mRNA-Seq Library Prep Kit
User Guide

Catalog Numbers:
004 (SENSE mRNA-Seq Library Prep Kit for SOLiD)
005A-H (External Barcode Kit for SOLiD, Sets A-H)
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 SOLiD-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 an integrated poly(A) selection, so prior rRNA depletion is not required. Optional multiplexing of libraries can be carried out using up to 96 external barcodes. Libraries are compatible with both single-end and paired-end sequencing reagents.

The SENSE protocol comprises a highly specific bead-based poly(A) selection step which removes almost all traces of rRNA, tRNA, and non-polyadenylated RNA. Information regarding input RNA requirements can be found in Appendix A (p.18).

Library production is initiated by the random hybridization of starter/stopper heterodimers to the poly(A) RNA still bound to the magnetic beads. These starter/stopper heterodimers contain SOLiD-compatible linker sequences (SOLiD RNA adapters). A single-tube reverse transcription and ligation reaction extends the starter to the next hybridized heterodimer, where the newly-synthesized cDNA insert is ligated to the stopper. As the insert size is determined by the distance between starter/stopper binding sites RNA fragmentation is not required. Therefore spurious second strand synthesis from the 5’ ends of fragments is absent providing the basis for the excellent strand-specificity of the SENSE protocol.

Second strand synthesis is performed to release the library from the beads, and the library is then amplified, introducing the sequences required for colony formation (see Appendix E, p.25, for a schematic representation of the finished library). Library quantification can be performed with standard protocols and is further discussed in Appendix B (p.21). Libraries are compatible with single-end or paired-end sequencing. Barcodes can be introduced as standard external barcodes (Appendix D, p.23). Data can be analyzed with a number of standard bioinformatic pipelines. Special considerations for the analysis of SENSE data, such as read orientation, are presented in Appendix F (p.26).
Figure 1. Schematic overview of the SENSE workflow.
2. Kit Components and Storage Conditions

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, and CW, which may precipitate during shipping. If a white precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

Cat. No. 004.08 (8 preps): Add 8 ml absolute ethanol to CW and shake to combine.
Cat. No. 004.24 (24 preps): Add 24 ml absolute ethanol to CW and shake to combine.

![Figure 2. Location of kit contents.](image-url)
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).
- 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 Biocline; for 96-well plates: 96S Super Magnet Plate, article# A001322 from Alpaqua.
- Benchtop centrifuge (rotor compatible with 1.5 ml tubes or 96-well plates).
- Calibrated single-channel pipettes for handling 1 µl to 1000 µl volumes.
- Thermomixer for 1.5 ml tubes or 96-well plates (dry bath incubator with shaking function).
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).

**Optional Equipment**

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

**Labware**

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

The complete set of materials, reagents, and labware necessary for RNA extraction and quality control is not listed. Consult Appendix A (p.18) for more information on RNA quality. Consult Appendix B (p.21) 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 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 containing the beads has to stay in close contact with the magnet. Do not remove the tube from the magnetic stand when removing the supernatant, as the absence of the magnet will cause the beads to go into suspension again.
- In general, beads should not be centrifuged during the protocol. However, should liquid
condense (e.g., after step 16) or become entrapped in the cap or drops of fluid stay on the side of the reaction tube, centrifugation at 2,000 x g for 30 sec should be carried out before placing the tube on the magnetic rack.

- Allowing the beads to dry out can damage them. Always keep the beads in suspension except for the short period after withdrawing the supernatant but before adding the next reagent. Beads can be resuspended by vortexing but make sure that beads are not deposited on the tube walls above the level of the liquid, where they can dry during incubation. If necessary, stuck beads can be collected by centrifuging the tube briefly with a benchtop centrifuge.

**General**

- 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 right up 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.

**Pipetting and Handling of (Viscous) Solutions**

- Enzyme mixes and RTS 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** (\(= 8 \mu l \times 24\) preps \( \times 1.1 \))

+ 52.8 µl **E2** (\(= 2 \mu l \times 24\) preps \( \times 1.1 \))

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**

<table>
<thead>
<tr>
<th>Aliquot and Wash Beads</th>
<th>Denature RNA</th>
<th>Hybridize mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB – thawed at RT</td>
<td>Total RNA – thawed on ice</td>
<td>BW – stored at RT</td>
</tr>
<tr>
<td>HYB – thawed at RT</td>
<td>H$_2$O – thawed at RT</td>
<td></td>
</tr>
<tr>
<td>BW – stored at RT</td>
<td>Thermocycler</td>
<td>Thermomixer set to 25 °C 1,250 rpm</td>
</tr>
<tr>
<td>Magnetic rack / plate</td>
<td>60 °C, 1 min 25 °C, ∞</td>
<td></td>
</tr>
</tbody>
</table>

**Aliquot and Wash Beads**

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

1. Mix the beads (MB) well. Transfer 10 µl of the resuspended beads per library preparation into a new 1.5 ml tube. Beads can be washed as a batch if multiple library preparations are required.

2. Place the tube in a magnetic rack and let the beads collect for 5 minutes or until the supernatant is completely clear (depends on the magnet strength). Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.

3. Remove the tube from the magnetic rack and add 200 µl Bead Wash Buffer (BW) per library preparation. Resuspend the beads and transfer the tube to the magnetic rack. Let the beads collect for 5 minutes; remove and discard the supernatant.

4. Repeat this washing step once (for a total of two washes).

5. Resuspend the beads in 10 µl RNA Hybridization Buffer (HYB) per library preparation. Pipette and mix carefully to avoid introducing air bubbles.

**Denature RNA**

RNA samples are briefly heated to resolve secondary structures and promote efficient hybridization. For information on appropriate amounts of total RNA input as well as RNA quantification and quality control see Appendix A (p.19).

6. Dilute 500 ng to 2 µg of total RNA to a volume of 10 µl with RNase-free Water.

7. Denature RNA samples using a thermocycler at 60 °C for 1 minute and then hold at 25 °C. Do not cool samples excessively or place denatured RNA on ice.
Hybridize mRNA

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

8 Add the 10 µl of denatured RNA to 10 µl of washed beads and incubate using a thermomixer at 25 °C for 20 minutes with 1,250 rpm agitation.

9 Transfer the tube onto a magnetic rack and let the beads collect for 5 minutes (or until the supernatant is clear). Remove and discard the supernatant.

10 Remove the tube from the magnetic rack and add 100 µl Bead Wash Buffer (BW). Resuspend the beads and mix well. Incubate using a thermomixer at 25 °C for 5 minutes with 1,250 rpm agitation. Collect the beads by placing the tube onto a magnetic stand for 5 minutes. Remove and discard the supernatant.

11 Repeat this washing step once (for a total of two washes).
5.2 Library Generation

Preparation

<table>
<thead>
<tr>
<th>Reverse Transcription and Ligation</th>
<th>Second Strand Synthesis</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTS – thawed on thermomixer, 5 MIN 25 °C, 1,250 RPM</td>
<td>SSM – thawed at RT E2 – keep on ice or at -20 °C</td>
<td>CBS – stored at RT CW – stored at RT EB – thawed at RT</td>
</tr>
<tr>
<td>ST – thawed at RT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1 – keep on ice or at -20 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW – stored at RT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2O – thawed at RT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermomixer set to 25 °C 1,250 rpm Magnetic rack / plate</td>
<td>Thermocycler 98 °C, 90 sec 65 °C, 60 sec 72 °C, 5 min 25 °C, ∞</td>
<td>Benctop centrifuge set to 18°C Column (1 per sample) Collection tubes (2 per sample)</td>
</tr>
</tbody>
</table>

Reverse Transcription and Ligation

The starter/stopper heterodimer mix is hybridized to the RNA, and reverse transcription and ligation is performed, generating short cDNA fragments with linker sequences at either end.

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

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

14 Incubate at 25 °C for 5 minutes using a thermomixer with 1,250 rpm agitation. **REMARK:** For low input RNA (< 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 and resuspend 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 (H2O).
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 O).

21. Add 1 µl Enzyme Mix 2 (E2 O) 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.

23. Add 160 µl Column Binding Buffer (CBS) 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. Do not discard the collection tube.

25. Apply 20 µl Elution Buffer (EB O) 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 160 µl Column Binding Buffer (CBS) to the eluted 20 µl, mix well, and reload the solution onto the same purification column. Place the purification column back into 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 O) 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 O) to the column. For further details please refer to Appendix A (p.18).

31. After elution, libraries can be stored at -20 °C for later amplification.
5.3 Library Amplification

Preparation

<table>
<thead>
<tr>
<th>PCR</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR – thawed at RT</td>
<td>CBS – stored at RT</td>
</tr>
<tr>
<td>E2 – keep on ice or at -20 °C</td>
<td>EB – thawed at RT</td>
</tr>
<tr>
<td></td>
<td>CW – stored at RT</td>
</tr>
<tr>
<td>Thermocycler</td>
<td>Benchtop centrifuge set to 18 °C</td>
</tr>
<tr>
<td>98 °C, 30 sec</td>
<td>Column (1 per sample)</td>
</tr>
<tr>
<td>98 °C, 10 sec</td>
<td>Collection tubes (2 per sample)</td>
</tr>
<tr>
<td>65 °C, 20 sec</td>
<td></td>
</tr>
<tr>
<td>72 °C, 30 sec</td>
<td></td>
</tr>
<tr>
<td>72 °C, 2 min</td>
<td></td>
</tr>
<tr>
<td>10 °C, ∞</td>
<td></td>
</tr>
</tbody>
</table>

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.

Transfer 10 µl of the eluted library to a PCR tube or plate containing 8 µl PCR Mix (PCR0) or 8 µl of the respective External Barcode Mix (PCR01-96) if multiplexing of libraries is intended. **REMARK:** External Barcode Mixes (005.08 A-H and 005.24 A-H) are sold separately and contain all reagents necessary for the PCR with the exception of the enzyme mix.

Add 2 µl of Enzyme Mix 2 (E2) and mix thoroughly.

Conduct 8 cycles of PCR with the following program: Initial denaturation at 98 °C for 30 seconds, 8 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:** If a qPCR is intended to determine the exact cycle number of the endpoint PCR, perform 20 or more cycles in the initial PCR using only half of the template and then use the appropriate cycle number determined according to Appendix A (p.18) for the remaining half of the template. For further details please refer to Appendix A (p.18).
Purification

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

35. Add 160 µl Column Binding Buffer (CBS) 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.

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 B, p.21), pooling (for multiplexed SENSE libraries; see Appendix D, p.23), and colony formation.
6. Short Procedure

**ATTENTION:** Spin down solutions before opening tubes or plates!

### 60 min Poly(A) Selection

- **Wash** 10 µl beads (MB) twice with 200 µl BW.
- Resuspend beads with 10 µl HYB.
- Dilute 500 ng to 2 µg total RNA in a volume of 10 µl H₂O.
- Incubate for 1 min at 60 °C, hold at 25 °C.
- Add RNA (10 µl) to beads (10 µl).
- Incubate for 20 min at 25 °C / 1,250 rpm.
- Wash 2 x for 5 min at 25 °C / 1,250 rpm with 100 µl BW.
- Withdraw supernatant.

### 120 min Library Generation

- Add 15 µl RTS and resuspend beads.
- Add 2 µl ST and incubate for 5 min at 25 °C / 1,250 rpm.
- Add 3 µl E1 and incubate for 2 min at 25 °C / 1,250 rpm.
- Raise temp. to 37 °C and incubate for 1 h / 1,250 rpm.
- Wash twice with 100 µl BW.
- Resuspend beads with 10 µl H₂O.
- Add 9 µl SSM and add 1 µl E2.
- Incubate: 98 °C / 90 sec, 65 °C / 60 sec, 72 °C / 5 min.
- Add 160 µl CBS, apply to column, centrifuge 1 min.
- Exchange collection tube with 1.5 ml tube.
- Add 20 µl EB to column, incubate 1 min at RT, centrifuge 2 min.
- Add 160 µl CBS to eluate, reload onto same column, transfer column into 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.

### 60 min Library Amplification

- Add 8 µl PCR and 2 µl E2 to 10 µl of the eluted library, mix.
- PCR: 98 °C, 30 sec
  - 98 °C, 10 sec
  - 65 °C, 20 sec
  - 72 °C, 30 sec
  - 72 °C, 1 min
- 8 x (see p.18) **ATTENTION:** Increase cycle number for low input RNA and samples with low mRNA content!
- 10 °C, ∞
- Add 160 µl CBS, centrifuge 1 min.
- Add 200 µl CW, centrifuge 1 min, repeat once.
- Exchange 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.
RNA 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 (universal human reference RNA-UHRR and brain 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 SOLiD single-end or paired-end sequencing with 8 cycles of library amplification.

The input requirements for your particular experiment may be different, and we have included extra reagents for library amplification and purification to assist with optimization. If RNA input is not sufficient, either due to naturally low poly(A) RNA content or degraded RNA, additional cycles of library amplification may be necessary. However, as additional cycles of library amplification may increase the proportion of PCR duplicates, it is more desirable to increase the amount of input RNA (if possible for your application) rather than to rely on extra PCR cycles to increase library yield.

As a starting point, we recommend performing the protocol initially with 500 ng or 2 µg of total RNA according to the expected poly(A) content. After purifying the second strand synthesis reaction (p.14), 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 1:50 SYBR Green I dilution (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 cycles or even more if little input material was used), and then determine the fluorescence value at which the fluorescence reaches a plateau. Calculate where the fluorescence is at 33 % of the maximum; this is the cycle number you should use for the endpoint PCR using 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).
Option II – Endpoint PCR with One Additional Cycle and Bioanalyzer Quantification

Insert 10 µl (of the eluted 23 µl double stranded library, step 30) into the PCR reaction and perform 9 cycles of library amplification instead of 8. 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 8 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.

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 be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. We do not recommend DNase treatment as the extended incubation with divalent cations can lead to RNA hydrolysis and decrease RNA integrity. If samples must be DNase treated, heat inactivation should be avoided and the enzyme deactivated by other means such as phenol/chloroform extraction or silica column purification.

**RNA Storage**

If immediate RNA extraction is not possible, tissue samples can be either flash-frozen with liquid nitrogen or submerged in RNAlater (Life Technologies Inc.) and stored at -80 °C. After extraction, RNA can be stored at -20 °C or -80 °C in 10 mM Tris pH 7.0. Avoid frequent freeze/thaw cycles as RNA might be sheared.

**SIRVs Spike-in RNA Variant Control Mixes**

Lexogen offers a set of artificial spike in transcripts called SIRVs (Spike-In RNA Variant Control Mixes, Cat. No. 025.03), which provide for the first time a comprehensive set of transcript variants to validate the performance of isoform-specific RNA-Seq workflows, and to serve as a control and anchor set for the comparison of RNA-Seq experiments. The SIRV sequences enable bioinformatic algorithms to accurately map, assemble, and quantify isoforms, and provide the means for the validation of these algorithms in conjunction with the preceding RNA-Seq pipelines. The SIRVs consist of 69 artificial RNA transcripts with no identity to any known genomic sequences hence they can be spiked into any RNA. Each SIRVs box contains 3 tubes labeled E0, E1, or E2. Each mix contains all 69 SIRV transcripts, but at different molar concentrations to each other. SIRV Mixes can be used as single spike-ins, or as a combination of two or three different SIRV mixes for the assessment of differential gene expression.

**ERCC RNA Spike-in Controls**

To enable the hypothesis-neutral calculation of strandedness, we highly recommend the addition of artificial transcripts of known strand orientation and concentration such as the ERCC RNA spike-in controls (Ambion Inc.). These sets of RNAs have a known strand orientation and no antisense transcripts, so the calculation of strandedness based on ERCC sequences is more accurate than calculations based on reads aligning to the genome.
8. Appendix B: 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.

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 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.
9. Appendix C: Typical Results

SENSE SOLiD libraries have an average library size of 244 bp with a size range of 125 - 700 bp (see Figure 3). Of this length, adapter sequences (including a 10 nt external barcode) consume 93 bp. Total insert size therefore ranges from approximately 32 bp to 607 bp for standard SENSE libraries, with 64 % of fragments having an insert of 100 bp or greater.

Typical concentration after 8 cycles of PCR amplification is approximately 12 nM (1.7 ng/µl), which is well suited for colony formation without further processing. A shorter side-product caused by the direct ligation of starter/stopper heterodimers to one another is sometimes visible at ~112 bp 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 on page 18 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.

<table>
<thead>
<tr>
<th>Sequencing length</th>
<th>Library*</th>
<th>Insert</th>
<th>Library yield</th>
<th>Recommended PCR cycles</th>
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<tr>
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<td>Start [bp]</td>
<td>End [bp]</td>
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<td>Mean size &gt;50 nt</td>
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<td>125</td>
<td>700</td>
<td>244</td>
<td>151</td>
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*Libraries with 10 bp external barcodes, introduced during the PCR reaction. Linker sequences plus external barcodes are in total 93 bp.
SR: Single-Read Sequencing PE: Paired-End Sequencing

Figure 3. Bioanalyzer traces of a SENSE SOLiD library. The library peaks at 200 bp, of which 93 nt are adapter sequences (including a 10 nt external barcode).
10. Appendix D: Multiplexing

SENSE SOLiD libraries are offered with an external barcoding option. External barcodes can be introduced during library amplification with the SENSE External Barcode Kits (Cat. No. 005.08 or 005.24, 12 Barcodes per set, 8 Sets (A-H)), allowing up to 96 samples to be sequenced per SOLiD flow chip lane. External barcodes are 10 nt long.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
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<tr>
<td>PCR02: AGGGAGCTGTG</td>
<td>PCR14: GTGGCCAGA</td>
<td>PCR26: CGGAGGAGG</td>
<td>PCR38: GTTGTCTGAG</td>
<td>PCR50: AAGGGCTGAG</td>
<td>PCR62: AAGGTAGAGG</td>
<td>PCR74: GAGGCCGAG</td>
<td>PCR86: AGTACCAGA</td>
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</table>

In general we recommend using a complete set of 12 barcodes for multiplexing (e.g., PCR01-12 or PCR13-24, and so on). However, SOLiD external barcodes can be used in sets of 4 (PCR01-PCR04; PCR05-PCR08; PCR09-PCR12, and so on) if fewer barcodes are required.

When choosing subsets of barcodes it is important to make sure they are color balanced. SOLiD uses a two base color code scheme. Two base encoding provides higher system accuracy and built-in error checking capability to discriminate between measurement error and true sequence variation. Since each base is interrogated twice the information about each base is included in two adjacent pieces of color space data. SENSE external barcode kits have all been color balanced within sets of 4. Some examples of subsets of barcodes are listed on page 24.
Two samples per lane: Replace the standard 8 µl PCR Mix (PCR O) with 4 µl PCR01 and 4 µl PCR02 for one sample and 4 µl PCR03 and 4 µl PCR04 for the second. Here each sample is barcoded twice in order to preserve the balance.

Four samples per lane: Replace the standard 8 µl PCR Mix (PCR O) with 8 µl PCR01 for one sample, 8 µl PCR02 for the second, 8 µl PCR03 for the third, and 8 µl PCR04 for the fourth. Apply only one PCR mix to each sample.

Eight samples per lane: Replace the standard 8 µl PCR Mix (PCR O) with 8 µl PCR01 through PCR08. Apply only one PCR mix to each sample.

Twelve samples per lane: Replace the standard 8 µl PCR mix (PCR O) with 8 µl PCR01 through PCR12. Apply only one PCR mix to each sample.

If multiplexing fewer than 12 samples per lane it is also possible to assign a specific set of barcodes to each lane mix, in which case sequencing results can be unequivocally associated with their corresponding biological samples regardless of miscommunications or mix-ups between lanes while sequencing. Various multiplexing options are available depending on your experimental design, but care should be taken to always use sets of barcodes which are color balanced. Furthermore, the individual libraries within a lane should be mixed in an equimolar ratio to ensure this balance.
11. Appendix E: Sequencing*

General
SENSE is offered with SOLiD RNA adapters or SOLiD4 DNA adapters. Please take into consideration that SOLiD 5500 DNA adapters differ in P2 adapters from the sequences shown here.

For sequencing on SOLiD 5500xl Wildfire please use the 5500 W Conversion Primers Kit sold by Life Technologies. The conversion kit contains primers to amplify the library and enable the library to be amplified on the 5500 W FlowChip for sequencing. The kit also includes an enzyme to modify the library prior to deposition of the library into the 5500 W FlowChip.

We recommend using 8 cycles of amplification with the SENSE kit and then use 5 cycles of amplification with the conversion kit on the purified SENSE libraries.

All SENSE libraries can be sequenced with standard SOLiD sequencing primers. A schematic representation of the libraries is shown below.

Libraries without Barcodes
Here the standard PCR mix (PCR o) supplied with the basic kit (Cat. No. 004.08, 004.24) is used.

5'CCACTACGCTCCGCTTCTCCCTCTCTATGGGCAGTCGGTGAT-(Insert…
3'GGTGATGCGGAGGCGAAAGGAGAGATACCCGTCAGCCACTA-(Insert…

...Insert)- CGCCTTGCCGTACAGCAGAATGAGGAACCCGGGGCAG 3'
...Insert)- GCGGAACCGGCGATCGTCCTCTCTTACTCCCTTGGGGCCCGTC 5'

Libraries with External Barcodes
External barcodes (10 nt) are introduced during PCR (step 32). The standard PCR mix (PCR o) supplied with the basic kit is replaced by PCR Mixes (PCR01-96) from the external barcode kit (Cat. No. 005.08, 005.24, sets A-H).

5'CCACTACGCTCCGCTTCTCCCTCTCTATGGGCAGTCGGTGAT-(Insert…
3'GGTGATGCGGAGGCGAAAGGAGAGATACCCGTCAGCCACTA-(Insert…

...Insert)- CGCCTTGCCGTACAGCAG-barcode-AGAGAATGAGGAACCCGGGGCAG 3'
...Insert)- GCGGAACCGGACATCGTCCTCTTACCTTGGGGCCCGTC 5'

*Note: Some nucleotide sequences shown in Appendix E may be copyrighted by Applied Biosystems, now Life Technologies.
12. Appendix F: Data Analysis

For data analysis we recommend using LifeScope Genomic Analysis Software by Applied Biosystems, now Life Technologies.

In contrast to most other library preparation protocols, SENSE libraries generate reads in a strand orientation opposite to the genomic reference during Read 1. We do not recommend reverse-complementing the reads before aligning as the leading base would be lost. We recommend either generating a reverse complement on the output BAM-file (e.g., using awk-scripts or SAMTOOLS) or simply inverting the directionality flag in the alignment files after mapping.

Trimming

From our experience trimming of the reads is not necessary when using LifeScope. However, 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 hence it may be beneficial to trim these nucleotides. Trimming can be done with the same work-flow for both reads in a paired end dataset. Six nucleotides need to be removed from both reads (Read 1 and 2). With LifeScope it is possible to move the seed to position 6. For example to map 50 bp reads use: first.map.scheme.unmapped.50=25.2:6:15. For more information please consult the LifeScope Advanced User Guide (p.148ff).

While trimming the first nucleotides introduced by the starter/stopper can decrease the number of reads of suitable length, the absolute number of mapping reads usually increases due to the improved read quality. Reads which are too short or have generally low quality scores should be removed from the set.

Alignment

At this point the filtered and trimmed reads can be aligned using LifeScope to the reference genome or assembled de novo. For visualization purposes the directionality flag in the alignment files after mapping must be inverted for Read 1.

Transcriptome Modeling

The resulting alignment files are used to model the transcriptome and assess transcript abundance. Further analyses are experiment-specific and can include differential expression, differential splicing, and promoter usage.
## 13. Appendix G: Revision History

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<th>Publication No.</th>
<th>Change</th>
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<td>Magnetic Plate and tube recommendations.</td>
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<td>Recommendations for low input RNA in step 14 and 16.</td>
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<td>Increase cycle number for low input RNA and low mRNA content.</td>
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<td>Lowered SYBR Green I concentration in qPCR.</td>
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SENSE mRNA-Seq Library Prep Kit · User Guide

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