LEXOGEN

Enabling complete transcriptome sequencing

SENSE™

Making sense of RNA sequencing

FFPE 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 V1.2)
039 (Poly(A) RNA Selection Kit)
042 (SENSE Total RNA-Seq Library Prep Kit for Illumina with RiboCop)
047 (i5 Dual Indexing Add-on Kit for QuantSeq/SENSE for Illumina)

009UG102V0111

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ATTENTION: Barcodes rearranged!

009UI093V0300

The SENSE Total RNA-Seq Library Prep Kit protocols have been updated!

Major changes of the updates:

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

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

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

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1. Overview

This SENSE Total RNA-Seq kit is a library preparation protocol designed to generate Illumina-compatible libraries from RNA extracted from Formalin-Fixed Paraffin-Embedded (FFPE) tissue or samples within 3.5 hours.
FFPE RNA is often heavily degraded due to the fixation process. As a result of this degradation 28S and 18S rRNA peaks are often completely absent from such RNA samples and hence the calculation of a meaningful RIN value is not possible anymore. A more commonly used reference value for FFPE and degraded RNA is the Distribution Value 200 (DV_{200}), which is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is. SENSE Total was used to successfully generate RNA-Seq libraries from as little as 0.7 ng FFPE RNA with a DV_{200} of only 44 % (RIN 1.6).

Due to the heavily degraded status of FFPE RNA, oligodT priming is not a good option for RNA-Seq library preps, as this will result in an extreme 3' bias. Additionally, the poly(A) tails of FFPE mRNA can be heavily modified with mono-methylol groups and might not anneal well with oligodT beads [1]. Information regarding input RNA requirements can be found in Appendix A (p.18). We recommend using Lexogen's RiboCop rRNA Depletion Kit V1.2 (Cat. No. 037.24 and 037.96), which efficiently eliminates 28S, 18S, 5.8S, 45S, 5S, mt16S, and mt12S rRNA from human, mouse, and rat samples even if the RNA input is degraded. SENSE Total RNA-Seq is also available as a bundle with RiboCop (Cat. No. 042.08, 042.24, and 042.96).

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 (>99.9 %) of the SENSE protocol and the enables the discovery and quantification of antisense transcripts and overlapping genes.

Second strand synthesis is performed to degrade the RNA, and the library is then amplified, introducing the sequences required for cluster generation (see Appendix G, p.29, for a schematic representation of the finished library). Library quantification can be performed with standard protocols and is further discussed in Appendix D, p.24. Libraries are compatible with single-read or paired-end sequencing. Up to 96 i7 indices are included in the kit and are introduced during the PCR amplification step (Appendix F, p.27). An i5 Dual Indexing Add-on Kit (Cat. No. 047) is also available for unique indexing of up to 384 libraries. Data can be analyzed with a number of standard bioinformatic pipelines. Special considerations for the analysis of SENSE data, such as read orientation, are presented in Appendix H, p.30.

[1] Masuda, N. et al., Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res. 1999 Nov 15;27(22):4436-43.

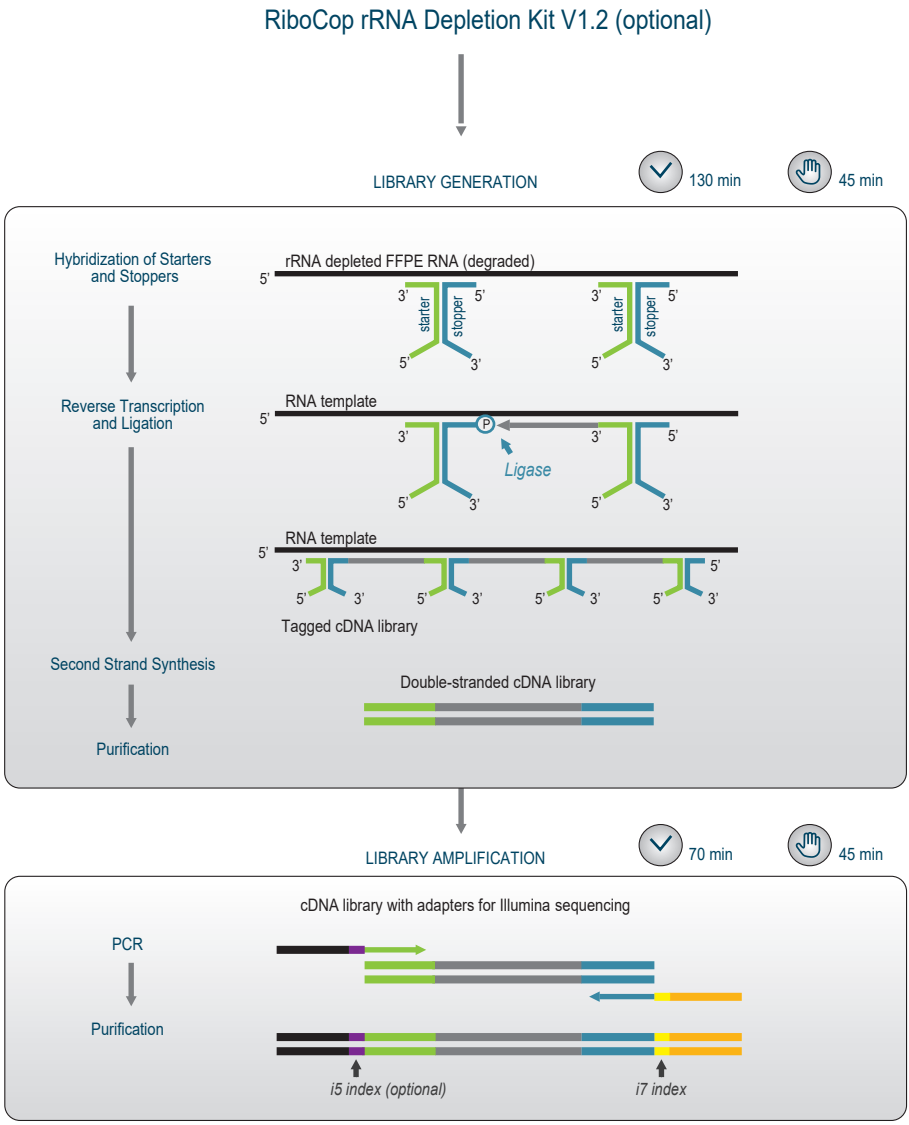


Figure 1. Schematic overview of the SENSE workflow.

2. Kit Components and Storage Conditions

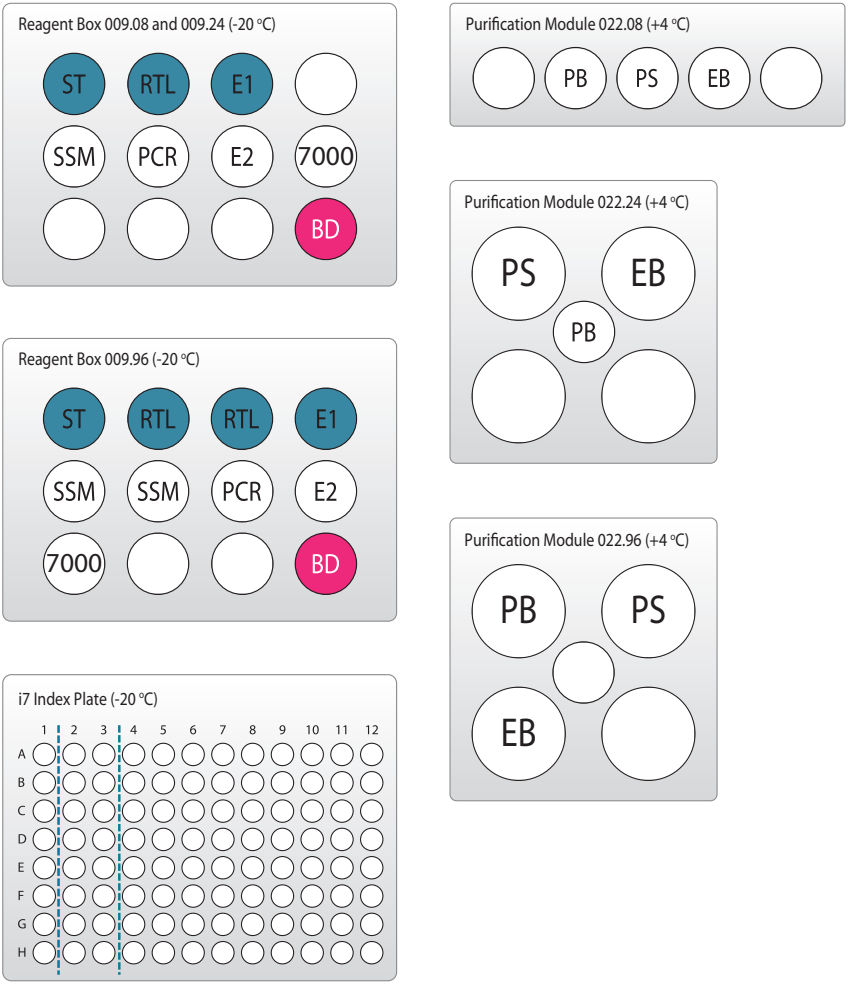


Figure 2. Location of kit contents. Each kit consists of a Reagent Box (to be stored at -20 °C) and a corresponding Purification Module (to be stored at +4 °C). For Cat. No. 009.08, 8 preps and Cat. No. 009.24, 24 preps, the included i7 Index Plate is only filled in Column 1 (indices 7001-7008) and Column 1-3 (indices 7001-7024), respectively. For Cat. No. 009.96, 96 preps all 96 indices 7001-7096 are included (to be stored at -20 °C).

Kit Component	Tube Label	Volume*			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	1,480 µl	-20 °C
Enzyme Mix 1	**E1** ●	27 µl	80 µl	317 µl	-20 °C
Second Strand Synthesis Mix	**SSM** ○	159 µl	476 µl	1,901 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
P7 Primer	**7000** ○	5 µl / reaction (for 8 qPCR rxns)			-20 °C
i7 Index Plate (96-well plate)		5 µl / reaction			-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**	370 µl	1,109 µl	4,435 µl	+4 °C
Purification Solution	**PS**	942 µl	2,825 µl	11,300 µl	+4 °C
Elution Buffer**	**EB**	880 µl	2,640 µl	10,560 µl	+4 °C / -20 °C

*including 10 % surplus

**including additional volume for 8 qPCR reactions

Upon receiving the SENSE kit, store the Purification Module (Cat. No. 022.96), containing **PB**, **PS**, and **EB** at +4 °C and the rest of the kit in a -20 °C freezer. **REMARK**: **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), 10,000x in DMSO for qPCR.
- 300 mM Tris, pH8.0 (for **ST** ● dilution).

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 1,000 µl volumes.
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

Labware

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

Optional Equipment

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

The complete set of materials, reagents, and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A (p.18) for more information on RNA quality.
Consult Appendix D (p.24) for information on library quantification methods.

4. Guidelines

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.

RNA Handling

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

eliminate RNase contamination. Well before starting a library preparation, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions. **ATTENTION:** Do not forget to rinse off any RNaseZap residue with RNase-free water after usage! Residues of RNaseZap may damage the RNA.

- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

Bead Handling

- Beads are stored at +4 °C and must be resuspended before usage. Beads can be resuspended by pipetting up and down several times or by vortexing. When properly resuspended, the solution should have a uniform brown color with no visible clumping on the walls or 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.

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 2. 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. Keep in mind to include a 10 % surplus when preparing the mastermix. Please contact info@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, prepare a fresh 1:5 dilution! **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 **EB** – thawed at RT or stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!**
Thermocycler: 25 °C, 15 min 25 °C, 2 min 37 °C, 1 h 70 °C, 5 min 25 °C, ∞ 96-well PCR plate PCR sealing films Plate centrifuge	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. Dilute an appropriate amount of **ST** ● 1:5 with 300 mM Tris, pH 8.0 to prevent extensive linker-linker formation (see Appendix B, p.21). 5 µl 1:5 diluted **ST** ● will be needed per reaction. **REMARK:** If more than 8 µl of RNA should be inserted into the library prep, **ST** ● may be diluted differently, e.g., 10.5 µl RNA + 2.5 µl **1:2.5 diluted ST** ●.

2. Mix up to 8 µl of your RNA with 5 µl **1:5 diluted ST** ● and 14 µl Reverse Transcription and Ligation Mix (**RTL** ●) in a PCR plate or 8-well strip. 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.

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

Purification

The double-stranded library is purified using magnetic beads to remove second strand synthesis reaction components. The Purification Module (**PB**, **PS**, **EB**) 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.

10 Add 15 µl Purification Beads (**PB**) and 22 µl of Purification Solution (**PS**) to the completed second strand synthesis reaction. Mix well by pipetting and vortexing. Incubate for 5 minutes at room temperature.

11 Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear (depends on the magnet strength).

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 reprecipitate 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 2 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 C (p.23).
22	☞ Safe stopping point. After elution, libraries can be stored at -20 °C for later amplification.

5.2 Library Amplification - Single Indexing (i7 only)

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

Preparation

PCR	Purification
PCR ○ – thawed at RT **E2** ○ – keep on ice or at -20 °C **i7 Index Plate** – thawed at RT; **spin down before opening!**	**PB** – stored at +4 °C **EB** – thawed at RT or stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user, **prepare fresh!**
Thermocycler 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 21 - 23x see Appendix B, p.21 72 °C, 1 min 10 °C, ∞ PCR plates, PCR sealing films, Plate centrifuge	96-well magnetic plate 96-well PCR plates PCR sealing films Plate centrifuge

PCR

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

NOTE: At this point we recommend placing the Purification Module (**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 i7 index (**7001-7096**, in 96-well plate). Mix well by pipetting. Seal the PCR plate and quickly spin down to make sure all liquid is collected at the bottom of the well. **ATTENTION:** Spin down the i7 Index Plate before opening! Visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired indices. Avoid cross contamination! Reseal opened wells of the i7 primer plate after usage to prevent cross contamination! **REMARK:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, use 5 µl of the P7 Primer (**7000** ○) per reaction. The qPCR option is available for a total of 8 samples. For further details please refer to Appendix C (p.23).

26 Conduct 21 to 23 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 21 to 23 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 your input RNA quality and amount. Please refer to the table in Appendix B, p.21 for some reference values generated with different FFPE RNAs. Other RNA inputs may require different cycle numbers, hence we recommend taking advantage of the qPCR assay as described in Appendix C (p.23).
☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

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

27	Add 25 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 29 µl of Purification Solution (**PS**) to the beads / **EB** mix to reprecipitate 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 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.
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.24), pooling (for multiplexing see Appendix F, p.27), and cluster generation. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

6. Short Procedure

ATTENTION: Spin down solutions before opening tubes or plates!

130 min Library Generation

☐	Mix 8 µl FFPE RNA, 5 µl 1:5 diluted **ST** ● (in 300 mM Tris, pH8.0) and 14 µl **RTL** ●.	Reverse Transcription and Ligation
☐	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. ☞ Safe stopping point.	
☐	Pre-mix 18 µl **SSM** ○ and 2 µl **E2** ○ per reaction, mix well.	Second 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 / ∞. ☞ Safe stopping point.	
☐	Add 15 µl **PB** + 22 µl **PS**, mix well, incubate 5 min at RT.	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.23 for qPCR). ☞ Safe stopping point.	

70 min Library Amplification

☐	Pre-mix 7 µl **PCR** ○ and 1 µl **E2** ○ per reaction, mix well.	PCR
☐	Add 8 µl **PCR** / **E2** mix to 17 µl of each purified library.	
☐	Add 5 µl i7 primer (**7001-7096**, from the 96-well plate), mix well. **ATTENTION:** Reseal opened index wells after usage!	
☐	PCR: 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec } 21- 23x (see p.21) 72 °C, 30 sec 72 °C, 1 min 10 °C, ∞. ☞ Safe stopping point.	
☐	Add 25 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 29 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. ☞ Safe stopping point.	

7. Appendix A: RNA Requirements

Potential Contaminants

RNA samples should be free of salts, metal ions, and organic solvents which can be carried over from RNA extraction. Several sources of contamination can be detected with a UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio should 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 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. DNA from FFPE samples will also be heavily degraded and hence, would not be easily distinguishable from the RNA based on size. We highly recommend using an FFPE RNA extraction kit that also contains DNase I treatment. However, heat inactivation of DNase I should be avoided at all costs. We highly recommend phenol extraction and EtOH precipitation with a carrier such as linear polyacrylamide (LPA) or silica column purification for DNase I removal. SENSE libraries generated from samples containing gDNA may have an increased number of intergenic reads and/or lower strandedness.

RNA Integrity - FFPE RNA

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. RNA extracted from Formalin-Fixed Paraffin-Embedded (FFPE) tissue is often heavily degraded due to the fixation process. As a result of this degradation 28S and 18S rRNA peaks are often completely absent from such RNA samples and hence the calculation of a meaningful RIN value is not possible anymore. A more commonly used reference value for FFPE and degraded RNA is the Distribution Value 200 (DV_{200}), which is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is. An example of an FFPE RNA and DV_{200} calculation is shown in Figure 3.

SENSE Total was used to successfully generate RNA-Seq libraries from as little as 0.7 ng FFPE RNA with a DV200 of only 44 %.

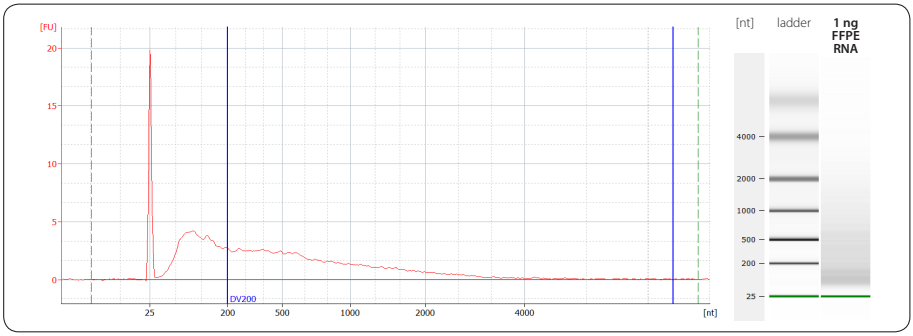


Figure 3. Bioanalyzer traces of 1 ng FFPE total RNA from normal human liver tissue (Pico RNA6000 chip). RNA is heavily degraded (absence of 28S and 18S rRNA peaks). A RIN of 2.0 is calculated for this sample by the software. With the smear analysis tool a region from 200 nt to 6000 nt can be selected, which gives the percentage of fragments larger than 200 nt, i.e., the DV_{200}. For this particular FFPE RNA, DV_{200} was 58 %.

rRNA Removal

Ribosomal RNAs (rRNAs) should be removed before starting a SENSE Total RNA-Seq library preparation as otherwise they will consume the majority of the sequencing space. However, for very limited amounts of RNA, sequencing a total RNA sample deeper may be considered as an alternative. For fragmented and FFPE RNA, rRNA should be removed by rRNA depletion as poly(A) selection would result in a massive 3' bias and a potential distortion of the gene expression profile as not all RNAs are fragmented to the same extent.

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 for than 40 % of the reads. For human / mouse / rat samples, we recommend using Lexogen's rRNA Depletion Kit V1.2 (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.

Best practice is to check the RNA before rRNA depletion as well as afterwards mainly for concentration assessment. Depending on the sample and tissue used the non-ribosomal RNA fraction recovered is between 1 - 5 % of the total RNA. Significantly higher recovery rates may point to incomplete rRNA depletion. If RNA is extremely limited, the post depletion check may be skipped.

RNA Storage

RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Avoid frequent freeze / thaw cycles to prevent further damage of the RNA. Addition of an RNase inhibitor, e.g., RNasin from Promega at a final concentration of 0.4 U/µl, is recommended.

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. For degraded RNA samples such as FFPE RNA a reduction of spike-in transcripts may be considered (e.g., only 1/10 of the recommended amount) to prevent the intact spike-in transcripts from taking up too much of the sequencing space.

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 RNA Spike-in Controls (Ambion Inc.). These sets of RNAs, just as the SIRVs, have a known strand orientation and no (or in case of the SIRVs only known) antisense transcripts, so the calculation of strandedness based on spike-in sequences is more accurate than calculations based on reads aligning to the genome. ERCC spike-in transcripts are however monoexonic hence for SENSE we recommend using SIRVs. For degraded RNA samples such as FFPE RNA a reduction of spike-in transcripts (e.g., only 1/10 of the recommended amount) may be considered to prevent the intact spike-in transcripts from taking up too much of the sequencing space.

8. Appendix B: RNA Input

General Considerations - Starter/Sstopper Mix Dilutions

RNA isolated from Formalin-Fixed Paraffin-Embedded (FFPE) samples is often heavily degraded. Using degraded RNA input will also result in shorter insert sizes in the resulting NGS library compared to using intact RNA. The short insert size has to be taken into consideration when choosing the sequencing length of an NGS-run. DV_{200} is the percentage of RNA fragments larger than 200 nucleotides. The lower the DV_{200}, the more degraded the RNA is. Degraded RNA input will require the dilution of the Starter/stopper heterodimers (**ST** ●). Diluting **ST** ● will prevent the generation of too short inserts and unwanted linker-linker side-products. As a starting point we recommend **ST** ● dilutions of 1:5. For a DV_{200} below 50 %, it may be beneficial to use higher **ST** ● dilutions, e.g., 1:10, if the amount of unwanted side product is too prominent. Higher **ST** ● dilutions may also require an increase in cycle number (at least +1 cycle, for more accurate determination please use the qPCR assay, see Appendix C, p.23).

FFPE RNA Input Amount and Quality

For determining the input RNA amount, we highly recommend using an RNA6000 Pico chip or if such a device is not available use Qubit measurements using the RNA High Sensitivity Kit. **ATTENTION:** Do not use NanoDrop quantification as with limited RNA amounts the NanoDrop tends to severely overestimate the amount of RNA.

Typical inputs between 0.7 ng and 2 ng rRNA depleted FFPE RNA generate libraries for single-read 50 nt (SR50) or 75 nt (SR75) sequencing with 21 - 23 cycles of library amplification. SENSE Total RNA-Seq was tested on several mouse (Mm) and human (Hs) FFPE RNAs with different DV_{200}. For instance, 2 ng DNase I treated and RiboCop rRNA depleted FFPE Mm Spleen RNA with a DV_{200} of 51 % using an **ST** ● dilution of 1:5 in step 1 generated 10.5 nM library with 23 cycles of amplification. Exemplary data of SENSE libraries generated from FFPE RNA from a variety of tissues and RNA quality are depicted in the table below.

RiboCop rRNA Depleted FFPE RNA from	DV_{200}	ng Inserted into SENSE	Step 1: ST dilution	Step 10: PS	Library*			Insert				Library Yield		PCR Cycles for FFPE RNA**
					Start [bp]	End [bp]	Mean Size*	Mean Size	> 50 nt	> 100 nt	> 200 nt	ng / µl	nM	
Hs Liver	58 %	1.8 ng	ST ● 1:5	22 µl	150	600	300	178	100 %	84 %	21 %	1.4	8.0	21
Mm Brain	65 %	1.6 ng			150	600	271	149	100 %	74 %	9 %	1.8	10.4	21
Mm Lung	55 %	2 ng			150	600	269	147	99 %	73 %	8 %	1.3	7.5	22
Mm Spleen	51 %	2 ng			150	600	268	146	99 %	72 %	8 %	1.7	10.5	23
Mm Liver	47 %	2 ng			150	600	254	132	98 %	62 %	5 %	1.5	9.2	23
Mm Heart	44 %	0.7 ng			150	600	250	128	98 %	58 %	4 %	0.7	4.5	23

*For multiplexed libraries including a 6 nt index adapter sequences are 122 bp long. For non-indexed samples (P7 Primer **7000**) the adapter sequences are 116 bp long.

****ATTENTION:** Cycle numbers in the table above are specific to the RiboCop rRNA depleted FFPE RNA samples listed here. **Other FFPE RNAs, RNAs with different DV_{200}, or different input amounts may require different cycle numbers**. We 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 C (p.23).

The input requirements for your particular experiment may be different, therefore we have included extra reagents (**PCR** ○, **E2** ○, and P7 Primer **7000** ○) for 8 qPCR assays to help with cycle optimization. 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.

As a starting point, we recommend performing the protocol initially with 2 ng of DNAse I treated and rRNA depleted FFPE RNA. 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).

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. **EXAMPLE:** For the Mm Heart FFPE RNA sample, 42 % of the inserts are below 100 nt, which means in a SR100 sequencing run already 42 % of the reads will contain adapter sequences.

Extremely degraded RNA and insufficient **ST** ● dilution may lead to unwanted side-products at ~145 bp. For further information please refer to Appendix D, p.24 and Appendix E, p.26.

NOTE: FFPE RNA may also be contaminated with fragmented DNA, which may result in an overestimation of inserted RNA and/or in a high number intergenic reads in NGS samples. For FFPE RNA it may therefore be advisable to perform a DNase I treatment or at least distinguish between RNA and DNA when quantifying your input material.

9. Appendix C: qPCR and Reamplification

qPCR to Determine the Exact Cycle Number of Your Endpoint PCRs

Dilute the samples you want to check by qPCR by adding 2 µl of Elution Buffer (**EB**) or RNase-free water to the 17 µl of your eluted library from step 21. For determining the cycle number of your endpoint PCR, please use 5 µl of the P7 Primer without index (**7000** ○ included in this kit for 8 reactions or **7000** ● from the PCR Add-on Kit for Illumina (Cat. No. 020.96)) in step 25 of the protocol. Insert 1.7 µl (of the diluted 19 µl double-stranded library, step 21) into a qPCR reaction. Simply add SYBR Green I (or an equivalent fluorophore) to the PCR reaction to a final concentration of 0.1x. For SYBR Green I use 1.2 µl of a 2.5x SYBR Green I solution (1:4000 SYBR Green I dilution, diluted in DMSO). Fill up the total PCR reaction volume to 30 µl with water or **EB**. Alternatively, if 8 or more qPCRs are run at the same time, best practice would be to prepare a mastermix with 0.15 µl of a 20x SYBR Green I solution (1:100 SYBR Green I dilution in DMSO) per reaction. SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually. Overcycle this initial qPCR (30 cycles or even more if little input material was used; include a no template control!). Determine the fluorescence value at which the fluorescence reaches the plateau. Calculate where **the fluorescence is at 50 % of the maximum** and determine at which cycle these 50 % of fluorescence are reached. As in the endpoint PCR 10x more cDNA will be used compared to the qPCR, three cycles can be subtracted from the determined cycle number. This is the cycle number you should use for the endpoint PCR using the remaining 17 µl of the template. There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer. **EXAMPLE:** 0.7 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 (50 % of maximum fluorescence) was 26 cycles. The remaining 17 µl template (i.e.,10 times more cDNA than in the qPCR, hence -3 cycles) should be amplified with 23 cycles. The undiluted cDNA of library 2 can be amplified with 23 cycles as well. **REMARK:** If the amount of unwanted side-product is too high (e.g., due to heavily degraded RNA and insufficient **ST** ● dilution), the qPCR results may be driven mainly by the side product and the final amplification may still result in lower library yield than intended. **ATTENTION:** The qPCR approach described here is valid regardless if dual or single indexed endpoint PCRs are intended.

Reamplification of i7 Indexed Libraries (i7 only)

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

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

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

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 the equipment available. 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, e.g., Qubit dsDNA HS assay. However, most UV-Vis spectrophotometers are not sensitive enough at these concentrations to accurately quantify NGS libraries and cannot discern between unwanted side-products and actual library and should therefore be avoided.

Typical Results

Typical concentrations of SENSE Total RNA-Seq libraries generated from FFPE RNA are between 4.5 - 10.5 nM (0.7 - 1.8 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 indices) or ~145 bp (with single indexing, i7), and should compose no more than 3 % of the total library. Higher proportions of this side-product could occur if the RNA is too degraded (DV_{200} below 40 %) and/or if the starter/stopper heterodimers (**ST** ●) were too concentrated. By preparing a lane mix and carrying out an additional purification of the lane mix with 0.9x **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 such libraries can still be used for sequencing (see also Appendix E, p.26).

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. Performing the qPCR reaction to determine the cycle number of your endpoint PCR as recommended in Appendix C (p.23) 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 i7 indexed libraries be undercycled, reamplification using Lexogen's PCR Add-on Kit for Illumina (Cat. No. 020.96) is possible. For more details please refer to Appendix C (p.23) as well as the PCR Add-on Kit Instruction Manual.

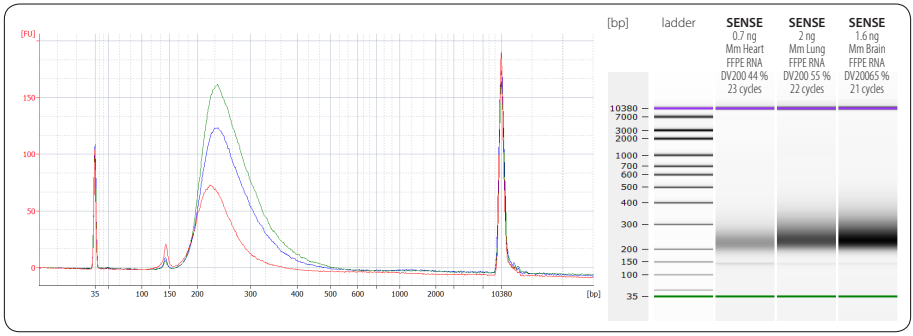


Figure 4. Bioanalyzer traces of SENSE libraries synthesized with different FFPE RNA amounts and qualities. Red trace: 0.7 ng Mm Heart FFPE RNA (DV_{200} = 44 %), 23 cycles; dark blue trace: 2 ng Mm Lung FFPE RNA (DV_{200} = 55 %), 22 cycles; green trace: 1.6 ng Mm Brain FFPE RNA (DV_{200} = 65 %), 21 cycles. For all libraries ST ● was diluted 1:5. The small peak around 145 bp is an unwanted side-product generated by the ligation of starter/stopper heterodimers and should not comprise more than 3 % of the total library (red trace: 9%, dark blue trace: 2 % and green trace 1 %). The amount of side-product correlates with the DV_{200} value. The more degraded the RNA i.e., the lower the DV_{200} value, the higher the amount of unwanted side product. To prevent this, higher ST dilutions e.g., 1:10 are recommended for FFPE RNAs with a DV200 below 50 %. Alternatively, a repurification of the lane mix containing libraries with a higher percentage of side product would be highly recommended (see also Appendix E, Removal of Artifacts, p.26).

11. Appendix E: Removal of Artifacts

FFPE RNA is often heavily degraded. As the starter/stopper heterodimers hybridize along the RNA, degraded RNA input will require the dilution of the starter/stopper heterodimers (**ST** ●). Diluting **ST** ● will prevent the generation of too short inserts and unwanted linker-linker side-products. As a starting point we recommend **ST** ● dilutions of 1:5.
For samples with a DV_{200} below 50 % it may be beneficial to use higher **ST** ● dilutions, e.g., 1:10, if the amount of unwanted side product is too prominent. Higher **ST** ● dilutions may also require an increase in cycle number (at least 1 cycle more, for more accurate determination please use the qPCR assay, see Appendix C, p.23).

If the amount of unwanted side-product is much higher than 3 % an additional purification step should be included. If multiple samples are prepared for a NGS sequencing, we highly recommend preparing a lane mix first and then performing the repurification. Repurification of the lane mix will provide a more efficient clean up than repurifying only the individual artifact contaminated library.
For preparing the lane mix, equal molar amounts of each individual library should be mixed. Here it is important that the molarity of the actual library and not the unwanted side product is used for the calculation, so we do not recommend preparing the lane mix according to NanoDrop, Qubit, or qPCR measurements. On microcapillary electrophoresis devices such as the Bioanalyzer, size ranges can be selected with the smear analysis tool. In order to completely exclude the linker-linker artifacts a size range from 155 bp to the library end (for FFPE samples this will be around 600 bp) should be selected. Equal amounts of each library within this size selection should be mixed (e.g., 10 fmol of each library). Afterwards the lane mix should be purified using 0.9x **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.

12. Appendix F: Multiplexing

SENSE libraries can be multiplexed. i7 indices (included in the kit) are introduced during the PCR amplification step (step 25). The i5 Dual Indexing Add-on Kit (Cat. No. 047) enables dual indexing (also introduced during the PCR amplification step) and allows for up to 384 different indexing combinations. For details on i5 indexing and sample sheet entry, please refer to the i5 Dual Indexing Add-on Kit Instruction Manual (047IM109).

i7 Indices

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

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

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

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

REMARK: If an 8 nt i7 index (Index 1) needs to be entered into an Illumina sample sheet, e.g., if SENSE libraries are multiplexed with 8 nt indexed libraries from other NGS-kit suppliers, add two nucleotides from the Illumina adapter sequence to the 3' end of the index. **EXAMPLE: 7001** would become CAGCGT**AT**, **7002** would become GATCAC**AT** and so on. These additional nucleotides are identical for all indices as they are derived from the Illumina adapter.

Some examples for subsets of indices are listed below.

Two samples per lane: In step 25 use 5 µl of an equimolar mix of **7001-7004** for one sample and 5 µl of an equimolar mix of **7005-7008** for the second. Here four indices are applied to each sample in order to provide a perfect nucleotide balance of the index read-out. Alternatively, two indices can be applied per sample but check the color balance using the evaluation tool at www.lexogen.com. For instance, use 2.5 µl of **7006** and 2.5 µl of **7008** for one sample and 2.5 µl of **7023** and 2.5 µl of **7096** for the second. Here two indices are applied to each sample in order to balance the red and green laser signals.

Four samples per lane: In step 25 use 5 µl of an equimolar mix of **7001-7002** for one sample, 5 µl of an equimolar mix of **7003-7004** for the second, 5 µl of an equimolar mix of **7005-7006** for the third, and 5 µl of an equimolar mix of **7007-7008** for the fourth. Here two indices are applied to each sample in order to provide a perfect nucleotide balance of the index read-out. Alternatively, when using only 1 index per sample we recommend checking the color balance with the evaluation tool provided at www.lexogen.com. Indices **7006**, **7008**, **7023**, and **7096** are examples of four well-balanced indices.

Eight samples per lane: In step 25 use 5 µl of indices **7001-7008** (column 1 of the i7 Index Plate). Apply only one index to each sample.

Twelve samples per lane: In step 25 use indices **7001-7008** (column 1 of the i7 Index Plate) plus indices **7009-7012** (first 4 indices of column 2 of the i7 Index Plate). Apply only one index to each sample.

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

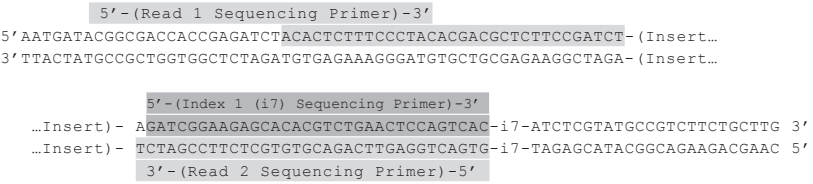
13. Appendix G: Sequencing*

General

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. Each sequencing facility has slightly different preferences of how much to load. From our experience a good starting point is to load between 7 to 14 pM of a SENSE library onto the flow cell. All SENSE libraries can be sequenced using the standard Illumina Multiplexing Read 1 and Read 2 Sequencing Primers.
Multiplexing indices (i7) 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 i7 Indexing

i7 indices (6 nt) are introduced during PCR (step 25).



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

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

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

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

14. Appendix H: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of SENSE NGS data and is kept as general as possible for integration with your standard pipeline. For more information please also check our website (www.lexogen.com/sense-data-analysis/).
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.

Demultiplexing

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

Trimming

As SENSE is based on random priming, there may be a higher proportion of errors at the first nucleotides of the insert due to non-specific hybridization of the starter/stopper heterodimer to the RNA. These mismatches can lead to a lower percentage of mappable reads when using a stringent aligner (such as TopHat), in which case it may be beneficial to trim these nucleotides. Trimming can be done with the same workflow 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.

15. Appendix I: Revision History

Publication No.	Change	Page
009UG102V0111	Safe stopping points added.	15-17
009UG102V0110	Referral to i5 Dual Indexing Add-on Kit (Cat. No. 047) for up to 384 unique indexing options.	4, 27
	Update of Figures (optional dual indexing). Kit Contents: i7 Index Plate, BC00 renamed to 7000.	5-7, 23
	Barcode plate (BC) was rearranged for improved balance and renamed to i7 Index Plate (7001-7096). Previous BC05: TAATCG replaced by 7025: TTTATG to avoid overlap with Illumina-specific indices.	6, 15, 21, 27-28
	Rinse with RNAse-free water after RNaseZap usage!	10
	qPCR endpoint determination using only 1.7 µl template and set to 50 % FU (previously 33 %). Subtract 3 cycles from determined endpoint (EP) when using 10x as much template (17 µl in EP, 1.7 µl in qPCR).	23
	Evaluation tool for color balance of index subsets at www.lexogen.com.	27-28
009UG102V0100	Special User Guide for FFPE RNA.	

This User Guide is dedicated to Scott Tighe.

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SENSE FFPE Total RNA-Seq Library Prep Kit · User Guide

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