LEXOGEN

Enabling complete transcriptome sequencing

SENSE™

Making sense of RNA sequencing

mRNA-Seq Library Prep Kit

User Guide

Catalog Numbers:
006 (SENSE mRNA-Seq Library Prep Kit for Ion Torrent)
007A (SENSE Barcode Kit for Ion Torrent, Set A)
007B (SENSE Barcode Kit for Ion Torrent, Set B)
021 (PCR Add-on Kit for Ion Torrent)
022 (Purification Module with Magnetic Beads)
025 (SIRVs Spike-in RNA Variant Control Mixes)

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1. Overview

This SENSE mRNA-Seq kit is an all-in-one library preparation protocol designed to generate Ion Torrent-compatible libraries from total RNA within 4 hours. The SENSE protocol maintains strand-specificity (>99.9 %) and allows the mapping of reads to their corresponding strand on the genome, enabling the discovery and quantification of antisense transcripts and overlapping genes. SENSE includes poly(A) selection step, hence prior rRNA depletion is not required. Insert size can be varied during the library preparation protocol itself, meaning that size selection with additional kits is not necessary. Optional multiplexing of libraries can be carried out using up to 24 in-line barcodes.

The SENSE protocol starts with a highly specific bead-based poly(A) selection step which removes almost all traces of rRNA, tRNA, and non-polyadenylated RNA. Information regarding input RNA requirements can be found in Appendix A (p.17).

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

Second strand synthesis is performed to release the library from the oligodT beads, and the library is then amplified, introducing the complete sequences required for colony formation (see Appendix E, p.25, for a schematic representation of the finished library). SENSE only comprised two silica-based purification steps - one post second strand synthesis and one post PCR. Library quantification can be performed with standard protocols and is further discussed in Appendix C (p.22). Barcodes can be introduced as In-line barcodes at the beginning of each read (Appendix D, p.24). Data can be analyzed with a number of standard bioinformatics pipelines. Special considerations for the analysis of SENSE data, such as read orientation, are presented in Appendix F (p.26).

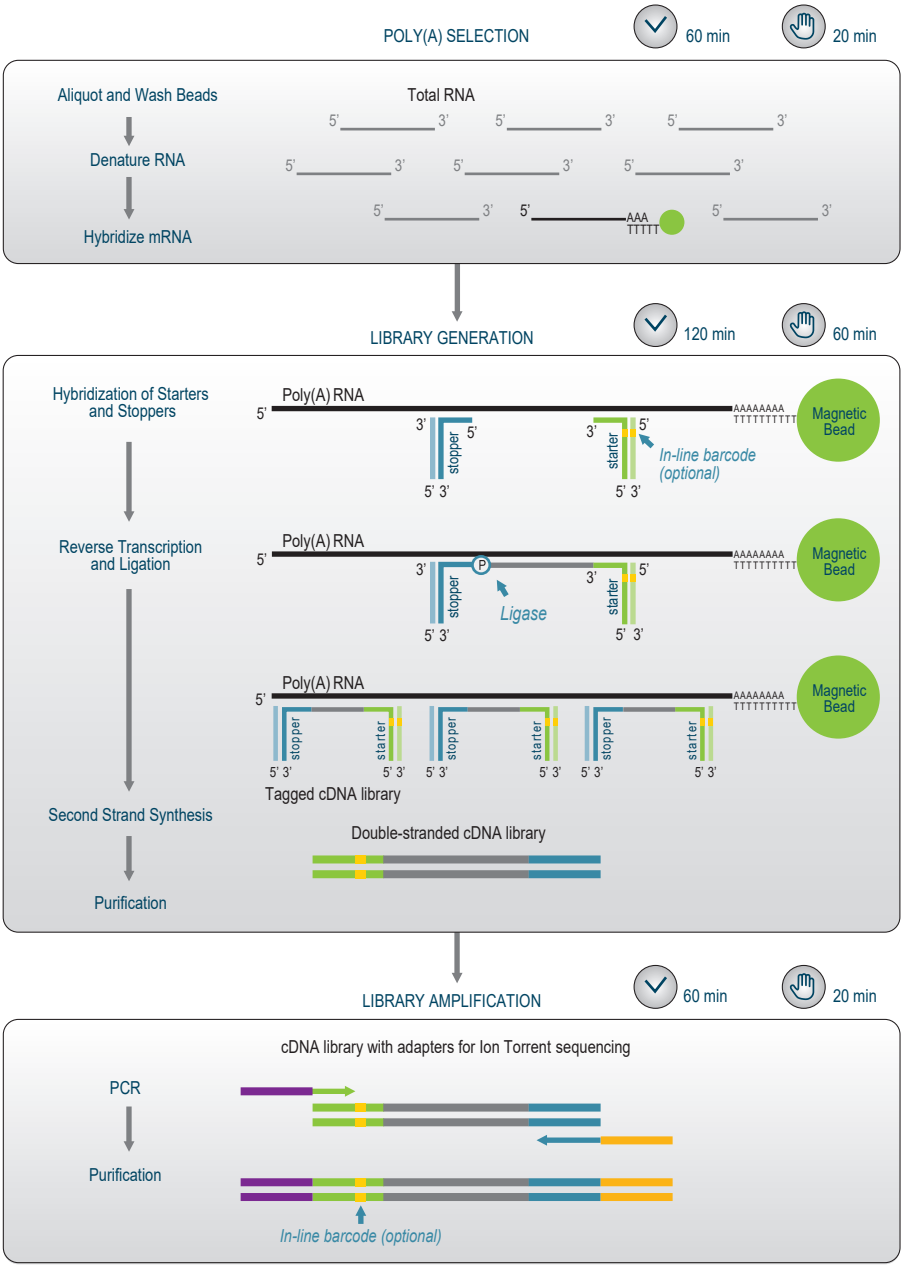

Figure 1. Schematic overview of the SENSE workflow.

2. Kit Components and Storage Conditions

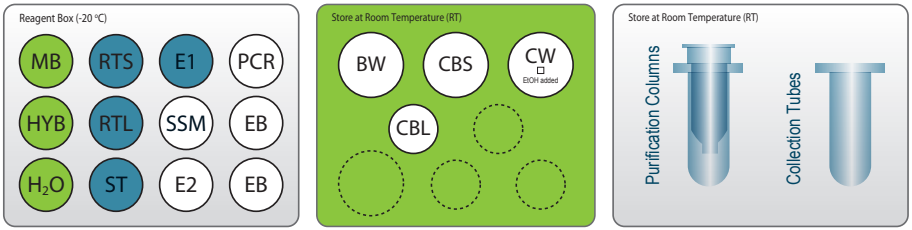

Figure 2. Location of kit contents in a 24 prep kit.

Kit Component	Tube Label	Volume* provided		Storage
		8 preps	24 preps	
Magnosphere MS150 / oligodT Beads	**MB** ●	88 µl	264 µl	-20 °C
RNA Hybridization Buffer	**HYB** ●	88 µl	264 µl	-20 °C
Molecular Biology Grade Water	**H_2O** ●	900 µl	900 µl	-20 °C
Reverse Transcription and Ligation Mix Short	**RTS** ●	321 µl	396 µl	-20 °C
Reverse Transcription and Ligation Mix Long	**RTL** ●	132 µl	396 µl	-20 °C
Starter/Stopper Mix (No In-line Barcode)	**ST** ●	17.6 µl	52.8 µl	-20 °C
Enzyme Mix 1	**E1** ●	26.4 µl	79.2 µl	-20 °C
Second Strand Synthesis Mix	**SSM** ○	79.2 µl	237.6 µl	-20 °C
Enzyme Mix 2**	**E2** ○	44 µl	132 µl	-20 °C
PCR Mix**	**PCR** ○	140.8 µl	422.4 µl	-20 °C
Elution Buffer	**EB** ○	510.4 µl	1531.2 µl	-20 °C
Bead Wash Buffer	**BW**	7.04 ml	21.12 ml	RT
Column Binding Buffer Short	**CBS**	4.22 ml	12.67 ml	RT
Column Binding Buffer Long	**CBL**	2.82 ml	8.45 ml	RT
Column Wash Buffer	**CW**	6.4 ml***	19.2 ml***	RT

*including 10 % surplus **including additional volume for one qPCR /reaction ***including ethanol (to be added by user)

Upon receiving the SENSE kit, remove the smaller inner box and store it in a -20 °C freezer. The rest of the kit components should be stored at room temperature and protected from light. Before use, check the contents of **BW**, **CBS**, **CBL**, and **CW**, which may precipitate during shipping. If a white precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

Cat. No. 006.08 (8 preps): Add 8 ml absolute ethanol to **CW** and shake to combine.
Cat. No. 006.24 (24 preps): Add 24 ml absolute ethanol to **CW** and shake to combine.

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

- Absolute ethanol, add to Column Wash Buffer (**CW**).
- Optional: SYBR Green I (Sigma-Aldrich, Cat. No. S9430), 10,000 x in DMSO for qPCR.

Equipment

- Magnetic rack / plate e.g., for 1.5 ml tubes: BcMag Magnetic separator-24, article# MS-03 from Bioclone; for 96-well plates: 96S Super Magnet Plate, article# A001322 from Alpaqua.
- Benchtop centrifuge (12,000 x g, rotor compatible with 1.5 ml tubes or 96-well plates).
- Calibrated single-channel pipettes for handling 1 µl to 1000 µl volumes.
- Thermomixer for 1.5 ml tubes or 96-well plates (dry bath incubator with shaking function).
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes)

Optional Equipment

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

Labware

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

The complete set of materials, reagents, and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A (p.17) for more information on RNA quality.
Consult Appendix C (p.22) 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 -20 °C and must be resuspended after thawing. 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 tube / plate in 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 line on the wall of the tube.
- To remove the supernatant the tube / plate containing the beads has to stay in close contact with the magnet. Do not remove the tube / plate from the magnetic stand when removing the supernatant, as the absence of the magnet will cause the beads to go into suspension again.

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

General

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

Pipetting and Handling of (Viscous) Solutions

- Enzyme mixes, **RTS** ●, and **RTL** ● 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 20 and 32 of the SENSE mRNA-Seq protocol mastermixes of enzymes and reaction buffers can be prepared. When preparing mastermixes and when using multi-channel pipettes, always include a 10 % surplus per reaction in order to have enough solution available for all reactions.

EXAMPLE: Step 32 for 24 preps: use 211.2 µl **PCR** ○ (= 8 µl x 24 preps x 1.1)
+ 52.8 µl **E2** ○ (= 2 µl x 24 preps x 1.1)
resulting in a total of 264 µl, which is well enough for multi-channel pipetting.
All reagents of the SENSE mRNA-Seq Kit include a 10 % surplus.

5. Detailed Protocol

5.1 Poly(A) Selection

Preparation

Aliquot and Wash Beads	Denature RNA	Hybridize mRNA
MB – thawed at RT **HYB** – thawed at RT **BW** – stored at RT	**Total RNA** – thawed on ice **H_2O** – thawed at RT	**BW** – stored at RT
Magnetic rack / plate	Thermocycler 60 °C, 1 min 25 °C, ∞	Thermomixer set to 25 °C 1,250 rpm

Aliquot and Wash Beads

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

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

Denature RNA

RNA samples are briefly heated to resolve secondary structures and promote efficient hybridization. For information on appropriate amounts of total RNA input as well as RNA quantification and quality control see Appendix A (p.17).

6. Dilute 500 ng to 2 µg of total RNA to a volume of 10 µl with RNase-free Water (**H_2O** ●).
7. Denature RNA samples using a thermocycler at 60 °C for 1 minute and then hold at 25 °C. Do not cool samples excessively or place denatured RNA on ice.

Hybridize mRNA

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

8. Add the 10 µl of denatured RNA to 10 µl of washed beads and incubate using a thermomixer at 25 °C for 20 minutes with 1,250 rpm agitation.
9. Transfer the tube onto a magnetic rack and let the beads collect for 5 minutes (or until the supernatant is clear). Remove and discard the supernatant.
10. Remove the tube from the magnetic rack and add 100 µl Bead Wash Buffer (**BW**). Resuspend the beads and mix well. Incubate using a thermomixer at 25 °C for 5 minutes with 1,250 rpm agitation. Collect the beads by placing the tube onto a magnetic stand for 5 minutes. Remove and discard the supernatant.
11. Repeat this washing step once (for a total of two washes).

5.2 Library Generation

Preparation

Reverse Transcription and Ligation	Second Strand Synthesis	Purification
RTS } thawed on thermomixer, **RTL** } **5 MIN 25 °C, 1,250 RPM** **ST** - thawed at RT **E1** - keep on ice or at -20 °C **BW** - stored at RT **H_2O** - thawed at RT	**SSM** - thawed at RT **E2** - keep on ice or at -20 °C	**CBS** - stored at RT **CBL** - stored at RT **CW** - stored at RT **EB** - thawed at RT
Thermomixer set to 25 °C 1,250 rpm Magnetic rack / plate	Thermocycler 98 °C, 90 sec 65 °C, 60 sec 72 °C, 5 min 25 °C, ∞	Benchtop centrifuge set to 18 °C Column (1 per sample) Collection tubes (2 per sample)

Reverse Transcription and Ligation

The starters and stoppers are hybridized to the RNA, and reverse transcription and ligation is performed, generating short cDNA fragments with linker sequences at either end.

12. After removing the supernatant from the last wash, add 15 µl Reverse Transcription and Ligation Mix **RTS** ● or **RTL** ●. **ATTENTION: RTS** ● is used for sequencing runs of up to 100 nt; **RTL** ● is used for sequencing runs ≥200 nt. Please also consult Appendix B: Adjusting Library Size (p.21).

13 Add 2 µl Starter/Stopper Mix (**ST** ●). For multiplexed libraries with in-line barcoding, replace **ST** ● with **ST01** ● through **ST24** ● (sold separately, see also Appendix D, p.24). Mix by vortexing.

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

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

16 Raise the temperature on the thermomixer to 37 °C and incubate for one hour with 1,250 rpm agitation. **OPTIONAL:** This step can be extended to 2 hours to increase the yield, e.g., for low input RNA.

17 Apply 100 µl Bead Wash Buffer (**BW**) to the RT/ligation reaction and mix thoroughly. Collect the beads with a magnetic rack for 5 minutes. Remove and discard the supernatant.

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

19 After removing the supernatant from the second wash, resuspend the beads in 10 µl RNase-free Water (**H_2O** ●).

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.

20 Transfer the resuspended beads to a PCR tube or plate containing 9 µl Second Strand Synthesis Mix (**SSM** ○). **OPTIONAL:** A mastermix of 9 µl **SSM** ○ and 1 µl **E2** ○ per reaction may be prepared beforehand.

21 Add 1 µl Enzyme Mix 2 (**E2** ○) and mix well.

22 Conduct one cycle of thermocycling with the following program: 98 °C for 90 seconds, 65 °C for 60 seconds, 72 °C for 5 minutes, hold at 25 °C.

Purification

The double-stranded library is column-purified to remove the magnetic beads and second strand synthesis reaction components.
ATTENTION: Two different column binding buffers **CBS** and **CBL** are provided to further refine library size during column purification. For appropriate mixing of **CBS** and **CBL,** please consult Appendix B: Adjusting Library Size (p.21).

23	Add a total of 160 µl Column Binding Buffer (x µl **CBS** and y µl **CBL**, see table in Appendix B, p.21) to the reaction, mix well, and transfer the solution to a purification column placed in a 2 ml Collection Tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.
24	Transfer the purification column into a new 1.5 ml tube. Discard the flow-through but do not discard the collection tube as it will be needed again in step 26.
25	Apply 20 µl Elution Buffer (**EB** ○) to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000 x g at 18 °C to elute the library.
26	Add a total of 160 µl fresh Column Binding Buffer (same mixing ratio as used in step 23: x µl **CBS** and y µl **CBL**, see table in Appendix B, p.21) to the eluted 20 µl, mix well, and reload the solution onto the same purification column. Place the purification column back into in the original collection tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.
27	Apply 200 µl of Column Wash Buffer (**CW**) to the column and centrifuge for 1 minute at 12,000 x g at 18 °C.
28	Repeat this washing step once (for a total of two washes).
29	Transfer the column to a fresh collection tube. Centrifuge for 2 minutes at 12,000 x g at 18 °C to dry the column.
30	Transfer the column to a new 1.5 ml tube and apply 13 µl Elution Buffer (**EB** ○) to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000 x g at 18 °C to elute the library. **ATTENTION:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, apply 23 µl Elution Buffer (**EB** ○) to the column. **REMARK:** This SENSE kit contains enough reagents to perform a qPCR for each reaction. For further details please refer to Appendix A (p.17).
31	After elution, libraries can be stored at -20 °C for later amplification.

5.3 Library Amplification

Preparation

PCR	Purification
PCR – thawed at RT **E2** – keep on ice or at -20 °C	**CBS** – stored at RT **EB** – thawed at RT **CW** – stored at RT
Thermocycler 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec 72 °C, 30 sec } 9 -11 x see Appendix B, p.21 72 °C, 2 min 10 °C, ∞	Benchtop centrifuge set to 18 °C Column (1 per sample) Collection tubes (2 per sample)

PCR

The library is amplified to add the complete adapter sequences required for colony formation and to generate sufficient material for quality control and sequencing.

32	Prepare a mastermix of 8 µl PCR Mix (**PCR** ○) and 2 µl Enzyme Mix 2 (**E2** ○) per reaction.
33	Add 10 µl of the **PCR** / **E2** mastermix to 10 µl of the eluted library in a PCR tube or plate. Mix thoroughly.
34	Conduct 9 to 11 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 9 to 11 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 2 minutes, hold at 10 °C. **ATTENTION:** Cycle numbers vary depending on the **CBS/CBL** mixture used in steps 23 and 26. Please refer to the table in Appendix B (p.21).

Purification

The finished library is purified from PCR components that can interfere with quantification.

35	Add 160 µl of Column Binding Buffer (**CBS**) to the reaction, mix well, and transfer the solution to a column placed in a 2 ml collection tube. Centrifuge for 1 minute at 12,000 x g at 18 °C.
36	Apply 200 µl of Column Wash Buffer (**CW**) to the column and centrifuge for 1 minute at 12,000 x g at 18 °C.
37	Repeat this washing step once (for a total of two washes).
38	Remove the column and transfer to a fresh collection tube. Centrifuge for 2 minutes at 12,000 x g at 18 °C to dry the column.
39	Transfer the column to a new 1.5 ml tube and apply 15 µl Elution Buffer (**EB** ○) to the column. Incubate at room temperature for 1 minute and centrifuge for 2 minutes at 12,000 x g at 18 °C to elute the library.
40	At this point, the libraries are finished and ready for quality control (Appendix C, p.22), pooling (for multiplexed SENSE libraries; see Appendix D, p.24), and colony formation.

6. Short Procedure

ATTENTION: Spin down solutions before opening tubes!
All centrifugation steps are at 12,000 x g and 18 °C!

60 min Poly(A) Selection

☐☐	Wash 10 µl beads (**MB** ●) twice with 200 µl **BW**.	Aliquot and Wash Beads
☐	Resuspend beads with 10 µl **HYB** ●.	
☐	Dilute 500 ng to 2 µg total RNA in a volume of 10 µl with **H_2O** ●.	Denature RNA
☐	Incubate for 1 min at 60 °C, hold at 25 °C.	
☐	Add RNA (10 µl) to beads (10 µl).	Hybridize mRNA
☐	Incubate for 20 min at 25 °C / 1,250 rpm.	
☐☐	Wash 2 x for 5 min with 100 µl **BW** at 25 °C / 1,250 rpm.	
☐	Withdraw supernatant.	

120 min Library Generation

☐	Add 15 µl **RTS** ● or **RTL** ● (see p.21) and resuspend beads.	Reverse Transcription and Ligation
☐	Add 2 µl **ST** ● (or 2 µl **ST01** ● to **ST24** ●, sold separately) and incubate for 5 min at 25 °C / 1,250 rpm. **REMARK:** For low input RNA (< 50 ng) incubate 20 min.	
☐	Add 3 µl **E1** ● and incubate for 2 min at 25 °C / 1,250 rpm.	
☐	Raise temp. to 37 °C and incubate for 1 h / 1,250 rpm. **OPTIONAL:** This step may be extended to 2 hours to increase the yield, e.g., for low input RNA.	
☐☐	Wash twice with 100 µl **BW**.	
☐	Resuspend beads with 10 µl **H_2O** ●.	
☐	Add 9 µl **SSM** ○ and 1 µl **E2** ○.	2nd Strand Synthesis
☐	Incubate: 98 °C / 90 sec, 65 °C / 60 sec, 72 °C / 5 min, 25 °C / ∞.	
☐	Add .....µl **CBS +** .....µl **CBL** (see p.21) per rxn, mix well, apply to column, centrifuge 1 min.	Purification
☐	Exchange collection tube with 1.5 ml tube.	
☐	Add 20 µl **EB** ○ to column, incubate 1 min at RT, centrifuge 2 min.	
☐	Add .....µl **CBS +** .....µl **CBL** (same ratio as before, see p.21) to eluate, mix,well, reload onto same column, transfer column into original collection tube, centrifuge 1 min.	
☐☐	Add 200 µl **CW**, centrifuge 1 min, repeat once.	
☐	Transfer column into a fresh collection tube, centrifuge 2 min.	
☐	Exchange collection tube with 1.5 ml tube.	
☐	Add 13 µl **EB** ○ to column, incubate 1 min at RT, centrifuge 2 min (for qPCR use 23 µl **EB** ○, see p.17).	

60 min Library Amplification

☐	Prepare a mastermix of 8 µl **PCR** ○ and 2 µl **E2** ○ per reaction. Mix well.	PCR
☐	Add 10 µl of the **PCR** / **E2** mastermix to 10 µl purified cDNA library, mix well.	
☐	PCR: 98 °C, 30 sec 98 °C, 10 sec 65 °C, 20 sec } 9 - 11 x (see p.21) 72 °C, 30 sec 72 °C, 2 min 10 °C, ∞	
☐	Add 160 µl **CBS**, mix well, centrifuge 1 min.	Purification
☐☐	Add 200 µl **CW**, centrifuge 1 min, repeat once.	
☐	Exchange collection tube with a new collection tube, centrifuge 2 min.	
☐	Exchange collection tube with 1.5 ml tube.	
☐	Add 15 µl **EB** ○ to column, incubate 1 min at RT, centrifuge 2 min.	

7. Appendix A: RNA Requirements - PCR Cycles

RNA Input Amount

High quality mRNA-Seq data relies on high quality input RNA. The amount of total RNA required for SENSE depends on the poly(A) RNA content of the sample in question. This protocol was tested extensively with various mouse tissues and human reference RNA. Typical inputs of 500 ng total RNA for mRNA-rich tissues (such as kidney, liver, and brain) or 2 µg total RNA for tissues with lower mRNA content (such as lung and heart) generate high quality libraries for single-end 200 nt sequencing (SR200) with 10 cycles of library amplification. For other library sizes PCR cycles need to be adjusted as described in the table of Appendix B (p.21).

The input requirements for your particular experiment may be different, and we have included extra reagents for library amplification and purification to assist with optimization. If RNA input is not sufficient either due to naturally low poly(A) RNA content or degraded RNA, additional cycles of library amplification may be necessary. However, as additional cycles of library amplification may increase the proportion of PCR duplicates, it is more desirable to increase the amount of input RNA (if possible for your application) rather than to rely on extra PCR cycles to increase library yield. **ATTENTION:** If using 50 ng total RNA input or less, we recommend increasing the **ST** ● hybridization time (step 14) from 5 minutes to 20 minutes. For reduced RNA inputs we also strongly recommend performing a qPCR assay (see below) to determine the appropriate cycle number for the endpoint PCR.

As a starting point, we recommend performing the protocol initially with 500 ng or 2 µg of total RNA according to the expected poly(A) content. After purifying the second strand synthesis reaction (p.13), elute with 23 µl Elution Buffer (**EB** ○) instead of 13 µl. To determine the exact cycle number needed for your endpoint PCRs, you have two options:

Option I – qPCR to Determine the Exact Cycle Number of Your Endpoint PCRs

Insert 10 µl (of the eluted 23 µl double stranded library, step 30) into a qPCR reaction. Simply add SYBR Green I (or an equivalent fluorophore) to the PCR-reaction to a final concentration of 0.1 x. For SYBR Green I use 1 µl of a 2x SYBR Green I solution (1:5000 diluted in DMSO). The total PCR reaction volume will be 21 µl. SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines has to be adjusted manually. Overcycle this initial qPCR (20 or 35 cycles 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-Calculate where the fluorescence is 33 % of the maximum, and use the corresponding cycle number for the endpoint PCR with the remaining 10 µl of the template. The SENSE kit is provided with enough **PCR** Mix ○ and **E2** ○ to perform 2 PCR reactions for each library. There is no need to purify or analyze the overcycled PCR reaction on a Bioanalyzer. Please be aware that the

post-PCR purification columns are only intended for the endpoint PCRs and not for the qPCR reactions (eight post-PCR purification columns, plus two extra columns for the 8 prep kit and 24 post-PCR purification columns plus six extra columns for the 24 prep kit).

EXAMPLE: 500 ng input RNA was used for generating two libraries. The cDNA of library 1 was diluted and inserted into the qPCR assay. The cycle number determined in the overcycled qPCR (33 % of the maximum fluorescence) was 10 cycles. The remaining half of the template should be amplified with 10 cycles, whereas the undiluted cDNA of library 2 can be amplified with 9 cycles, as here double the amount of template is inserted into the PCR.

Option II – Endpoint PCR with One Additional Cycle and Bioanalyzer Quantification (if qPCR is not available)

Insert 10 µl (of the eluted 23 µl double stranded library, step 30) into the PCR reaction and perform 11 cycles of library amplification instead of 10 (or one more cycle than listed in the table of Appendix B (p.21) depending on the column binding buffer (**CBS/CBL**) that was used for library purification. If the library yield is as described in Appendix C (p.22), performing the protocol on similar samples as described in the manual (with 13 µl elution buffer and 10 cycles of amplification) should generate sufficiently complex libraries. If yield is insufficient amplify the remaining 10 µl of the purified second strand synthesis reaction with 2 - 4 additional cycles (until an acceptable yield is reached), and increase the total RNA input accordingly in future experiments. Extra reagents for two (8 prep kit) or six (24 prep kit) additional library purifications are included. Lexogen also offers a PCR Add-on Kit for Ion Torrent (Cat. No. 021.96) for additional PCR reactions to establish the correct cycle number for amplification.

RNA Integrity

The integrity of an RNA sample can be assessed with a variety of methods. We recommend the use of a microfluidics assay such as the RNA6000 series for the 2100 Bioanalyzer (Agilent Technologies Inc.), although RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available. Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN), and we recommend a RIN score of 8 or greater for optimal sequencing results. Typically, such samples have easily detectable rRNA peaks and a comparatively low abundance of short RNAs, which can arise from both intact short transcripts as well as from RNA degradation. Libraries can also be generated from lower quality RNA, but this may lead to 3'-bias in sequencing results.

Potential Contaminants

RNA samples should be free of salts, metal ions, and organic solvents which can be carried over from RNA extraction. Several sources of contamination can be detected with a UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260/A280 ratio between 1.8 and 2.1. The A260/A230 ratio should be approximately 2. Several common contaminants including proteins, chaotropic salts, and phenol absorb strongly between 220 and 230 nm and can

often be identified as peaks in this region. Contamination with any of these generates a lower A260/230 ratio. Phenol also has an absorption maximum between 250 and 280 nm which overlaps that of nucleic acid, so high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts.

Genomic DNA Contamination

Depending on the RNA extraction protocol used, samples may also contain significant amounts of gDNA, which is indistinguishable from RNA on a spectrophotometer. Furthermore, as many of the dyes used in RNA microfluidics assays stain single-stranded nucleic acids much more intensely 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 also 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 bioinformatics 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 SIRVs or the ERCC RNA spike-in controls (Ambion Inc.). These sets of RNAs have a known strand orientation and no antisense transcripts, so the calculation of strandedness based on ERCC sequences is more accurate than calculations based on reads aligning to the genome.

8. Appendix B: Adjusting Library Size

The size of SENSE libraries can be adjusted to the desired sequencing length. This is accomplished by modulating the insert range of the library generated during RT/ligation and by using different size cut-offs during purification. SENSE is offered with two different reverse transcription and ligation mixes to be used in step 12 of library generation. As shown in the table below **RTS** ● will produce libraries with shorter mean insert sizes, while **RTL** ● generates libraries with longer inserts. Additionally, the desired library size can be further fine-tuned by varying the ratio of short (**CBS**) to long (**CBL**) column binding buffer in steps 23 and 26. Please refer to the table below to see which column binding buffer (**CBS**, **CBL**, and mixtures thereof) is appropriate for your desired read length. The required volumes of **CBS** and **CBL** can be added directly to the sample after second strand synthesis. The µl listed refer to the volumes needed per sample to be purified.
Depending on your selected insert range the number of PCR cycles during library amplification varies slightly (from 9 to 11 cycles). Check the table to see which cycle number is required to obtain > 6 nM of library for your selected read length.
All reference values shown here refer to 500 ng total RNA starting material (UHRR).
ATTENTION: If using lower RNA input amounts further cycles need to be added (for RNA amount and establishing PCR cycle number see also Appendix A, p.17).

Sequencing length		Ratio of CB in steps 23 + 26		Library*			Insert					library yield		PCR cycles
		CBS x	CBL y	Start [bp]	End [bp]	Mean size*	Mean size	>100 nt	> 200 nt	>300 nt	> 400 nt	ng/µl	nM	
SR100	RTS	160 µl		100	700	203	132	51 %	5 %	5 %	1 %	1.0	7.8	9
		120 µl	40 µl	100	700	232	161	71 %	10 %	2 %	1 %	1.3	8.7	11
		80 µl	80 µl	120	700	260	189	92 %	23 %	3 %	1 %	1.3	8.4	11
SR200	RTL	120 µl	40 µl	115	1,700	340	269	90 %	34 %	13 %	5 %	1.9	10.8	10
		80 µl	80 µl	130	1,700	355	284	97 %	45 %	16 %	6 %	1.6	8.1	10
SR300		40 µl	120 µl	150	1,700	403	332	99 %	64 %	26 %	11 %	1.4	6.1	10
SR400			160 µl	170	1,700	453	382	100 %	90 %	48 %	21 %	1.6	6.2	11

*For non-multiplexed libraries. Libraries prepared with in-line barcodes are 13 bp longer. SR: Single-Read Sequencing

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

ATTENTION: DO NOT USE **CBL** ALONE in step 23 or 26 if the library was synthesized with **RTS** ● as this may result in severe decrease in library yield.

9. Appendix C: Library Quality Control

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

Quality Control Methods

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

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

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

Typical Results

SENSE kits are provided with 2 different reaction buffers **RTS** ● and **RTL** ● which generate libraries with different size ranges. Additionally, the library size can be varied depending on the column binding buffers used in steps 23 and 26. For a detailed overview regarding library size, insert range, and yield please refer to the table in Appendix B: Adjusting Library Size (p.21).

Typical concentrations are between 6 - 11 nM (1.0 - 1.9 ng/µl), which are well suited for template preparation without further processing. A shorter side-product caused by the direct ligation of starters and stoppers to one another is sometimes visible at ~83 bp (~96 bp if barcodes are used) 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. This might have an impact on library quantification and PCR duplication rate. Performing the qPCR reaction to determine the cycle number of your endpoint PCR as recommended in Appendix A, p.17 should prevent overcycling. Still, even overcycled PCRs can be used for subsequent sequencing reactions without significantly compromising your results. However, for further experiments using the same input RNA please adjust your cycle number accordingly or take advantage of the qPCR option.

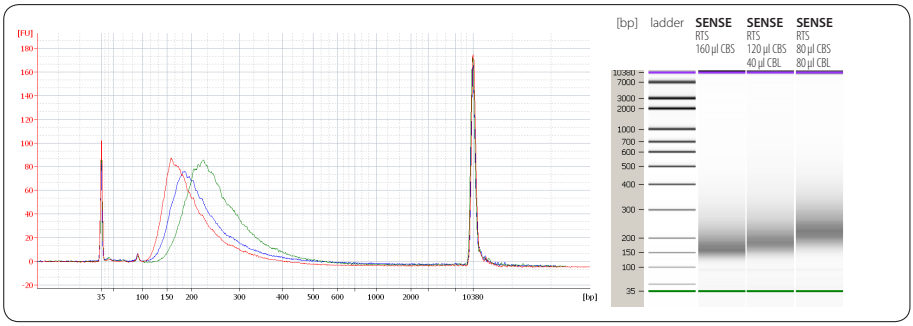

Figure 3. Bioanalyzer traces of RTS synthesized SENSE libraries from 500 ng Universal Human Reference RNA, purified with varying combinations of CBS and CBL. Red trace: 160 µl CBS, dark blue trace: 120 µl CBS + 40 µl CBL, and green trace: 80 µl CBS + 80 µl CBL.

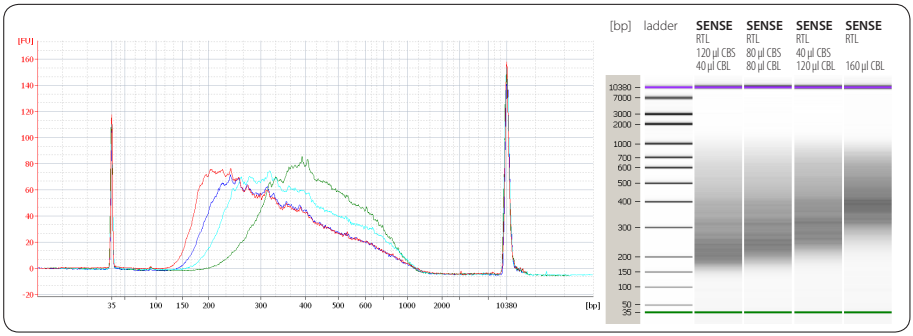

Figure 4. Bioanalyzer traces of RTL synthesized SENSE libraries from 500 ng Universal Human Reference RNA, purified with varying combinations of CBS and CBL. Red trace: 120 µl CBS + 40 µl CBL, dark blue trace: 80 µl CBS + 80 µl CBL, light blue trace: 40 µl CBS + 120 µl CBL, green trace: 160 µl CBL.

10. Appendix D: Multiplexing

SENSE libraries can be multiplexed. Barcodes can be introduced as in-line barcodes at the beginning of each read during the RT/ligation step.

In-line Barcodes

In-line barcodes can be introduced during library preparation with the SENSE In-line Barcode Kits (Cat. No. 007.08A, 007.24A , 007.08B, or 007.24B), allowing up to 24 samples to be sequenced on a single Ion chip. Indexing is performed by replacing the Starter/Stopper Mix (**ST** ●) used during reverse transcription and ligation (step 13, p.13) with Starter/Stopper Mixes supplied with the Barcode Kit (**ST01** ● to **ST24** ●).

Barcodes are 9 nt long (+ 4 nt CGAT) and are located within the first 17 nucleotides of the read (TCAG - barcode - CGAT).

Barcoded libraries should be mixed at an equimolar ratio. Ion Torrent sequencers do not have any prerequisites regarding barcode mixing. Hence, any barcode combination can be used if fewer than 12 or 24 samples are to be multiplexed.

Barcode Set A Starter/Stopper Mix;	Barcode sequence, Set A
ST01	CTAAGGTAA
ST02	TAAGGAGAA
ST03	AAGAGGATT
ST04	TACCAAGAT
ST05	CAGAAGGAA
ST06	CTGCAAGTT
ST07	TTCGTGATT
ST08	TTCCGATAA
ST09	TGAGCGGAA
ST10	CTGACCGAA
ST11	TCCTCGAAT
ST12	TAGGTGGTT

Barcode Set B Starter/Stopper Mix;	Barcode sequence, Set B
ST13	TCTAACGGA
ST14	TTGGAGTGT
ST15	TCTAGAGGT
ST16	TCTGGATGA
ST17	TCTATTCGT
ST18	AGGCAATTG
ST19	TTAGTCGGA
ST20	CAGATCCAT
ST21	TCGCAATTA
ST22	TTCGAGACG
ST23	TGCCACGAA
ST24	AACCTCATT

Barcodes sequences are available for download at www.lexogen.com. Care should be taken that the individual libraries are mixed in an equimolar ratio.
The standard Starter/Stopper Mix (**ST** ●) included in the basic kit does not contain a barcode.

11. Appendix E: Sequencing*

General

The amount of library required for the template preparation depends on which system is used for sequencing, the Ion PGM System or the Ion Proton System.
We recommend adhering the template concentration recommended by Life Technologies, which is 20 pM for the Ion PGM System (template preparation kit for the Ion OneTouch 2 System), 14 pM for the Ion PGM System (template preparation kit for the Ion OneTouch DL System), and 11 pM for the Ion Proton System (template preparation kit for the Ion OneTouch 2 System).

Libraries without Barcodes

Here the standard Starter/Stopper Mix (**ST** ●) supplied with the basic kit (Cat. No. 006.08, 006.24) is used.

```
5’CCATCTCATCCCTGCGTGTCTCCGACTCAG- Insert -ATCACCGACTGCCCATAGAGAGGAAAGCGGAGGCGTAGTGG 3’
3’GGTAGAGTAGGGACGCACAGAGGCTGAGTC- Insert -TAGTGGCTGACGGGTATCTCTCCTTTCGCCTCCGCATCACC 5’
```

Libraries with In-line Barcodes

In-line barcodes are 9 nt long followed by an additional 4 nt constant sequence (CGAT) and compose the first 17 nucleotides of the read (TCAG - barcode - CGAT). These barcodes are introduced during reverse transcription and ligation (step 13, p.13). The standard starter/stopper mix (**ST** ●) supplied with the basic kit is replaced by the starter/stopper mixes (**ST01** ● to **ST24** ●) supplied with the In-line Barcode Kits (Cat. No. 007.08A, 007.24A , 007.08B, or 007.24B). No separate read-out of the index is required.

```
5’CCATCTCATCCCTGCGTGTCTCCGACTCAG- barcode- CGAT- Insert…
3’GGTAGAGTAGGGACGCACAGAGGCTGAGTC- barcode- GCTA- Insert…

   …Insert -ATCACCGACTGCCCATAGAGAGGAAAGCGGAGGCGTAGTGG 3’
   …Insert -TAGTGGCTGACGGGTATCTCTCCTTTCGCCTCCGCATCACC 5’
```

*Note: Some nucleotide sequences shown in Appendix E may be copyrighted by Life Technologies.

12. Appendix F: Data Analysis

This section describes a basic bioinformatics workflow for the analysis of SENSE NGS data and is kept as general as possible for integration with your standard pipeline. In contrast to most other library preparation protocols, SENSE libraries generate reads in a strand orientation opposite to the genomic reference. Most tools and programs can deal with reverse complement reads. For visualization purposes, however, reads can be re-oriented, either by conversion into their reverse complement before mapping, or simply by inverting the directionality flag in the alignment files after mapping.

De-multiplexing (optional)

SENSE In-line barcodes: Barcodes are automatically demultiplexed by the machine.

The barcode is contained within the first 17 nucleotides of the read (4 nt lead sequence TCAG, 9 nt barcode plus 4 nt constant region CGAT), and the entire 17 nucleotides should be removed after de-multiplexing but before alignment.

Processing Raw Reads - 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 starters and stoppers to the RNA. These mismatches can lead to a lower percentage of mappable reads when using a stringent aligner, in which case it may be beneficial to trim these nucleotides. The first six nucleotides of the insert should be removed from Read 1 (starter side). In case of a non-multiplexed library this means the first 10 nt (TCAG plus 6 nt of the starter) of the read should be trimmed. If the insert size was smaller than the sequencing length, it might be beneficial to also trim the last 6 nt (introduced by the stopper) before reading into the P1 adapter sequence.

While trimming the first nucleotides introduced by the starters (or stoppers if sequencing already reached the P1 adapter sequence) 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 trimming we recommend using the FASTX-toolkit available from the Hannon lab (CSHL) or the trimming functions of the bbmap suite http://sourceforge.net/projects/bbmap/.

Quality Assessment

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.

SENSE starters are not entirely random. The hexamer starter is GNNNNG. Without trimming the entire starter sequence (flanked by the TCAG lead sequence and the CGAT constant sequence) is seen in the FASTQC reports.

Alignment

At this point the filtered and trimmed reads can be reverse complemented and aligned with a short read aligner to the reference genome or assembled de novo. Alternatively, reads can be mapped first without conversion to the reverse complement and the reverse complement is then generated on the output BAM-file. Also, by simply inverting the directionality flag in the alignment files, generating reverse complements can be avoided entirely.
For mapping we recommend using STAR aligner or alternatively, the TMAP mapping program, as this program is ideal for aligning reads of variable length. It includes three algorithms that may be run together (mapall) or individually (map1, map2, and map3). For RNA-Seq seed lengths of 18 nucleotides and employing the default number of allowable mismatches per seed are commonly used.

13. Appendix G: Revision History

Revision date YYYY-MM-DD	Publication No.	Change	Page
2016-04-11	006UG007V0106	Updated Figure 1, Figure 2, and Kit Contents Table.	5, 6
		Prepare mastermixes in step 20 and step 32.	13, 14
		Extended qPCR description (Example added, SYBR Green I usage).	17
2016-02-01	006UG007V0105	Consistency changes. Consitent labeling (ST01- ST24).	13, 23, 24
2015-11-26	006UG007V0104	Spike-in RNA Variant Control Mixes, Cat. No. 025.03.	0, 20
		Recommendation for Preparation of Mastermixes.	10
		Extended incubation time at steps 14 and 16 for higher yield.	13
		Endpoint PCR set at 33 % of the maximum qPCR fluorescence.	18
2015-06-01	006UG007V0103	Changes to Front Page - available Kits and Modules.	0
		Increased volumes for CW requiring more EtOH addition.	6
		Recommendation for SYBR Green I.	7
		Lowered SYBR Green I concentration in qPCR.	18
		Revision of Barcode Set B table.	24
2014-11-26	006UG007V0102	Consistency changes. Including Barcode Set B.	24
2014-02-01	006UG007V0101	Consistency changes.	
2013-08-30	7	Initial Release.	

LEXOGEN

Enabling complete transcriptome sequencing

SENSE mRNA-Seq Library Prep Kit · User Guide

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