

SMALL RNA-SEQ

Small RNA-Seq Library Prep Kit User Guide

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052 (Small RNA-Seq Library Prep Kit for Illumina)
135 (Small RNA-Seq Library Prep Kit for Illumina with TraPR)

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When describing a procedure for publication using this product, please refer to it as Lexogen's Small RNA-Seq Library Pep Kit.

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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.21 for more details.

Input RNA (see Appendix B, p.21) 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.

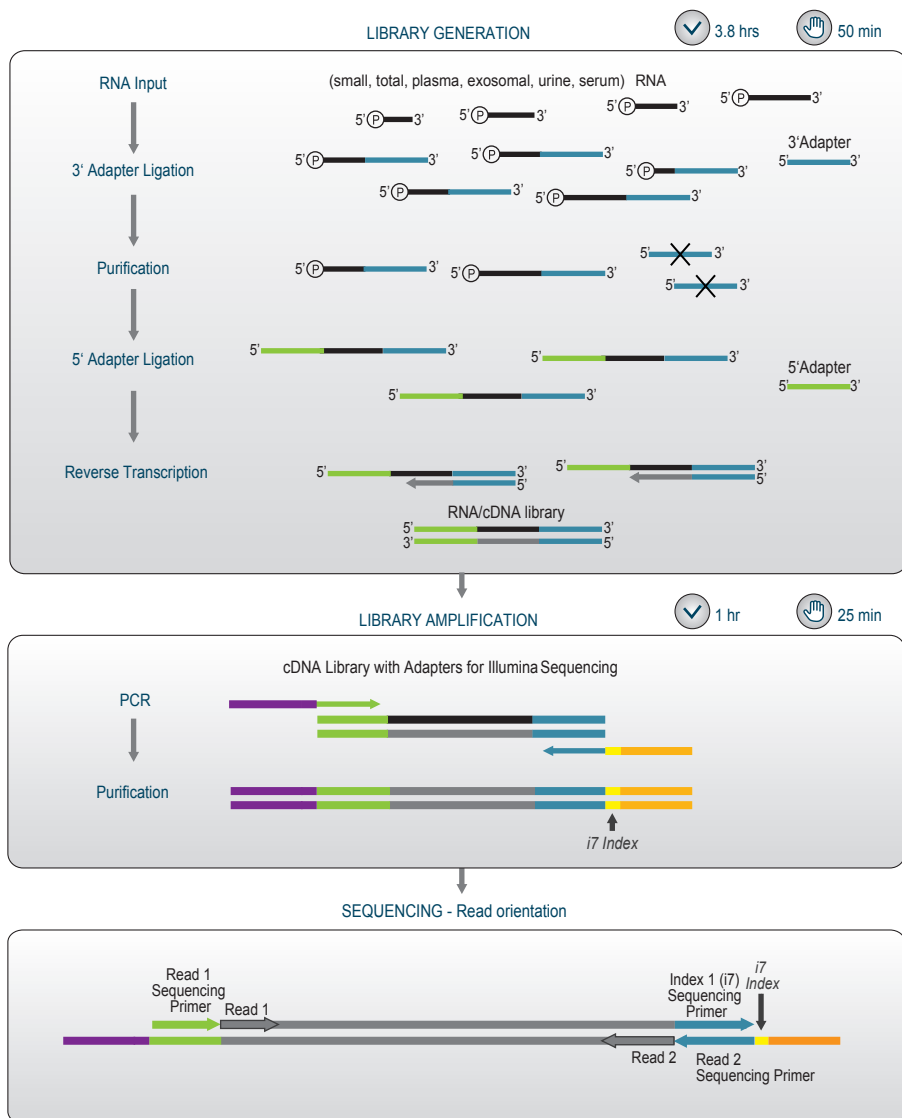


Figure 1. Schematic overview of the Small RNA-Seq Library Prep workflow.

2. Kit Components and Storage Conditions

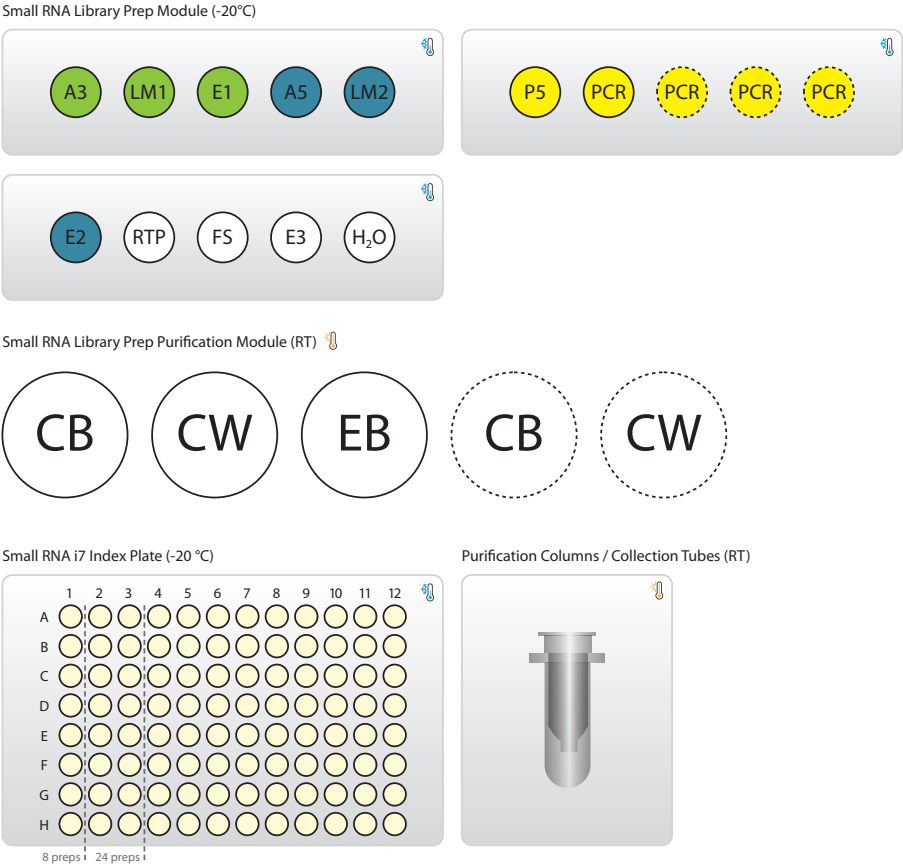




















Figure 2. Location of kit contents. For the 8 and 24 prep kits, the dotted PCR, CB and CW tubes are missing. The Small RNA i7 Index Plate is filled to the first dotted line with 8 unique i7 indices for the 8 prep kit, to the second dotted line with 24 unique indices for the 24 prep kit, and 96 unique indices are provided for the 96 prep kit.

Kit Components	Label	Volume ¹			Storage
		8 preps	24 preps	96 preps	
3' Adapter	A3 ●	8.8 µl	26.4 µl	105.6 µl	 -20 °C
Ligation Mix 1	LM1 ●	105.6 µl	316.8 µl	1,267.2 µl	 -20 °C
Enzyme Mix 1	E1 ●	8.8 µl	26.4 µl	105.6 µl	 -20 °C
5' Adapter	A5 ●	8.8 µl	26.4 µl	105.6 µl	 -20 °C
Ligation Mix 2	LM2 ●	96.8 µl	290.4 µl	1,161.6 µl	 -20 °C
Enzyme Mix 2	E2 ●	8.8 µl	26.4 µl	105.6 µl	 -20 °C
Reverse Transcription Primer	RTP ○	8.8 µl	26.4 µl	105.6 µl	 -20 °C
First Strand cDNA Synthesis Mix	FS ○	70.4 µl	211.2 µl	844.8 µl	 -20 °C
Enzyme Mix 3	E3 ○	8.8 µl	26.4 µl	105.6 µl	 -20 °C
Molecular Biology Grade Water	H ₂ O ○	1,400 µl	1,400 µl	1,400 µl	 -20 °C
PCR Mix	PCR ●	440 µl	1,320 µl	4x 1,320 µl	 -20 °C
P5 Primer	P5 ●	26.4 µl	79.2 µl	316.8 µl	 -20 °C
Small RNA i7 Index Primer	SRi	3 µl each (one i7 Index / rxn)			 -20 °C
Column Binding Buffer	CB	5.5 ml	16 ml	2x 32 ml	 RT
Column Wash Buffer	CW	60 ml ²	128 ml ²	2x 128 ml ²	 RT
Elution Buffer	EB	380 µl	1 ml	4 ml	 RT
Purification Columns	PC	16	48	192	 RT
Collection Tubes	CT	16	48	192	 RT

¹ Including ≥10 % surplus ² Including ethanol (to be added by user)

Upon receiving the Small RNA-Seq Kit, store the Small RNA Library Prep Module and Small RNA Index Plate in a -20 °C freezer. The rest of the kit components (**CB**, **CW**, **EB**, 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 (Thermo Fisher Scientific, Inc. #EC6265BOX).
- SYBR® Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Inc. #S11494).
- 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 work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per the manufacturer's instructions. **ATTENTION:** Do not forget to rinse off any RNaseZap residue with RNase-free water after usage! Residues of RNaseZap may damage the RNA.
- Protect all reagents and your RNA samples from RNases on your skin by wearing a clean lab coat and fresh gloves. Change gloves after making contact with equipment or surfaces outside of the RNase-free zone.
- Avoid speaking above opened tubes. Keep reagents closed when not in use to avoid airborne RNase contamination.

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

3' Adapter Ligation	Purification - Removal of Excess 3' Adapter	5' Adapter Ligation	Reverse Transcription of Ligated RNA
H₂O ○ – thawed at RT A3 ● – thawed at RT LM1 ● – thawed at RT E1 ● – keep on ice or at -20 °C	CB – stored at RT 100 % EtOH – provided by user CW – stored at RT add EtOH! EB – thawed at RT	A5 ● – thawed at RT LM2 ● – thawed at RT E2 ● – keep on ice or at -20 °C	RTP ○ – thawed at RT FS ○ – thawed at RT E3 ○ – keep on ice or at -20 °C
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** ●) are briefly heated to resolve secondary structures before the ligation is performed. For information on RNA quantification and quality control see Appendix A, p.19.

ATTENTION: Please refer to Appendix B, p.21 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** ○) and add 1 µl 3' Adapter (**A3** ●). **ATTENTION:** For RNA input amounts ≤100 ng pre-dilute **A3** ● according to the table in Appendix B, p.21.
- 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** ●) and 1 µl Enzyme Mix 1 (**E1** ●) 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!

Transfer the 3' adapter ligation reaction (20 μ l) into a 1.5 ml tube and add 300 μ l Column Binding Buffer (**CB**), mix well by pipetting. Add 50 μ l of 100 % EtOH to the reaction, mix well. Transfer the solution onto a Purification Column placed in a 2 ml Collection Tube.

- 5 **ATTENTION:** Do not prepare mastermixes containing the 100 % EtOH with (**CB**) before adding to the reaction. **REMARK:** For inputs >100 ng, 100 μ l of 100 % EtOH may be added (see Appendix B, p.21), which increases the recovery of ligated miRNA fragments but may also increase the amount of linker-linker artifacts 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 μ l 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 μ l 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.21.
- 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.
- 14 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** ○).

- 15 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.21.
- 16 Incubate for 2 minutes at 70 °C in a pre-heated thermocycler. Place the tube on ice. Spin down before opening the tube.
- 17 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.
- 18 Add 9 µl of Mastermix 3 to each reaction from step 16. Mix well and spin down.
- 19 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

PCR		Purification
PCR ●	– thawed at RT	CB – stored at RT
P5 ●	– thawed at RT	CW – stored at RT
SRi7001-7096	– thawed at RT	EB – thawed or stored at RT
H ₂ O ○	– thawed at RT	
Thermocycler	98 °C, 30 sec <div><div>98 °C, 10 sec 60 °C, 30 sec 72 °C, 15 sec</div><div>} 12 - 22x Appendix B, p.21</div></div> 72 °C, 10 min 10 °C, ∞	

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.21) 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.21.

🔒 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**), mix well by pipetting. Add 50 µl 100 % EtOH to the reaction, mix well. Transfer the solution onto a Purification Column placed in a 2 ml Collection Tube. **ATTENTION:** Do not prepare mastermixes containing the 100 % EtOH with (**CB**) before adding to the reaction.

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.22).

 Safe stopping point. Libraries can be stored at -20 °C at this point.

6. Short Procedure

ATTENTION: All centrifugation steps are performed at 18 °C

3.8 hrs




Library Generation


Total RNA (>100 ng)	Total RNA / Enriched Small RNA (≤100 ng)
3' Adapter Ligation	
<input type="checkbox"/> Skip!	<input type="checkbox"/> Pre-dilute A3 ● to 0.5x or 0.3x (Appendix B, p.21).
<input type="checkbox"/> Mix 6 µl RNA with 1 µl A3 ●.	
<input type="checkbox"/> Incubate for 2 min at 70 °C. Place on ice.	
<input type="checkbox"/> Prepare a mastermix of 12 µl LM1 ● and 1 µl E1 ● per reaction. Mix well.	
<input type="checkbox"/> Add 13 µl LM1 / E1 mastermix per reaction, mix well.	
<input type="checkbox"/> Incubate for 1 hr at 28 °C. 🛑 Safe stopping point.	
Purification	
<input type="checkbox"/> Add 300 µl of CB , then 50 µl (or 100 µl) 100 % EtOH to ~20 µl 3' ligated RNA, mix well, and apply to column.	<input type="checkbox"/> Add 300 µl of CB , then 50 µl 100 % EtOH to ~20 µl 3' ligated RNA, mix well, and apply to column.
<input type="checkbox"/> Centrifuge 1 min at 3,500 x g. Discard the flow-through.	
<input type="checkbox"/> Add 600 µl of CW , centrifuge 1 min at 14,000 x g.	
<input type="checkbox"/> Discard flow-through, centrifuge 2 min at 14,000 x g.	
<input type="checkbox"/> Exchange Collection Tube with 1.5 ml tube.	
<input type="checkbox"/> Add 12 µl EB , centrifuge 1 min at 200 x g, and 2 min at 14,000 x g.	
5' Adapter Ligation	
<input type="checkbox"/> Skip!	<input type="checkbox"/> Pre-dilute A5 ● to 0.5x or 0.3x (Appendix B, p.21).
<input type="checkbox"/> Denature 1.1 µl A5 ● per reaction for 2 min at 70 °C. Place on ice.	
<input type="checkbox"/> Prepare a mastermix of 1 µl denatured A5 ●, 11 µl LM2 ●, and 1 µl of E2 ● per reaction. Mix well.	
<input type="checkbox"/> Add 13 µl A5 / LM2 / E2 mastermix per reaction. Mix well.	
<input type="checkbox"/> Incubate for 1 hr at 28 °C.	
Reverse Transcription of Ligated RNA	
<input type="checkbox"/> Skip!	<input type="checkbox"/> Pre-dilute RTP ○ to 0.5x or 0.3x (Appendix B, p.21).
<input type="checkbox"/> Add 1 µl RTP ○ per reaction. Mix well.	
<input type="checkbox"/> Incubate for 2 min at 70 °C. Place on ice.	
<input type="checkbox"/> Prepare a mastermix of 8 µl FS ○ and 1 µl E3 ○ per reaction. Mix well.	
<input type="checkbox"/> Add 9 µl FS / E3 mastermix per reaction. Mix well.	
<input type="checkbox"/> Incubate for 1 hr at 50 °C. 🛑 Safe stopping point.	

1 hr


Library Amplification

PCR

- ☐ Prepare a mastermix of 50 μ l **PCR** , 3 μ l **P5** , and 11 μ l **H₂O**  per sample. Mix well.
- ☐ Add 64 μ l of **PCR / P5 / H₂O** to ~33 μ l sample.
- ☐ Add 3 μ l **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, 30 sec
 - 98 °C, 10 sec
 - 60 °C, 30 sec
 - 72 °C, 15 sec

12 - 22x
 Appendix B, p.21
- 72 °C, 10 min
- 10 °C, ∞  Safe stopping point.

Purification

- ☐ Add 300 μ l of **CB**, then 50 μ l 100 % EtOH, mix, and apply to column. Centrifuge 1 min at 3,500 x g. Discard the flow-through.
- ☐ Add 600 μ l 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 μ l **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-enriched 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/ μ l.

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

Ribosomal depletion may not be required for small RNA-Seq library prep. However, 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 can 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. For more information please consult the online Frequently Asked Questions: <https://www.lexogen.com/small-rna-seq-library-prep-kit/#smallRNAfaq>.

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:

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

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).

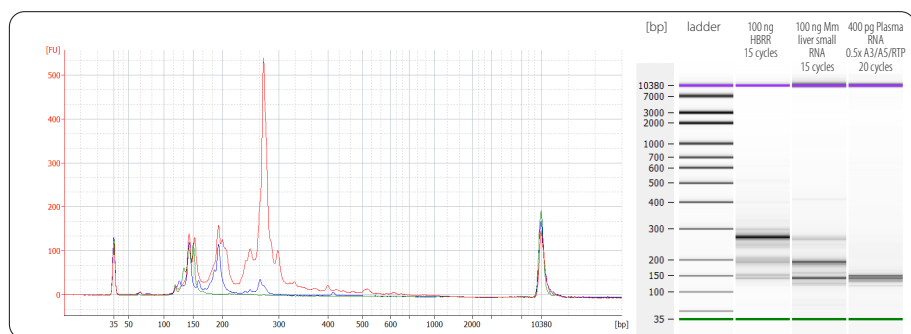


Figure 3. Bioanalyzer trace of Small RNA-Seq libraries synthesized from 100 ng Human Brain Reference RNA (HBRR, red trace), 100 ng mouse (Mm) liver small RNA (blue trace), and 400 pg human plasma RNA (green trace). HBRR and enriched small RNA input (isolated with the SPLIT RNA Extraction Kit from Mm liver tissue) were amplified with 15 cycles. Libraries were synthesized using 0.5x A3 adapter, A5 adapter and RTP, respectively. 50 µl EtOH was added in step 5 for all preps. Libraries from plasma RNA were amplified with 20 cycles. 1 µl of each library was loaded onto a High Sensitivity DNA chip. Peaks at 143 bp correspond to the miRNA library, a peak at 153 bp represents piRNA, the peak at ~195 bp corresponds to tRNA, and the peak at ~270 bp originates from snRNA. Peaks at 120 bp are linker-linker artifacts, which can occur at lower RNA input amounts (≤1 ng). In this case, an additional clean-up of the lane mix with magnetic beads as described in Appendix G, p.26 is recommended. Furthermore, for libraries synthesized from total RNA a size selection using magnetic beads as described in Appendix G, p.26 (see Figure 4, p.23) or gel extraction as described in Appendix H, p.29, may be performed.

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.26. An example of such a size selection is depicted in Figure 4.

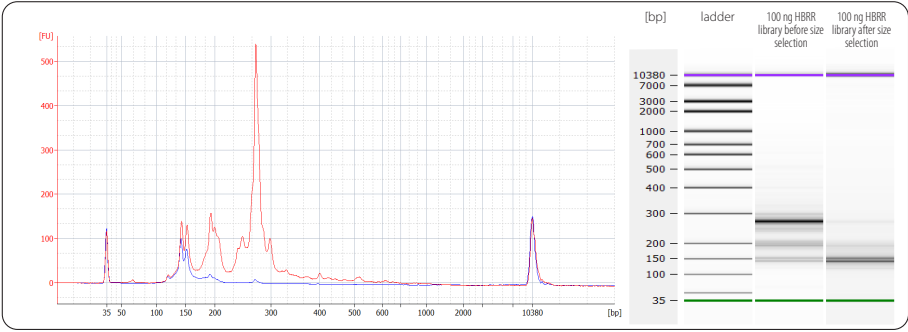


Figure 4. Bioanalyzer trace of small RNA-Seq library synthesized from 100 ng Human Brain Reference total RNA (HBRR) input (50 µl EtOH added in step 5) was amplified for 15 cycles: before (red trace) and after (blue trace) size selection with magnetic beads as described in Appendix G, p.26. In the red trace, peaks at 143 bp correspond to the miRNA library, a peak at 153 bp represents piRNA, the peak at ~195 bp corresponds to tRNA, 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)) or via gel purification (see Appendix H, p.29). 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.26) is depicted in Figure 5.

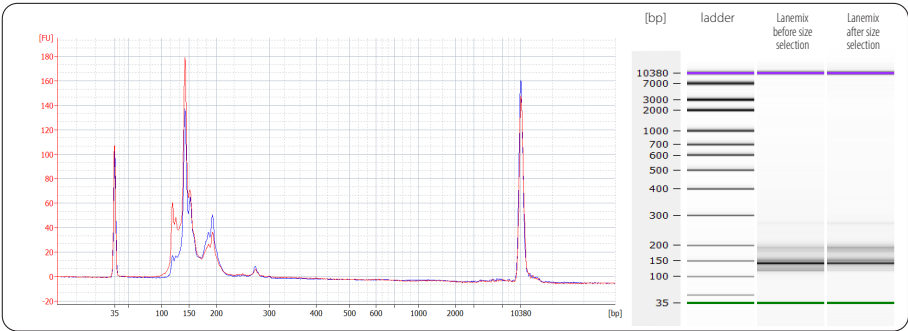


Figure 5. Bioanalyzer trace of a lane mix before (red trace) and after (blue trace) repurification of linker-linker-contaminated lane mixes with magnetic beads as described in Appendix G, p.26. The lane mix was prepared from 16 libraries synthesized from small RNA-enriched and total RNA samples that already underwent size selection using magnetic beads as described in Appendix G, p.26. 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).

	1	2	3	4	5	6	7	8	9	10	11	12
A	SRI7001: CAGCGT	SRI7009: TCAGGA	SRI7017: TCTTAA	SRI7025: TTTATG	SRI7033: AGATAG	SRI7041: CTCTCG	SRI7049: GTGCCA	SRI7057: AGTACT	SRI7065: AAGCTC	SRI7073: GACATC	SRI7081: GCAGCC	SRI7089: CCGGGA
B	SRI7002: GATCAC	SRI7010: CGGTTA	SRI7018: GTCAGG	SRI7026: AACGCC	SRI7034: TTTGTA	SRI7042: TGACAC	SRI7050: TCGAGG	SRI7058: ATAAGA	SRI7066: GACGAT	SRI7074: CGATCT	SRI7082: ACTCTT	SRI7090: CCTGCT
C	SRI7003: ACCACT	SRI7011: TTAACT	SRI7019: ATACTG	SRI7027: CAAGCA	SRI7035: GTTACC	SRI7043: AAGACA	SRI7051: CACTAA	SRI7059: GGTGAG	SRI7067: TCGTTC	SRI7075: CGTCGC	SRI7083: TGCTAT	SRI7091: GCGCTG
D	SRI7004: TGCACG	SRI7012: ATGAAC	SRI7020: TATGTC	SRI7028: GCTCGA	SRI7036: CGCAAC	SRI7044: ACAGAT	SRI7052: GGTATA	SRI7060: TTCCGC	SRI7068: CCAATT	SRI7076: ATGGCG	SRI7084: AAGTGG	SRI7092: GAACCT
E	SRI7005: ACATTA	SRI7013: CCTAAG	SRI7021: GAGTCC	SRI7029: GCGAAT	SRI7037: TGGCGA	SRI7045: TAGGCT	SRI7053: CGCCTG	SRI7061: GAAGTG	SRI7069: AGTTGA	SRI7077: ATTGGT	SRI7085: CTCATA	SRI7093: TTCGAG
F	SRI7006: GTGTAG	SRI7014: AATCCG	SRI7022: GGAGGT	SRI7030: TGGATT	SRI7038: ACCGTG	SRI7046: CTCCAT	SRI7054: AATGAA	SRI7062: CAATGC	SRI7070: AACCGA	SRI7078: GCCACA	SRI7086: CCGACC	SRI7094: AGAATC
G	SRI7007: CTAGTC	SRI7015: GGCTGC	SRI7023: CACACT	SRI7031: ACCTAC	SRI7039: CAACAG	SRI7047: GCATGG	SRI7055: ACAACG	SRI7063: ACGTCT	SRI7071: CAGATG	SRI7079: CATCTA	SRI7087: GGCCAA	SRI7095: AGGCAT
H	SRI7008: TGTGCA	SRI7016: TACCTT	SRI7024: CCGCAA	SRI7032: CGAAGG	SRI7040: GATTGT	SRI7048: AATAGC	SRI7056: ATATCC	SRI7064: CAGGAC	SRI7072: GTAGAA	SRI7080: AACAAG	SRI7088: AGACCA	SRI7096: ACACGC

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 support@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.

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' AATGATACGGCGACCACCGAGATCTACACGTTCTAGAGTTCTACAGTCCGACGATC -(Insert...
3' TTAGTATGCGCTGGTGGCTCTAGATGTGCAAGTCTCAAGATGTCAGGCTGCTAG -(Insert...

5'-(Index 1 (i7) Sequencing Primer)-3'
...Insert)- TGAATTCTCGGGTGCCAAGGAAGTCCAGTCAC-i7-ATCTCGTATGCCGCTTCTGCTTG 3'
...Insert)- ACCTTAAGAGCCACGGTTCCTTGAGGTGAGT-i7-TAGAGCATACGGCAGAGACGAAC 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.

Multiplexing with Other Library Types

We do not recommend multiplexing Lexogen libraries with libraries from other vendors in the same sequencing lane.

Though this is possible in principle, specific optimization of index combinations, library pooling conditions, and loading amounts may be required, even for advanced users. Sequencing complex pools that include different library types at different lane shares may have unpredictable effects on sequencing run metrics, read quality, read outputs, and / or demultiplexing performance. Lexogen assumes no responsibility for the altered performance of Lexogen libraries sequenced in combination with external library types in the same lane (or run).

Due to size differences, libraries prepared with the Lexogen Small RNA-Seq Library Prep Kit (or any other small RNA library prep kit) should not be sequenced together with QuantSeq, QuantSeq-Flex, or CORALL libraries. Please refer to the sequencing guidelines for each library type (library adapter details, loading amounts to use, and use of custom sequencing primers, etc), which are provided in our Library Prep Kit User Guides, and online Frequently Asked Questions (FAQs).

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.

13. Appendix G: Size Selection of NGS Libraries Using Magnetic Beads

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. 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.29). 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.

13.1 Purification of Lane Mixes to Remove Long Library Fragments

A right side cut-off is required to remove the long library fragments. 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.

1

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 μ l lane mix volume, add 20 μ l of **PB** to 42 μ l of supernatant.

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!

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.

Add 120 μ l 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.


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

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.

Add 15 μ l 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.

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

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

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.

13.2 Repurification of Linker-Linker Contaminated Lane Mixes

If linker-linker artifacts (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.

ATTENTION: The recommended approach to repurification depends on the degree of contamination:

- >25% of the library - gel extraction is strongly recommended (see Appendix H, p.29).
- 10 - 25% of the library - gel extraction (see Appendix H, p.29), **or** magnetic bead repurification (see protocol below) is recommended.
- <10% of the library - repurification is optional (gel extraction, or magnetic beads repurification), libraries may be sequenced directly.

NOTE: Repurification to remove linker-linker should best be performed on the lane mix.

1

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.

2

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

3

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.

4

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.

5

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

6

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.

7

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.


8

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

9

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

10

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.

14.1 Gel Extraction Module - Kit Components and Storage Conditions

ATTENTION: The Buffers, Tubes and Purification Columns supplied in the Gel Extraction Module (Cat.No. 054), are compatible with PAGE gel purification only (see 14.2, p.30). For agarose gel purification (see 14.3, p.33), the Ladders L1 and L2 may be used for electrophoresis. However, an alternative agarose gel extraction kit is required for final purification of the library or lanemix from the gel.

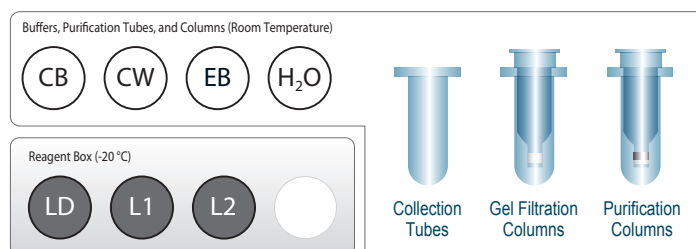


Figure 6. Location of kit contents.

Gel Extraction Module Components	Tube Label	Volume ¹	Storage
		24 preps	
Loading Dye	LD ●	264 µl	-20 °C
NGS MW Ladder	L1 ●	44 µl	-20 °C
NGS Control Ladder	L2 ●	44 µl	-20 °C
Column Binding Buffer	CB	5.5 ml	RT
Column Wash Buffer	CW	60 ml ²	RT
Elution Buffer	EB	528 µl	RT
Molecular Biology Grade Water	H ₂ O	5.5 ml	RT

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 buf-

fer 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.

14.2 Detailed Protocol: 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.

NOTE: Repurification to remove linker-linker peaks should best be performed on the lane mix.

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.

6 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.

7 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.

8 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.

9 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.

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

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

12 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.

13 Centrifuge at $\sim 14,000 \times g$ ($\sim 12,000$ rpm) for 2 minutes. Discard the Gel Filtration Column and retain the eluate in the 1.5 ml tube.

14 Add 200 μ l of Column Binding Buffer (**CB**). Mix by vortexing.

15 Add 200 μ l 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 $\geq 3,500 \times g$ ($\geq 6,000$ rpm) for 1 minute at 18 °C. Discard the flow-through.

18 Apply 600 μ l of Column Wash Buffer (**CW**, with EtOH added) to the column and centrifuge for 1 minute at $14,000 \times g$ ($\sim 12,000$ 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 $14,000 \times g$ ($\sim 12,000$ rpm) to dry the column.

21 Transfer the column to a new 1.5 ml tube and apply 10 - 20 μ l Elution Buffer (**EB**) to the column. Centrifuge for 1 minute at $200 \times g$ ($\sim 1,400$ rpm), then 2 minutes at $14,000 \times g$ ($\sim 12,000$ 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.22), pooling (if the gel purification was not performed on a lane mix already. For multiplexed Small RNA-Seq Libraries; see Appendix D, p.24), and cluster generation.

 Safe stopping point. Libraries can be stored at -20 °C at this point.

Typical Results

Small RNA libraries and lane mixes will produce a smear with distinct bands overlaid. The 140 bp band corresponds to the major miRNA peak, while the 120 bp band will be visible if there is linker-linker contamination. Ensure that you cut above the 120 bp band to exclude linker-linker from the gel piece for final purification.

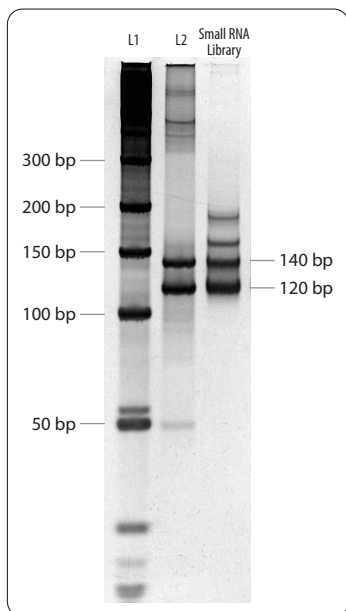


Figure 7. Non-denaturing polyacrylamide gel (6 %) of a small RNA-Seq library synthesized from 1 ng small RNA input (small RNA fraction of Mouse liver, extracted with the SPLIT RNA Extraction Kit, Lexogen). Lane 1 contains the L1 ladder, lane 2 the L2 ladder, and lane 3 shows the small RNA library containing 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.

14.3 Detailed Protocol: Agarose Gel Purification

Size selection or linker-linker repurification of small RNA-Seq samples (lane mix) can also be purified using agarose gel.

ATTENTION: The Gel Extraction Module (Cat. No. 054) should not be used for agarose gel extraction.

- 1 Prepare a 3% non-denaturing TBE agarose gel (with EtBr in the gel and running buffer).
- 2 Prepare an equimolar lane mix (calculated from the Bioanalyzer in the range from 135 – 150 bp, i.e., the miRNA fraction).
- 3 Load the lane mix on to the gel alongside dsDNA ladders L1 and L2 from the Gel Extraction Module (Cat. No. 054). Run the gel in TBE for 2 hours at 80 V.
- 4 Excise the desired band(s) and avoid recovering the ~120 bp linker-linker artifacts that contain no insert. **REMARK:** For final gel extraction, any commercially available gel extraction kit that will recover double-stranded DNA of ≥ 130 bp in size can be used

Typical Results

Small RNA libraries and lane mixes will produce a smear with distinct bands overlaid. The 140 bp band corresponds to the major miRNA peak, while the 120 bp band will be visible if there is linker-linker contamination. Ensure that you cut above the 120 bp band to exclude linker-linker from the gel piece for final purification.

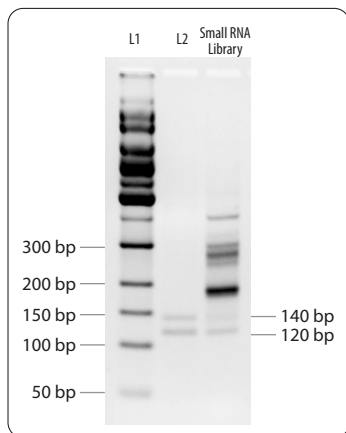


Figure 8. 3% non-denaturing TBE agarose gel (with EtBr in the gel and running buffer) of a small RNA-Seq library synthesized from 100 ng total RNA input (Human). Lane 1 contains the L1 ladder, lane 2 the L2 ladder, and lane 3 shows the small RNA library containing the linker-linker at 120 bp and the 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

Publication No. / Revision Date	Change	Page
052UG128V0113 Aug. 26, 2022	Updated Legal Disclaimer text.	2
	Updated Kit Components Figure 2 and Table to reflect current packaging and storage requirements.	6-7
	Added recommendations for multiplexing with other library types.	25
052UG128V0112 Sep. 19, 2019	General terms and conditions were updated.	2
	Short protocol reformatted.	16-17
	Appendix G: Repurification of Linker - Linker Contaminated Lane Mixes protocol recommendations using magnetic beads have been modified.	27
	Appendix H: Recommendations for agarose gel purification were updated.	28, 32
052UG128V0102 Jun. 19, 2018	Updated table in Appendix B.	20
	Added details for agarose gel extraction and example gel images to Appendix H.	28-31
052UG128V0101 Nov. 7, 2017	Recommendations to save beads and supernatant in Appendix G as a precaution. Corrected volumes for CB , CW , EB in table, Appendix H.	25-26, 28
052UG128V0100 Aug. 8, 2017	Initial Release.	

16. Notes

Associated Products:

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)
080 (Reamplification Add-on Kit for Illumina)
128 (TraPR sRNA Isolation Kit)

Small RNA-Seq Library Prep Kit · User Guide

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