Small RNA-Seq Library Prep Kit

User Guide

Catalog Numbers:
052 (Small RNA-Seq Library Prep Kit for Illumina)
054 (Gel Extraction Module)
058 (Small RNA-Seq Library Prep Kit for Illumina including Purification Module with Magnetic Beads)
008 (SPLIT RNA Extraction Kit)
022 (Purification Module with Magnetic Beads)
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1. Overview

The Small RNA-Seq Library Prep Kit for Illumina consists of all the reagents and components required to generate small RNA libraries to be used for Next Generation Sequencing on an Illumina platform. All molecular reagents including adapters, primers, enzyme mixes, and buffers are provided. A column-based purification is included for rapid purification of nucleic acid products generated during the workflow.

The library prep workflow could be used for different forms of input including purified total RNA or enriched small RNA, as well as RNA from low RNA content sample types such as plasma, serum, and urine. For enrichment of small RNAs we recommend using Lexogen’s SPLIT RNA Extraction Kit (Cat. No. 008).

The Small RNA-Seq Library Prep Kit for Illumina has a streamlined workflow that reduces the handling time, such that the entire library prep procedure can be completed in less than 5 hours.

Depending on the input RNA source and amount some protocol adjustments are recommended. Please refer to Appendix B, p.20 for more details.

Input RNA (see Appendix B, p.20) is first subjected to a 3’ adapter ligation. Excess 3’ adapter is then removed by column purification. This is followed by the ligation of 5’ adapters. The input RNA, flanked by 5’ and 3’ adapters, is then converted into cDNA. Multiplexing indices are introduced during the PCR amplification step, allowing multiplexing of up to 96 libraries. The library product is then subjected to a clean-up and concentration step. In most cases, particularly when the input RNA is enriched in microRNA (miRNA), the prepared library could be used directly for analysis on an Illumina sequencer.

Alternatively, a magnetic bead-based purification protocol may be used to either remove linker-linker artifacts (120 bp) or to separate the small RNA library from the total RNA library. For this purification we recommend using the Gel Extraction Module (Cat. No. 054) or the bundled version with Lexogen’s Purification Module with Magnetic Beads (Cat. No. 058).

Lexogen’s Small RNA-Seq libraries are compatible with single-read (SR) and paired-end (PE) sequencing reagents. In general, short read lengths (SR) are sufficient for sequencing small RNA-Seq libraries.
Figure 1. Schematic overview of the Small RNA-Seq Library Prep workflow.
2. Kit Components and Storage Conditions

Upon receiving the Small RNA-Seq Kit, remove the smaller inner box and store it in a -20 °C freezer. The rest of the kit components (CB, CW, columns, collection tubes) should be stored at room temperature (RT) and protected from light. Before use, check the contents of CB and CW. If a white precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

**ATTENTION:** For Cat. No. 052.08 (8 preps): Add 42 ml absolute ethanol to CW and shake to combine. For Cat. No. 052.24 (24 preps) and Cat. No. 052.96 (96 preps): Add 90 ml absolute ethanol to each CW bottle 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 with the protocol. All reagents, equipment, and labware must be free of nucleases and nucleic acid contamination.

Reagents

- Absolute (100 %) ethanol, add to Column Wash Buffer (CW) before usage.

Equipment

- Benchtop centrifuge (3,000 x g, rotor compatible with 96-well plates).
- Benchtop centrifuge (14,000 x g, rotor compatible with 1.5 ml and 2.0 ml tubes).
- Calibrated single-channel pipettes for handling 1 µl to 1,000 µl volumes.
- qPCR machine.
- Thermocycler.
- Vortex mixer.
- Ice bath or ice box, ice pellets, or benchtop cooler (-20 °C for enzymes).

Labware

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

Optional Equipment and Reagents

- Automated microfluidic electrophoresis station (e.g., Agilent Technologies 2100 Bioanalyzer).
- Agarose gels, dyes, and electrophoresis rig.
- Lexogen’s Purification Module with Magnetic Beads (Cat. No. 022).
- Lexogen’s Gel Extraction Module (Cat. No. 054).
- 6 % Novex® TBE PAGE Gel 1.0 mM 10-well (Life Technologies, Inc. #EC6265BOX).
- SYBR® Gold Nucleic Acid Gel Stain (Life Technologies, Inc. #S-11494).
- Small Pestil or Gel Breaker Tubes (IST Engineering #3388-100).
- Tubes (1.5 ml or 2 ml) for gel extraction.

The complete set of materials, reagents, and labware necessary for quality control is not listed.
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. Well before starting a library preparation, clean your workspace, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer’s instructions. **ATTENTION:** Do not forget to rinse off any RNaseZap residue with RNase-free water after usage! Residues of RNaseZap may damage the RNA.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

General
- In steps 3, 12, 17, and 20 of the Small RNA-Seq protocol, mastermixes of enzymes and reaction buffers should be prepared. When preparing mastermixes and using multi-channel pipettes always include a 10 % surplus per reaction in order to have enough solution available for all reactions. All reagents of the Small RNA-Seq Kit include a 10 % surplus.
- 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 and to avoid cross contamination a centrifugation step should be performed after incubations at elevated temperatures and before removing the sealing foil from PCR plates or opening tubes.
- Ensure that adequate volumes of all reagents and the necessary equipment are available before beginning the protocol.
- 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.
• When mixing by pipetting, set the pipette to a larger volume. For example after adding 9 µl in step 18 use a pipette set to 15 µl or 20 µl to ensure proper mixing.
• Before you start, check solutions for the formation of precipitate and if necessary, incubate at 37 °C until buffer components dissolve completely.
• If necessary the protocol can be stopped at certain points of the protocol (indicated as safe stopping points) and samples can be stored at -20 °C.
• Preheat lid to 105 °C, in case this has to be adjusted manually.

Pipetting and Handling of (Viscous) Solutions

• Enzyme mixes are viscous solutions that 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 in the tip 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. Afterwards, spin down the tube to ensure that all liquid is collected at the bottom 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 to facilitate pipetting.
Safety Information

Column Binding Buffer (CB) contains guanidine isothiocyanate, an irritant, which might also be present in the flow-through fractions. This chemical is harmful, as contact with acids liberates very toxic gas (hydrogen cyanide).

Do not add bleach or acidic solutions directly to solutions or sample preparation waste that contains guanidine isothiocyanate.

Solutions containing isopropanol or ethanol are considered flammable. Use appropriate precautions when using these chemicals.

For your protection, always wear a laboratory coat, gloves, and safety glasses when handling these chemicals.

Dispose of the buffers and chemicals in appropriate waste containers.

Consult the appropriate Material Safety Data Sheets (MSDS), available at www.lexogen.com, and contact your Environmental Health and Safety department for proper work and disposal guidelines.
5. Detailed Protocol

5.1 Library Generation

Preparation

<table>
<thead>
<tr>
<th>3' Adapter Ligation</th>
<th>Purification - Removal of Excess 3' Adapter</th>
<th>5' Adapter Ligation</th>
<th>Reverse Transcription of Ligated RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O Pane • — thawed at RT</td>
<td>CB • — stored at RT</td>
<td>A5 Pane • — thawed at RT</td>
<td>RTP Pane • — thawed at RT</td>
</tr>
<tr>
<td>A3 Pane • — thawed at RT</td>
<td>100 % EtOH • — provided by user</td>
<td>LM2 Pane • — thawed at RT</td>
<td>FConfirm • — thawed at RT</td>
</tr>
<tr>
<td>LM1 Pane • — thawed at RT</td>
<td>CW • — stored at RT add EtOH!</td>
<td>E2 Pane • — keep on ice or at -20 °C</td>
<td>EConfirm • — keep on ice or at -20 °C</td>
</tr>
<tr>
<td>E1 Pane • — keep on ice or at -20 °C</td>
<td>EB • — thawed at RT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

70 °C, 2 min place on ice 28 °C, 60 min

70 °C, 2 min place on ice 28 °C, 60 min

70 °C, 2 min place on ice 50 °C, 60 min

3’ Adapter Ligation

RNA samples and 3’ Adapter (A3 Pane •) are briefly heated to resolve secondary structures before the ligation is performed. For information on RNA quantification and quality control see Appendix A, p.18.

**ATTENTION:** Please refer to Appendix B, p.20 for information on appropriate amounts of input RNA and recommended protocol adjustments for RNA input amounts lower than 100 ng.

1. Dilute your input RNA (>100 ng) to a volume of 6 µl with Molecular Biology Grade Water (H₂O Pane •) and add 1 µl 3’ Adapter (A3 Pane •). **ATTENTION:** For RNA input amounts ≤100 ng pre-dilute A3 Pane • according to the table in Appendix B, p.20.

2. Incubate the mixture for 2 minutes at 70 °C in a pre-heated thermocycler. Place the tube on ice. Spin down before opening the tube.

3. Prepare Mastermix 1 containing 12 µl Ligation Mix 1 (LM1 Pane •) and 1 µl Enzyme Mix 1 (E1 Pane •) per reaction. Mix well and spin down. **REMARK:** When preparing mastermixes always include a 10 % surplus per reaction.

4. Add 13 µl of Mastermix 1 to the denatured RNA / A3 sample (from step 2). Mix well, spin down, and incubate the reaction in a thermocycler for 1 hour at 28 °C. ➡️ Safe stopping point. Reactions can be stored at -20 °C at this point.
Purification - Removal of Excess 3’ Adapter

Excess 3’ adapters are removed using a column-based purification. **ATTENTION:** Ensure that absolute ethanol has been added to the bottle of Column Wash Buffer (CW)! Avoid contamination! 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 to facilitate pipetting!

5
Transfer the 3’ adapter ligation reaction (20 µl) into a 1.5 ml tube and add 300 µl Column Binding Buffer (CB) and 50 µl of 100 % EtOH to the reaction, mix well. Transfer the solution onto a Purification Column placed in a 2 ml Collection Tube. **REMARK:** For inputs >100 ng, 100 µl of 100 % EtOH may be added (see Appendix B, p.20), which increases the recovery of ligated miRNA fragments but may also increase the amount of linker-linkers generated during the subsequent 5’ Adapter Ligation.

6
Centrifuge for 1 minute at 3,500 x g (~6,000 rpm) at 18 °C. Discard the flow-through.

7
Apply 600 µl of Column Wash Buffer (CW) to the column and centrifuge for 1 minute at 14,000 x g (~12,000 rpm) at 18 °C.

8
Discard the flow-through. Centrifuge for 2 minutes at 14,000 x g (~12,000 rpm) at 18 °C to dry the column.

9
Transfer the column to a new 1.5 ml tube and apply 12 µl of Elution Buffer (EB) to the column. Centrifuge for 1 minute at 200 x g (~1,400 rpm) and 2 minutes at 14,000 x g (~12,000 rpm) at 18 °C to elute the 3’ adapter-ligated RNA.

10 Transfer the eluate into an RNase-free PCR tube.

5’ Adapter Ligation

During this step the 5’ Adapter (A5 ●) is ligated to the RNA. Before ligation the 5’ Adapter is denatured.

11 Denature the required aliquot (1 µl per prep + a 10 % surplus) of the 5’ Adapter (A5 ●) for 2 minutes at 70 °C in a thermocycler. Place on ice afterwards. Spin down before opening the tube. **ATTENTION:** For ≤100 ng input RNA pre-dilute A5 ● according to the table in Appendix B, p.20.

12 Prepare Mastermix 2 containing 1 µl denatured 5’ Adapter (A5 ●), 11 µl Ligation Mix 2 (LM2 ●), and 1 µl of Enzyme Mix 2 (E2 ●) per reaction. Mix well and spin down. **REMARK:** When preparing mastermixes always include a 10 % surplus per reaction.

13 Add 13 µl of Mastermix 2 to each reaction from step 10. Mix well and spin down.
Incubate the reaction in a thermocycler for 1 hour at 28 °C.

**Reverse Transcription of Ligated RNA**

The 3’ and 5’ adapter-ligated RNA is now converted into cDNA using a Reverse Transcription Primer (RTP).

Add 1 µl Reverse Transcription Primer (RTP) to the finished reaction from step 14. Mix well and spin down. **ATTENTION:** For ≤100 ng input RNA, pre-dilute RTP according to the table in Appendix B, p.20.

Incubate for 2 minutes at 70 °C in a pre-heated thermocycler. Place the tube on ice. Spin down before opening the tube.

Prepare Mastermix 3 containing 8 µl First Strand cDNA Synthesis Mix (FS) and 1 µl of Enzyme Mix 3 (E3) per reaction. Mix well and spin down. **REMARK:** When preparing mastermixes always include a 10 % surplus per reaction.

Add 9 µl of Mastermix 3 to each reaction from step 16. Mix well and spin down.

Incubate the reaction in a pre-heated thermocycler for 1 hour at 50 °C. Spin down the reaction before proceeding. ⚠️ Safe stopping point. Libraries can be stored at -20 °C at this point.
5.2 Library Amplification

Preparation

<table>
<thead>
<tr>
<th>PCR</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>– thawed at RT</td>
</tr>
<tr>
<td>P5</td>
<td>– thawed at RT</td>
</tr>
<tr>
<td>SRi7001-7096</td>
<td>– thawed at RT</td>
</tr>
<tr>
<td>H₂O</td>
<td>– thawed at RT</td>
</tr>
<tr>
<td></td>
<td>CB – stored at RT</td>
</tr>
<tr>
<td></td>
<td>CW – stored at RT</td>
</tr>
<tr>
<td></td>
<td>EB – thawed or stored at RT</td>
</tr>
<tr>
<td>Thermocycler</td>
<td>98 °C, 30 sec</td>
</tr>
<tr>
<td></td>
<td>98 °C, 10 sec</td>
</tr>
<tr>
<td></td>
<td>60 °C, 30 sec</td>
</tr>
<tr>
<td></td>
<td>72 °C, 15 sec</td>
</tr>
<tr>
<td></td>
<td>72 °C, 10 min</td>
</tr>
<tr>
<td></td>
<td>10 °C, ∞</td>
</tr>
<tr>
<td></td>
<td>12 - 22 cycles (Appendix B, p.20)</td>
</tr>
</tbody>
</table>

Endpoint PCR

The complete Illumina P5 and P7 adapter sequences required for cluster generation are added here. PCR amplification generates sufficient material for quality control and sequencing and introduces unique indices for multiplexing. The Small RNA-Seq Kit includes 8, 24, or 96 unique Small RNA i7 Index Primers (SRi7001-7096), respectively. There is no purification step between reverse transcription and PCR.

REMARK: The PCR Mix (PCR) should not be freeze-thawed more than three times. We recommend preparing separate aliquots and storing these at -20 °C.

20 Prepare Mastermix 4 containing 50 µl of PCR Mix (PCR), 3 µl of the P5 Primer (P5), and 11 µl of Molecular Biology Grade Water (H₂O). Mix well and spin down. REMARK: When preparing mastermixes always include a 10 % surplus per reaction.

21 Add 64 µl Mastermix 4 to ~33 µl of sample (from step 19).

22 Add 3 µl of the respective Small RNA i7 Index Primer (SRi7001-7096). Mix well and spin down. Add only one index primer per sample. ATTENTION: Spin down the Small RNA i7 Index Plate before opening the wells! Visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired indices. Avoid cross contamination! Reseal opened wells after usage to prevent cross contamination! NOTE: Each well of the Small RNA i7 index Plate is intended for single use only!

23 Conduct 12 - 22 cycles (see Appendix B, p.20) of thermocycling with the following program: 98 °C for 30 seconds, 12 - 22 cycles of 98 °C for 10 seconds, 60 °C for 30 seconds, 72 °C for 15 seconds and a final extension at 72 °C for 10 minutes, hold at 10 °C.

ATTENTION: For ≤100 ng RNA input, the number of PCR cycles could be increased up to 22 cycles. Please refer to Appendix B, p.20. Safe stopping point. Libraries can be stored at -20 °C at this point.
Purification

The amplified libraries are purified to remove PCR components that can interfere with quantification and other downstream applications.

**ATTENTION:** Ensure that absolute ethanol has been added to the bottle of Column Wash Buffer (CW)! Avoid contamination! 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 to facilitate pipetting!

- **24** Transfer the finished PCR reaction (~100 µl) into a 1.5 ml tube and add 300 µl Column Binding Buffer (CB) and 50 µl 100 % EtOH to the reaction, mix well. Transfer the solution onto a Purification Column placed in a 2 ml Collection Tube.

- **25** Centrifuge for 1 minute at 3,500 x g (6,000 rpm) at 18 °C. Discard the flow-through.

- **26** Apply 600 µl of Column Wash Buffer (CW) to the column and centrifuge for 1 minute at 14,000 x g (~12,000 rpm) at 18 °C. Discard the flow-through.

- **27** Repeat this washing step once (for a total of two washes).

- **28** Discard the flow-through. Centrifuge for 2 minutes at 14,000 x g (~12,000 rpm) at 18 °C to dry the column.

- **29** Transfer the column to a new 1.5 ml tube and apply 20 µl of Elution Buffer (EB) to the column. Centrifuge for 1 minute at 200 x g (~1,400 rpm) and 2 minutes at 14,000 x g (~12,000 rpm) at 18 °C to elute the library.

- **30** At this point, the Small RNA-Seq library is finished and ready for quality control (Appendix C, p.21). Safe stopping point. Libraries can be stored at -20 °C at this point.
6. Short Procedure

All centrifugation steps are performed at 18 °C

<table>
<thead>
<tr>
<th>230 min</th>
<th>Library Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix 6 µl RNA with 1 µl A3 [ATTENTION]: Pre-dilute A3 for ≤100 ng input RNA (see p.20).</td>
<td>3' Adapter Ligation</td>
</tr>
<tr>
<td>Incubate for 2 min at 70 °C. Place on ice.</td>
<td></td>
</tr>
<tr>
<td>Pre-mix 12 µl LM1 [1] and 1 µl E1 per reaction. Mix well.</td>
<td></td>
</tr>
<tr>
<td>Add 13 µl LM1 / E1 mix per reaction, mix well.</td>
<td></td>
</tr>
<tr>
<td>Incubate for 1 hr at 28 °C. [Safe stopping point]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add 300 µl of CB and 50 µl 100 % EtOH to ~20 µl 3' ligated RNA, mix well, and apply to column. Centrifuge 1 min at 3,500 x g. Discard the flow-through. [OPTIONAL]: For input amounts &gt;100 ng use 100 µl 100 % EtOH (see p.20).</td>
</tr>
<tr>
<td>Add 600 µl of CW, centrifuge 1 min at 14,000 x g.</td>
</tr>
<tr>
<td>Discard flow-through, centrifuge 2 min at 14,000 x g.</td>
</tr>
<tr>
<td>Exchange Collection Tube with 1.5 ml tube.</td>
</tr>
<tr>
<td>Add 12 µl EB, centrifuge 1 min at 200 x g, and 2 min at 14,000 x g.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5' Adapter Ligation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denature 1.1 µl A5 per reaction for 2 min at 70 °C. Place on ice. [ATTENTION]: Pre-dilute A5 for ≤100 ng input RNA (see p.20).</td>
</tr>
<tr>
<td>Pre-mix 1 µl denatured A5 [1], 11 µl LM2 [1], and 1 µl of E2 per reaction. Mix well.</td>
</tr>
<tr>
<td>Add 13 µl A5 / LM2 / E2 mastermix per reaction. Mix well.</td>
</tr>
<tr>
<td>Incubate for 1 hr at 28 °C.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reverse Transcription of Ligated RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add 1 µl RTP per reaction. Mix well. [ATTENTION]: Pre-dilute RTP for ≤100 ng input RNA (see p.20).</td>
</tr>
<tr>
<td>Incubate for 2 min at 70 °C. Place on ice.</td>
</tr>
<tr>
<td>Pre-mix 8 µl FS and 1 µl E3 per reaction. Mix well.</td>
</tr>
<tr>
<td>Add 9 µl FS / E3 mix per reaction, mix well.</td>
</tr>
<tr>
<td>Incubate for 1 hr at 50 °C. [Safe stopping point]</td>
</tr>
</tbody>
</table>
60 min                   Library Amplification

- Pre-mix 50 µl PCR, 3 µl P5, and 11 µl H₂O.
- Add 64 µl of PCR / P5 / H₂O to ~33 µl sample.
- Add 3 µl SRi7001-7096. Use only 1 Index per sample. ATTENTION: Spin down before opening! Avoid cross contamination! Reseal opened index wells after usage!

**PCR:**
- 98 °C, 10 sec
- 60 °C, 30 sec
- 72 °C, 15 sec
- 72 °C, 10 min
- 10 °C, ∞ Safe stopping point.

- 12 - 22x
  - Appendix B, p.20

- Add 300 µl of CB and 50 µl 100 % EtOH, mix, and apply to column. Centrifuge 1 min at 3,500 x g. Discard the flow-through.
- Add 600 µl of CW, centrifuge 1 min at 14,000 x g, discard flow-through, repeat once.
- Centrifuge 2 min at 14,000 x g.
- Exchange Collection Tube with 1.5 ml tube.
- Add 20 µl EB, centrifuge 1 min at 200 x g, and 2 min at 14,000 x g. Safe stopping point.
7. Appendix A: RNA Requirements

RNA Integrity
Small RNA-Seq Library Preparation relies on high quality input RNA if microRNAs are of interest. With low quality RNA the small RNA-fraction is often contaminated with fragmented larger RNAs. 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.). Most microfluidics platforms will carry out an automated peak analysis and generate a quality score (RIN or RQN), in addition to the 28S / 18S rRNA ratio. RNA quality can also be assessed with denaturing agarose gel electrophoresis if such a device is not available.

RNA Input Considerations
Small RNAs are RNAs ≤200 nt in length, and include microRNA (miRNA), Piwi-interacting RNA (piRNA), small interfering RNA (siRNA), small nucleolar RNA (snoRNAs), tRNA-derived small RNA (tsRNA), small rDNA-derived RNA (srRNA), and small nuclear RNA commonly referred to as U-RNA. Ensure that RNA is isolated using a protocol that can recover small RNA (including miRNA). It should be noted that many products using silica-based purification technology may not recover any small RNA. Please consult manufacturer’s specification. We highly recommend using Lexogen’s SPLIT RNA Extraction Kit (Cat. No. 008, Protocol for Small RNA Fraction or Total RNA Isolation), which enables the direct extraction of small RNA (down to 17 nt). For bodily fluids such as plasma/serum, urine, or exosomes, isolation of total RNA is preferred. There is no need to fractionate for small RNA. For RNA isolations from tissue or cells, both total RNA or an enriched small RNA fraction can be used.

As any RNA fragment with a 5’ Phosphate and a 3’ OH can be used as template, Lexogen’s Small RNA-Seq Library Prep Kit may also be used for FFPE RNA samples, although here a removal of ribosomal RNA (rRNA) as well as a DNase I treatment is highly recommended before starting the Next Generation Sequencing (NGS) sample preparation.

Quantification of RNA can be performed by standard procedures including spectrophotometry (such as NanoDrop™), capillary electrophoresis (such as Agilent Bioanalyzer) or fluorescent-based detection (such as Qubit®). Best practice would be to determine the amount of small RNA using a Small RNA Analysis chip (i.e. for the 2100 Bioanalyzer, Agilent Technologies, Inc), which has a quantitative range between 50 - 2,000 pg/µl.

For RNA isolated from human plasma or serum, it is recommended to perform an abundant sequence depletion (using for instance Norgen’s Abundant Sequence Depletion Kit (Cat. No. 63540) in order to enhance the proportion of miRNA present in the input RNA.
Potential Contaminants

RNA samples should be free of salts, metal ions, and organic solvents that can be carried over from the RNA extraction. Several sources of contamination can be detected with a UV-Vis spectrophotometer. An acceptably pure RNA sample should have an A260 / A280 ratio between 1.8 and 2.1. The A260 / A230 ratio should also be approximately 2. Several common contaminants including proteins, chaotropic salts, and phenol absorb strongly between 220 and 230 nm and can often be identified as peaks in this region. Contamination with any of these substances results in a lower A260 / A230 ratio. Phenol also has an absorption maximum between 250 and 280 nm, which overlaps that of nucleic acid. Therefore high 230 nm absorbance combined with a biphasic or broad peak between 250 and 280 nm may indicate contamination with phenol rather than chaotropic salts. These contaminants may have a negative impact on the efficiency of the protocol.

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 well if relatively intact or as a high molecular weight smear if it has been sheared during extraction.

The best way to avoid gDNA contamination is to use an RNA extraction protocol that does not co-isolate gDNA, such as Lexogen’s SPLIT RNA Extraction Kit (Cat. No. 008). However, DNA can be removed from irreplaceable samples by acidic phenol extraction or DNase I digestion. 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. Removal of DNA may be required for accurate concentration determination of the RNA input material.

rRNA removal

Some ribosomal RNAs (rRNAs) are quite small, such as the 5S rRNA (120 nt), the 5.8S rRNA (160 nt), or the 4.5S rRNA (105 nt, from chloroplasts). An rRNA removal step of such small rRNAs will increase the read depth for miRNA and other small RNAs. Plant specific rRNA depletion kits are available from suppliers, but not all of them may remove the 4.5S rRNA fraction. Avoid rRNA depletion kits that remove small fragments in the subsequent purification steps.
8. Appendix B: Input RNA / Protocol Adjustments

Depending on the RNA source as well as the amount of input RNA we recommend the following protocol adjustments:

<table>
<thead>
<tr>
<th>Input RNA Amount</th>
<th>Examples of RNA Sources</th>
<th>Dilution Factors Used for 3' Adapter (A3 ●), 5' Adapter (A5 ○), and Reverse Transcription Primer (RTP ○)</th>
<th>Ethanol Amount Added in Step 5</th>
<th>Number of PCR Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 ng - 1,000 ng</td>
<td>Cellular Total or Enriched Small RNA (e.g., miRNA), Whole Blood RNA</td>
<td>1x</td>
<td>50 µl optional: 100 µl</td>
<td>12 - 15</td>
</tr>
<tr>
<td>1 ng - 100 ng</td>
<td>Plasma / Serum RNA, FFPE RNA</td>
<td>0.5x</td>
<td>50 µl</td>
<td>16 - 20</td>
</tr>
<tr>
<td>50 pg - 1 ng</td>
<td>Plasma / Serum RNA, Sorted Cell RNA, Urine RNA, Exosomal RNA, Saliva RNA, CSF RNA</td>
<td>0.5x to 0.3x</td>
<td>50 µl</td>
<td>20 - 22</td>
</tr>
</tbody>
</table>

The RNA sources listed are just some exemplary RNAs that are most likely to be available in the given concentration ranges. For enriched small RNA or total RNA inputs of 100 ng, it is also possible to use the 0.5x 3’ Adapter (A3 ●), 5’ Adapter (A5 ○), and Reverse Transcription Primer (RTP ○) dilutions recommended in the table above.

The minimum amount of total RNA input depends on the small RNA content of the sample in question. We recommend increasing the input material for total RNA samples with less than 10% small RNA content. Where possible, we would recommend using ≥100 ng of total RNA input from cells or tissues.
9. Appendix C: Quality Control

Quality control of the small RNA-Seq libraries is highly recommended and can be carried out with various methods depending on the available equipment. A thorough quality control procedure should include the analysis of concentration, size distribution, and banding pattern of the amplified products.

The concentration of the PCR products can be measured with a UV-Vis spectrophotometer. Visual control of the banding pattern and the size distribution as well as detection of side-products can be done by analyzing a small volume of sample with microcapillary electrophoresis. Several electrophoresis platforms are available from various manufacturers. For low- to medium-throughput applications, we recommend the Bioanalyzer 2100 with DNA 1000 Kit or High Sensitivity DNA chips (HS chips, Agilent Technologies, Inc.). Typically, 1 µl of the amplified sample is sufficient for analysis. However, for quantification using HS chips libraries may need to be diluted. For accurate library quantification, ensure that the loaded library is within the quantitative range of the Bioanalyzer chips.

Examples of Small RNA-Seq libraries generated from total RNA (Human Brain Reference RNA, HBRR), enriched small RNA from mouse liver and human plasma RNA are shown in Figure 3. Mouse (Mus musculus, Mm) liver small RNA was isolated with Lexogen’s SPLIT RNA Extraction Kit (Cat. No. 008, Protocol for Small RNA Fraction Purification).

In some cases, especially if total cellular RNA was used as input material, a size selection may be performed to remove fragments larger than 160 bp (tRNAs, mRNAs, snRNA), which otherwise could consume sequencing space. Lexogen offers a bundled version of the Small RNA-Seq Lib-
library Kit including Purification Module with Magnetic Beads (Cat. No. 058), or a separate Purification Module with Magnetic Beads (Cat. No. 022) that can be used for size selection as described in Appendix G, p.25. An example of such a size selection is depicted in Figure 4.

Figure 4. Bioanalyzer trace of small RNA-Seq library synthesized from 100 ng Human Brain Reference total RNA (HBRR) input (50 µl EtOH added in step 5) was amplified for 15 cycles: before (red trace) and after (blue trace) size selection with magnetic beads as described in Appendix G, p.25. In the red trace, peaks at 143 bp correspond to the miRNA library, a peak at 153 bp represents piRNA, the peak at ~195 bp corresponds to rRNA, and the peak at ~270 bp originates from snRNA. The peaks above 160 bp were efficiently removed by the size selection with magnetic beads.

If the Small RNA-Seq libraries show linker-linker artifacts (peak at 120 bp), an additional clean-up step may be required. We recommend removing these side-products before proceeding to Next Generation Sequencing using Lexogen’s Purification Module with Magnetic Beads (Cat. No. 022). Please note that the Small RNA-Seq Kit is also available as a bundled version with this module (Cat. No. 058). Best practice is to prepare an NGS lane mix with all the samples that should be included in the run. To ensure equimolar representation of each library within the lane mix, exclude the linker-linker peak from the calculations by setting the Bioanalyzer ranges accordingly (View/Setpoints/Advanced/Smear Analysis). **ATTENTION:** Ensure that the library is within the quantitative range of the Bioanalyzer chip! Repurification of a linker-linker-contaminated NGS lane mix (left cut-off only, see Appendix G, p.25) is depicted in Figure 5.

Figure 5. Bioanalyzer trace of a lane mix before (red trace) and after (blue trace) repurification of linker-linker-contaminated lane mixes with magnetic beads as described in Appendix G, p.25. The lane mix was prepared from 16 libraries synthesized from small RNA-enriched and total RNA samples that already underwent size selection using magnetic beads as described in Appendix G, p.25. The linker-linker and smaller adapter fragments visible in the red trace below 120 bp, were effectively reduced by the size selection with magnetic beads.
10. Appendix D: Multiplexing

i7 Indices are introduced during the PCR amplification with the Small RNA i7 Index Primers SRi7001-7096 (step 23). Each kit size contains 8, 24, or 96 different indices, respectively, provided in a 96-well plate.

i7 Indices

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

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SRi7001:</td>
<td>CAGCGT</td>
<td>SRi7009:</td>
<td>TACGGA</td>
<td>SRi7017:</td>
<td>TTCGAA</td>
<td>SRi7025:</td>
<td>TTTAGT</td>
<td>SRi7033:</td>
<td>AGATAG</td>
<td>SRi7041:</td>
<td>CTCTCG</td>
</tr>
<tr>
<td>B</td>
<td>SRi7002:</td>
<td>GATCAC</td>
<td>SRi7010:</td>
<td>CGGTGA</td>
<td>SRi7018:</td>
<td>GTCAAGG</td>
<td>SRi7026:</td>
<td>AACGAC</td>
<td>SRi7034:</td>
<td>TGGTGA</td>
<td>SRi7042:</td>
<td>TGCAAC</td>
</tr>
<tr>
<td>F</td>
<td>SRi7006:</td>
<td>GTGAGG</td>
<td>SRi7014:</td>
<td>AATCCG</td>
<td>SRi7022:</td>
<td>GAGGAT</td>
<td>SRi7030:</td>
<td>TGGAAG</td>
<td>SRi7038:</td>
<td>ACCGCC</td>
<td>SRi7046:</td>
<td>CTCCAT</td>
</tr>
<tr>
<td>H</td>
<td>SRi7008:</td>
<td>TACCTT</td>
<td>SRi7016:</td>
<td>TACGCA</td>
<td>SRi7024:</td>
<td>CCGGAC</td>
<td>SRi7032:</td>
<td>CGAAGG</td>
<td>SRi7040:</td>
<td>GATTGTG</td>
<td>SRi7048:</td>
<td>AATAGC</td>
</tr>
</tbody>
</table>

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

If fewer barcodes are required, care should be taken to always use indices that give a well-balanced signal in both lasers (red and green channels) for each nucleotide position. The individual libraries within a lane should be mixed at an equimolar ratio to ensure this balance. Make sure to select a range from 130 - 500 bp on the analysis software of the microfluidics device for determining the appropriate amount of each library to be pooled for the lane mix. An evaluation tool to check the color balance of index subsets is available at www.lexogen.com/support-tools/index-balance-checker/.

REMARK: If an 8 nt i7 index needs to be entered into an Illumina sample sheet, e.g., if libraries are multiplexed with other 8 nt index libraries, add two nucleotides from the Illumina adapter sequence to the 3’ end of the i7 index sequence. EXAMPLE: SRi7001 would become CAGCGTAT, SRi7002 would become GATCACAT and so on. These additional nucleotides are identical for all indices as they are derived from the Illumina adapter.

For more indexing options contact Lexogen at info@lexogen.com.
11. Appendix E: Sequencing*

**General**

The amount of library loaded onto the flow cell will greatly influence the number of clusters generated. Each sequencing facility has slightly different preferences of how much to load. For information on machine-specific loading amounts please see the online Frequently Asked Questions available at [www.lexogen.com](http://www.lexogen.com).

A schematic representation of the Small RNA-Seq library adapters (Cat. No. 052) is shown below.

**Small RNA-Seq Libraries with i7 Indexing**

i7 Indices (6 nt) are introduced during PCR (step 23). Read 1 directly corresponds to the miRNA sequence.

```
5’-(Read 1 Sequencing Primer)-3’
5’AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATC -(Insert...
3’TACTATAGGGCTGCTAGATGTGCAAGTCTCAAGATGTCAGGCTGCTAG -(Insert...

5’-(Index 1 (i7) Sequencing Primer)-3’
...Insert)= TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-17-ATCTCGATGCGTCTCTGCTTCTGCTTG 3’
...Insert)= ACCTTAAGAGCCCGGTGTGGCTAGGGGGGTAGCTGCTAGTG-17-TAGAGCATACGGCAGAAGACGAAC 5’
3’-(Read 2 Sequencing Primer)-5’
```

**NOTE**: Indicated sequencing primers are for illustrative purposes only and do not reflect the actual primer start and/or end sites.

**NOTE**: Paired-end sequencing is not necessary for Small RNA-Seq libraries.

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

12. Appendix F: Data Analysis

Information on the data analysis pipeline for Small RNA-Seq Library fastq sequencing files is available from Lexogen online: [www.lexogen.com/small-rna-seq-library-prep-kit](http://www.lexogen.com/small-rna-seq-library-prep-kit).

For total RNA as well as enriched small RNA inputs the final library will contain library inserts that are longer than the miRNA fraction. The adapters will also ligate to other RNA types present in total RNA (e.g., tRNAs, piRNAs, mRNAs, snRNA, and ribosomal RNA), hence long library fragments (200 - 1,000 bp) should be removed to focus the sequencing depth on the miRNA fraction. In addition, small RNA-Seq library preps from limited amounts of starting material may contain linker-linker artifacts (at 120 bp). We recommend the use of Lexogen’s Purification Module with Magnetic Beads (Cat. No. 022) for this size selection. A bundled version of the Small RNA-Seq Library Prep Kit including Purification Module with Magnetic Beads (Cat. No. 058) is also available from Lexogen.

ATTENTION: Performing a quality control of your libraries (e.g., using a Bioanalyzer or similar) is highly recommended before proceeding with the Magnetic Bead Purification. If you wish to purify a more specific size range, gel extraction can be used as an alternative to bead purification (see Appendix H, p.28). The Purification Module (PB, PS, EB) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (PB) may have settled and must be properly resuspended before adding them to the reaction. Prepare fresh 80 % ethanol (EtOH) for bead washing. NOTE: The Purification Solution (PS) is not used in this protocol.

Purification of Lane Mixes to Remove Long Library Fragments and Linker-Linker Artifacts

A double-sided cut-off is required to remove both the long library fragments and linker-linker artifacts. The following protocol is designed to remove library fragments > 150 - 160 bp in size. Best practice would be to prepare an equimolar lane mix (calculated from the Bioanalyzer in the range from 135 - 150 bp, i.e., the miRNA fraction) and perform the magnetic bead purification exclusively on the lane mix.

If total RNA was used as input material, add 1.2 volumes of properly resuspended Purification Beads (PB) to a lane mix or individual Small RNA-Seq libraries, mix well, and incubate for 5 minutes at room temperature. REMARK: If the volume of the lane mix or library is less than 20 µl, make up the total volume to 20 µl with Elution Buffer (EB) before adding PB.

EXAMPLE: Add 24 µl PB to 20 µl of a Small RNA-Seq lane mix from total RNA input.

Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear. Fragments ≥200 bp are now bound to the magnetic beads. Although this fraction is not of interest, we recommend saving those beads after transferring the supernatant into a separate tube until the final library is analyzed. ATTENTION: Do not discard the supernatant. It contains the small RNA fraction!
Transfer the supernatant to a new tube or well. Add one volume of properly resuspended Purification Beads (PB) equal to the original volume of the lane mix, to the newly transferred supernatant. Mix well and incubate for 5 minutes. 

**EXAMPLE:** For a 20 µl lane mix volume, add 20 µl of PB to 42 µl of supernatant.

**4** Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear. **ATTENTION:** Do not discard the beads as they now contain the small RNA fraction!

**5** Remove the clear supernatant without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed. **ATTENTION:** We strongly recommend saving the supernatant in a separate tube until you have analyzed the final lane mix.

**6** Add 120 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the plate in contact with the magnet as the beads should not be resuspended during this washing step. Remove and discard the supernatant.

**7** Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.

**8** Leave the plate in contact with the magnet and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and hence the resulting library yield.

**9** Add 15 µl of Elution Buffer (EB) per well, remove the plate from the magnet, and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.

**10** Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

**11** Transfer 12 - 15 µl of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.

**12** At this point, the libraries are finished and ready for quality control, pooling (for multiplexing), and cluster generation. Safe stopping point. Libraries can be stored at -20 °C at this point.
Repurification of Linker-Linker Contaminated Lane Mixes

If linker-linkers at 120 bp are present in the library, an additional purification of the lane mix may be required, to prevent adapter sequences dominating the sequencing results. We highly recommend the use of Lexogen’s Purification Module with Magnetic Beads (Cat. No. 022) for removing linker-linkers before proceeding to sequencing. **NOTE:** Repurification to remove linker-linker peaks should best be performed on the lane mix.

Add 1.3 volumes of properly resuspended Purification Beads (PB) to a lane mix, mix well, and incubate for 5 minutes at room temperature. **REMARK:** If the volume of the lane mix is less than 20 µl, make up the total volume to 20 µl with Elution Buffer (EB) before adding PB.

**EXAMPLE:** Add 26 µl PB to 20 µl of a Small RNA-Seq lane mix.

1. Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

2. Remove but save the clear supernatant in a fresh tube or well, without removing the PCR plate from the magnetic plate. Make sure that accumulated beads are not disturbed as the library is bound to the beads at this stage! **ATTENTION:** We strongly recommend saving the supernatant in a separate tube until you have analyzed the final lane mix.

3. Add 120 µl of 80 % EtOH to the beads and incubate for 30 seconds. Leave the plate in contact with the magnet as the beads should not be resuspended during this washing step. Remove and discard the supernatant.

4. Repeat this washing step once for a total of two washes. Make sure to remove the supernatant completely.

5. Leave the plate in contact with the magnet and let the beads dry for 5 - 10 minutes or until all ethanol has evaporated. **ATTENTION:** Dry the beads at room temperature only and do not let the beads dry too long (visible cracks appear), this will negatively influence the elution and hence the resulting library yield.

6. Add 20 µl of Elution Buffer (EB) per well, remove the plate from the magnet, and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.

7. Place the plate onto a magnetic plate and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

8. Transfer 15 - 17 µl of the supernatant into a fresh PCR plate. Make sure not to transfer any beads.

9. At this point, the lane mix is ready for quality control, and cluster generation. Safe stopping point. Libraries can be stored at -20 °C at this point.
14. Appendix H: Size Selection of NGS Libraries Using Gel Purification

Standard polyacrylamide (PAA) and agarose gel purification, or Pippin Prep can be performed to remove linker-linker artifacts and to select for Small RNA-Seq libraries. Best practice would be to prepare an equimolar lane mix (calculated from the Bioanalyzer in the range from 135 - 150 bp, i.e., the miRNA fraction) and perform the gel extraction exclusively on the lane mix. This ensures that all samples within the lane mix are treated equally and minimizes labor.

Polyacrylamide Gel Purification

The following protocol describes size selection on a 6 % polyacrylamide gel using Lexogen’s Gel Extraction Module (Cat. No. 054). The volumes of Loading Dye and Ladder reagents are sufficient for preparing and running 4 gels of 6 samples each.

![Figure 6. Location of kit contents.](image)

<table>
<thead>
<tr>
<th>Gel Extraction Module Components</th>
<th>Tube Label</th>
<th>Volume¹</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading Dye</td>
<td>LD</td>
<td>264 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>NGS MW Ladder</td>
<td>L1</td>
<td>44 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>NGS Control Ladder</td>
<td>L2</td>
<td>44 µl</td>
<td>-20 °C</td>
</tr>
<tr>
<td>Column Binding Buffer</td>
<td>CB</td>
<td>5.5 ml</td>
<td>RT</td>
</tr>
<tr>
<td>Column Wash Buffer</td>
<td>CW</td>
<td>60 ml</td>
<td>RT</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>EB</td>
<td>528 µl</td>
<td>RT</td>
</tr>
<tr>
<td>Molecular Biology Grade Water</td>
<td>H₂O</td>
<td>5.5 ml</td>
<td>RT</td>
</tr>
</tbody>
</table>

¹ Including 10 % surplus ² Including 42 ml ethanol (to be added by the user)

Store the box containing LD ●, L1 ●, and L2 ● in a -20 °C freezer. The rest of the kit components (CB, CW, columns) should be stored at room temperature (RT) and protected from light. Before use, check the contents of CB and CW. If a white precipitate is visible, incubate at 37 °C until buffer components dissolve completely.
ATTENTION: Before use, add 42 ml absolute ethanol to the CW bottle and shake to combine. Additional absolute ethanol (100 % EtOH) is also required for the column purification.

1. Prepare a 6 % non-denaturing polyacrylamide gel or set up a 10-well 6 % Novex® TBE PAGE gel for electrophoresis (Life Technologies, Inc.) according to manufacturer’s instructions. Up to 8 libraries or lane mixes can be loaded on each Novex® TBE PAGE gel.

2. Mix 10 µl of Loading Dye (LD) with up to 20 µl of an equimolar lane mix, or use the purified library obtained in step 30 of the Small RNA-Seq Library Prep Kit.

3. Load each of the samples prepared in step 2 in separate wells of the gel.

4. Load 10 µl of the NGS MW Ladder (L1) in one well of the gel.

5. Load 10 µl of the NGS Control Ladder (L2) in one well of the gel.

The Loading Dye (LD) contains a high MW dye with light blue color and a low MW dye with dark blue color. Run the gel at room temperature for 1 hour at 120 V or until the dark blue dye reaches the bottom of the gel. ATTENTION: Do not let the dark blue dye exit the gel.

6. Remove the gel from the apparatus and place in a clean container with 1x TBE spiked with either SYBR Gold or Ethidium Bromide. Incubate for 5 - 20 minutes (depending on the concentration of the staining solution) then view the gel on a UV transilluminator.

7. The adapter-ligated library fragments containing miRNA (~21 nt) are represented by a band at ~143 bp. The adapter-ligated library fragments containing the piRNA (~30 nt) are represented by a band at ~153 bp. Excise the desired band(s) accordingly. Avoid recovering the ~120 bp linker-linker artifacts that contain no insert.

8. Homogenize the gel piece from each sample by either crushing into small pieces inside a 1.5 ml or 2 ml tube with a small pestle. Alternatively place the gel piece in a Gel Breaker Tube (IST Engineering, user-provided) inside a 2 ml tube and centrifuge at 14,000 x g (~12,000 rpm) for 2 minutes.

9. Add 200 µl of Molecular Biology Grade Water (H₂O) to the homogenized gel piece. Mix by vortexing briefly.

10. Rotate end-to-end for at least 2 hours to overnight at room temperature.

11. Briefly centrifuge the tubes to collect all liquid at the bottom. Transfer the entire contents (eluate and the gel debris) to the top of a Gel Filtration Column placed in a 1.5 ml tube.

12. Centrifuge at ~14,000 x g (~12,000 rpm) for 2 minutes. Discard the Gel Filtration Column and retain the eluate in the 1.5 ml tube.

15. Add 200 µl of 100 % EtOH. Mix by vortexing.

16. Assemble a spin column with the provided collection tube. Transfer the mixture from step 15 to the top reservoir of the spin column.

17. Centrifuge at ≥3,500 x g (≥6,000 rpm) for 1 minute at 18 °C. Discard the flow-through.

18. Apply 600 µl of Column Wash Buffer (CW, with EtOH added) to the column and centrifuge for 1 minute at 14,000 x g (~12,000 rpm) at 18 °C. Discard the flow-through.

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

20. Remove the column and transfer to a fresh collection tube. Centrifuge for 2 minutes at 14,000 x g (~12,000 rpm) to dry the column.

21. Transfer the column to a new 1.5 ml tube and apply 10 - 20 µl Elution Buffer (EB) to the column. Centrifuge for 1 minute at 200 x g (~1,400 rpm), then 2 minutes at 14,000 x g (~12,000 rpm) at 18 °C to elute the small RNA-Seq library.

22. At this point, the libraries are finished and ready for quality control (Appendix C, p.21), pooling (if the gel purification was not performed on a lane mix already. For multiplexed Small RNA-Seq Libraries; see Appendix D, p.23), and cluster generation. Safe stopping point. Libraries can be stored at -20 °C at this point.

![Non-denaturing polyacrylamide gel (6 %) of a small RNA-Seq library synthesized from 1 ng small RNA input (small RNA fraction of Mouse liver, extracted with the SPLIT RNA Extraction Kit, Lexogen). Lane 1 contains the L1 ladder, lane 2 the L2 ladder, and lane 3 shows the small RNA library containing the linker-linker at 120 bp and the target miRNA (140 bp). The band at 153 bp represents piRNA and the band at ~195 bp corresponds to tRNA.](image-url)
Agarose Gel Purification

The size selection of the small RNA-Seq samples (lane mix) can also be done using a 3% non-denaturing TBE agarose gel (with EtBr in the gel and running buffer). Prepare an equimolar lane mix (calculated from the Bioanalyzer in the range from 135 – 150 bp, i.e., the miRNA fraction), load the lane mix alongside dsDNA ladder(s) (e.g., from the Gel Extraction Module (Cat. No. 054), and run the agarose and EtBr gel in TBE for 2 hours at 80 V. Excise the desired band(s) and avoid recovering the ~120 bp linker-linker artifacts that contain no insert. For final gel extraction, any commercially available gel extraction kit can be used.

![Figure 8. 3% non-denaturing TBE agarose gel (with EtBr in the gel and running buffer) of a small RNA-Seq library synthesized from 100 ng total RNA input (Human). Lane 1 contains the L1 ladder, lane 2 the L2 ladder, and lane 3 shows the small RNA library containing the linker-linker at 120 bp and the target miRNA (140 bp). The band at ~195 bp corresponds to tRNA and the band at ~270 bp originates from snRNA.]

15. Appendix I: Revision History

<table>
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<th>Publication No. / Revision Date</th>
<th>Change</th>
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<td>052UG128V0102 Jun. 19, 2018</td>
<td>Updated table in Appendix B. Added details for agarose gel extraction and example gel images to Appendix H.</td>
<td>20, 28-31</td>
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<td>052UG128V0101 Nov. 7, 2017</td>
<td>Recommendations to save beads and supernatant in Appendix G as a precaution. Corrected volumes for CB, CW, EB in table, Appendix H.</td>
<td>25-26, 28</td>
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<td>052UG128V0100 Aug. 8, 2017</td>
<td>Initial Release.</td>
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