PE100				13 µl	27 µl	150	1500	386	264	94 %	36 %	14 %	1.8	8.2	18
≥ PE150				13 µl	8 µl	200	1500	442	320	97 %	56 %	25 %	1.6	6.4	19
≥ PE250				13 µl	-	200	2000	510	388	100 %	90 %	59 %	1.2	4.2	19

*For multiplexed libraries including a 6 nt barcode adapter sequences are 122 bp long. For non-barcoded samples (BC00 ○) the adapter sequences are 116 bp long. PE: paired-end sequencing

****ATTENTION:** Cycle numbers given in the table above are for rRNA depleted Universal Human Reference RNA. **Other RNAs may require different cycle numbers!** For other RNA inputs we highly recommend taking advantage of the qPCR assay described in Appendix A (p.19).

The size selection recommended for the different sequencing lengths in the table above was chosen 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 (i.e., one that should provide insert sizes slightly below the chosen read length of the run). While losing some sequencing space by reading into the adapter sequences the coverage along the transcripts will improve.

If using different RNA input amounts, cycle numbers need to be adjusted accordingly (for RNA amount also refer to Appendix A, p.18). The table below depicts some examples of varying input RNA amounts of rRNA depleted Universal Human Reference RNA (UHR).

100 ng - 1000 ng total RNA (UHR) was used for RiboCop rRNA depletion. The amount of rRNA depleted Universal Human Reference RNA (UHR) was determined using a Bioanalyzer Pico RNA 6000 chip. The amount of rRNA depleted UHR inserted into the SENSE library preparation determines the required cycle number during the amplification step. For other RNAs, the recovery of rRNA depleted RNA may be different, hence the amount of total RNA given in this table is only to be seen as a reference value for UHR.

Input RNA amount Universal Human Reference RNA			µl added in step 10 (PE100)		Library*			Insert				Library yield		PCR cycles for UHR RNA**
Total RNA used in RiboCop	µl from RiboCop depletion	rRNA-depleted UHR	BD	PS	Start [bp]	End [bp]	Mean size*	Mean size	> 100 nt	> 200 nt	> 300 nt	ng / µl	nM	
1000 ng	8 µl	8.8 ng	13 µl	27 µl	150	1000	381	259	95 %	40 %	15 %	3.2	14.8	17
500 ng	8 µl	3.5 ng			150	1000	382	260	96 %	40 %	14 %	2.3	10.3	18
200 ng	8 µl	2 ng			150	1500	386	264	94 %	36 %	14 %	1.3	6.1	18
100 ng	8 µl	0.6 ng			150	1500	380	258	95 %	43 %	15 %	0.9	4.2	19

*For multiplexed libraries including a 6 nt barcode adapter sequences are 122 bp long. For non-barcoded samples (**BC00**) the adapter sequences are 116 bp long.

ATTENTION: Cycle numbers given in the table above are for RiboCop rRNA depleted Universal Human Reference RNA. **Other RNAs may require different cycle numbers. For other RNA inputs we highly recommend taking advantage of the qPCR assay described in Appendix A (p.19).

Please be aware that with reduced input RNA, the library size may decrease as Starter / Stopper heterodimers hybridize more frequently. Furthermore, the amount of unwanted side products caused by the direct ligation of Starter / Stopper heterodimers to one another may increase with low input RNA levels. This side-product is visible at ~139 bp (no barcodes) or ~145 bp (with external barcodes).

Optionally this side-product can be removed by purifying the final lane mix once again using 0.9 volumes of **PB** (e.g., 50 µl lanemix + 45 µl **PB**) and incubating 5 minutes at room temperature and repeating the purification steps 28 to 40.

9. Appendix C: Low Quality RNA - FFPE

RNA isolated from Formalin-Fixed Paraffin Embedded (FFPE) samples is often heavily degraded. While RiboCop can handle FFPE RNA input without protocol adjustments, for SENSE we recommend implementing the following modifications if degraded or FFPE RNA input is used. This will prevent the generation of too short inserts and unwanted linker-linker side products.

1.) Dilute **ST** ● 1:10 with 10 mM Tris, pH 8.0 before getting started and use 5 µl of this 1:10 dilution per sample prep in step 1.

2.) Skip step 2. Alternatively mix 8 µl RNA and 5 µl **ST** ●, denature for 1 minute at 60 °C before adding **RTL** ● at 25 °C. Mix properly, then continue with step 3.

3.) In step 10 use 13 µl **BD** ● and 27 µl **PS**. Do not use the PE50 cut-off as this may increase the amount of unwanted side products.

SENSE was tested with 1.8 ng RiboCop rRNA depleted FFPE RNA from Human Adult Normal Liver Tissue with the protocol adjustments described above. The FFPE total RNA input had a RIN of 2.0 (DV200 of 58 %). The DV200 value is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV200, the more degraded the RNA is. Other FFPE RNAs, RNAs with lower DV200 values, and lower FFPE RNA input amounts may require more PCR cycles, hence we would strongly recommend using Lexogen's PCR Add-On Kit for Illumina (Cat. No. 020.96) and **taking advantage of the qPCR assay** as described in Appendix A (p.19).

Human Adult Normal Liver Tissue FFPE RNA (rRNA depleted)	Step 1 and 2	µl added in step 10 (PE100)		Library*			Insert				library yield		PCR cycles for FFPE RNA **
		BD ●	PS	Start [bp]	End [bp]	Mean size*	Mean size	> 50 nt	> 100 nt	> 200 nt	ng / µl	nM	
1.8 ng	8 µl RNA + 5 µl ST ● 1:10 diluted + 14 µl RTL; skip step 2	13 µl	27 µl	150	1000	300	178	100 %	84 %	21 %	0.7	4.0	21

*For multiplexed libraries including a 6 nt barcode adapter sequences are 122 bp long. For non-barcoded samples (**BC00**) the adapter sequences are 116 bp long.

****ATTENTION:** Cycle numbers given in the table above are for RiboCop rRNA depleted FFPE RNA from Human Adult Normal Liver Tissue. **Other RNAs may require different cycle numbers**. For other RNA inputs we highly recommend taking advantage of the qPCR assay described in Appendix A (p.19).

ATTENTION: FFPE RNA is degraded RNA, hence the insert sizes are smaller than for non-degraded RNA samples. Keep this in mind when choosing your sequencing length.

If your FFPE RNA still generates a lot of linker-linker products, an additional purification of the lane mix with 0.9 x **PB** (e.g., 50 µl lane mix plus 45 µl **PB**), incubating 5 minutes at room temperature, and following the protocol from step 28 to step 40 again may be necessary.

10. Appendix D: 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 of this User Guide 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. The use of such an assay for quantification in combination with Bioanalyzer analysis for size distribution is highly recommended.

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 (~5 ng) 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 therefore be avoided.

Typical Results

SENSE Total RNA-Seq library sizes can be varied by using different volumes of **BD** / **PS** in step 10. For a detailed overview regarding library size, insert range, and yield please refer to the table in Appendix B: Adjusting Library Size, p.22.

Typical concentrations are between 4.2 - 14.8 nM (0.9 - 3.2 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 ~139 bp (no barcodes) or ~145 bp (with external barcodes), and should compose no more than 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. Performing the qPCR reaction to determine the cycle number of your endpoint PCR as recommended in Appendix A (p.19) should prevent overcycling. Still, even overcycled PCRs can be used for subsequent sequencing reactions. However, to guarantee accurate quantification of overcycled libraries for lane mixing we recommend performing a qPCR-based quantification method rather than relying on the Bioanalyzer quantification. For further experiments using the same input RNA please adjust your cycle number accordingly.

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.19) as well as the PCR Add-on Kit Instruction Manual.

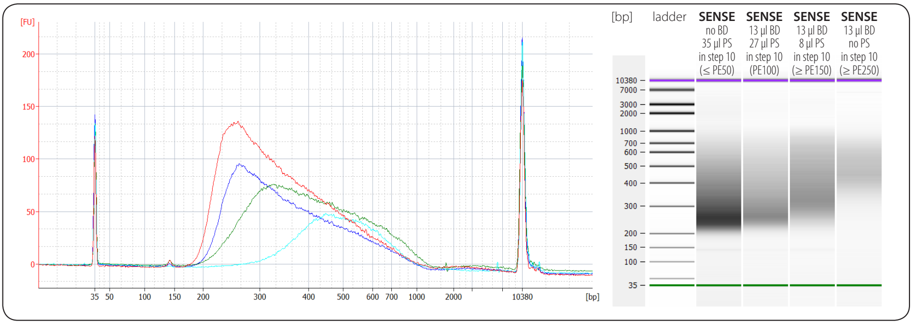

Figure 3. Bioanalyzer traces of SENSE libraries purified with different amounts of PS during step 10. Red trace: no BD, 35 µl PS, dark blue trace: 13 µl BD, 27 µl PS, green trace: 13 µl BD, 8 µl PS, and light blue trace: 13 µl BD, no PS. 200 ng total Universal Human Reference RNA (UHR) were inserted into a RiboCop depletion according to the User Guide. 2 ng (8 µl) of the resulting depleted UHR was inserted per SENSE library prep. Libraries were amplified for 18 and 19 cycles, respectively.

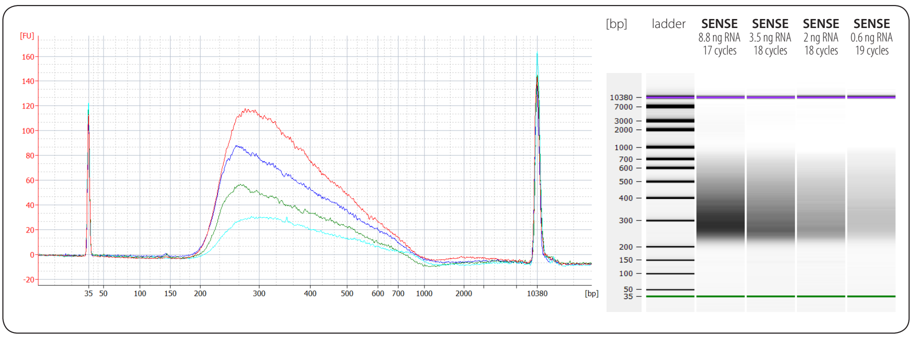

Figure 4. Bioanalyzer traces of SENSE libraries synthesized with different input RNA amounts. Input RNA was RiboCop depleted Universal Human Reference RNA. All samples shown here were purified using the settings for PE100 sequencing in step 10 (i.e., 13 µl BD and 27 µl PS). Red trace: 8.8 ng input RNA, 17 cycles; dark blue trace: 3.5 ng input RNA, 18 cycles; green trace: 2 ng input RNA, 18 cycles; light blue trace: 0.6 ng input RNA, 19 cycles.

11. Appendix E: Multiplexing

SENSE libraries can be multiplexed. Barcodes can be introduced as standard external barcodes during the PCR amplification step (step 25).

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.

ATTENTION: Avoid cross contamination! Spin down the barcode plate before removing carefully the seal. Tightly re-seal the plate for later use of the remaining barcodes.

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 or for NextSeq red laser: A/C and green laser: A/T) register a signal at each nucleotide position. Barcodes can be combined across rows or columns.

A schematic representation of the provided barcodes is shown in Figure 2, p.6.

The 8 reaction SENSE Total RNA-Seq kit (Cat. No. 009.08) includes Barcode Set 1 (BC01/BC13/BC25/BC37/BC49/BC61/BC73, and BC85).

The 24 reaction SENSE Total RNA-Seq kit (Cat. No. 009.24) 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. 009.96) includes all Barcode Sets (Set 1 - 12) and as mentioned previously barcodes can be combined here 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 on the following page.

Two samples per lane: In step 25 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 25 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 25 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 25 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. 009.24). Alternatively, if you are using the 96 reaction kit (Cat. No. 009.96) barcodes BC01 - 12 from row A can be used. Apply only one barcode to each sample.

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.

12. Appendix F: 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 and Read 2 Sequencing Primers.
External barcodes are included in the SENSE Total RNA-Seq kits (Cat. No. 009.08, Cat. No. 009.24, and Cat. No. 009.96). A schematic representation of those libraries is shown below.

Libraries with External Barcodes

External barcodes (6 nt) are introduced during PCR (step 25).

```
                           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'

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

13. Appendix G: 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. For more information please also check our website (www.lexogen.com).
In contrast to most other library preparation protocols, SENSE libraries generate reads in a strand orientation opposite to the genomic reference.

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.

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 (such as TopHat), 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. The first nine nucleotides need to be removed from Read 1 (starter side) and the first six nucleotides from Read 2 (stopper side). In case of any adapter contamination, trim also those sequences.
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. For paired-end reads remove both reads if one of the reads fails the QC. Alternatively, a less stringent aligner (e.g., STAR Aligner) could be used with the number of allowed mismatches being set to 10.

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***. Denote, the reads are in reverse complement to the transcript they derive from, hence reflect the cDNA not the mRNA. This information is important for downstream applications.

14. Appendix H: Revision History

Publication No.	Change	Page
009UG013V0122	Recommendations for low input and FFPE RNA (e.g, ST dilutions). New Appendix included for FFPE / low quality RNA. Renaming of subsequent Appendices. Page shifts.	12, 13, 16, 24
	Indication of safe stopping points.	13, 14, 17
	Re-seal used barcode wells to prevent contamination!	15
009UG013V0121	Pierce or cut open new barcode sealing. Consistency changes.	15
009UG013V0120	RiboCop as recommended depletion kit.	4, 18
	Changes to protocol (3 min 94 °C denaturation of RNA / **ST** ● / **RTL** ●). Protocol recommendations for fragmented RNA input.	12
	Changes to E1 formulation.	12
	Reduced volumes of **EB** / **PS** in steps 13 and 14, respectively.	13, 14
	Changed cut-offs in step 10.	13, 22
	PB volume post PCR reduced to 27 µl.	15
	Endpoint PCR set at 33 % of the maximum fluorescence.	19
	Re-Amplification Primer in PCR Add-on Kit.	19
	Updated figures and tables in Appendix B and C with SENSE libraries synthesized from RiboCop rRNA depleted UHR.	22, 23, 25
009UG013V0110	Renaming of PS1 ○ to BD ●.	4
	Figure 2 listing of Purification Modules 022.08., 022.24, and 022.96 (included in the kits).	5
	Extended recommendations regarding depletion kits.	11, 12
	ATTENTION: Reference values shown in Tables are for rRNA depleted UHR.	21, 22
009UG013V0101	Addition of PCR-Add-on Kit 020.96; 0.1x SYBR Green I recommendation.	18
009UG013V0100	Initial Release	

Enabling complete transcriptome sequencing

SENSE Total RNA-Seq Library Prep Kit · User Guide

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