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Reveal sRNA Potential

miRVEL Profiling Small RNA-Seq Library Prep Kit

User Guide

Catalog Numbers:
243 (miRVEL Profiling Small RNA-Seq Library Prep Kit for Illumina)
246 (miRVEL Profiling PCR Add-on and Reamplification Kit)

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Table of Contents

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1. Overview

The miRVEL Profiling Small RNA-Seq Library Prep Kit consists of all reagents and components required to generate small RNA libraries to be used for Next Generation Sequencing on an Illumina platform. The protocol does not require intermitted purification, and with only one magnetic bead based purification post PCR and is fully automation compatible.

The library prep workflow is suitable for a wide range of input RNA amounts from 10 ng - 1000 ng total RNA, and different sample types 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 TraPR (Cat. No. 128) or Lexogen's SPLIT RNA Extraction Kit (Cat. No. 008).

The miRVEL Profiling Small RNA-Seq Library Prep utilizes a streamlined workflow that reduces handling time and allows completion of the entire library prep procedure in less than 6 hours.

Depending on the input RNA source and amount some protocol adjustments are recommended for best performance. Please refer to Appendix B, p.17 for more details.

Input RNA (see Appendix B, p.17) is first subjected to a 3' adapter ligation followed by the addition of a blocking reagent and subsequent ligation of 5' adapters without the need for an additional purification step. The input RNA, flanked by 5' and 3' adapters, is then converted into cDNA. Unique Dual Indices (UDIs) are introduced during the PCR amplification step, allowing multiplexing of up to 96 libraries. The library product is then subjected to a magnetic bead-based 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.

Lexogen's miRVEL Profiling Small RNA-Seq libraries are compatible with single-read (SR) and paired-end (PE) sequencing reagents. In general, short read lengths of 50 nt in single-read mode are sufficient for sequencing small RNA-Seq libraries.

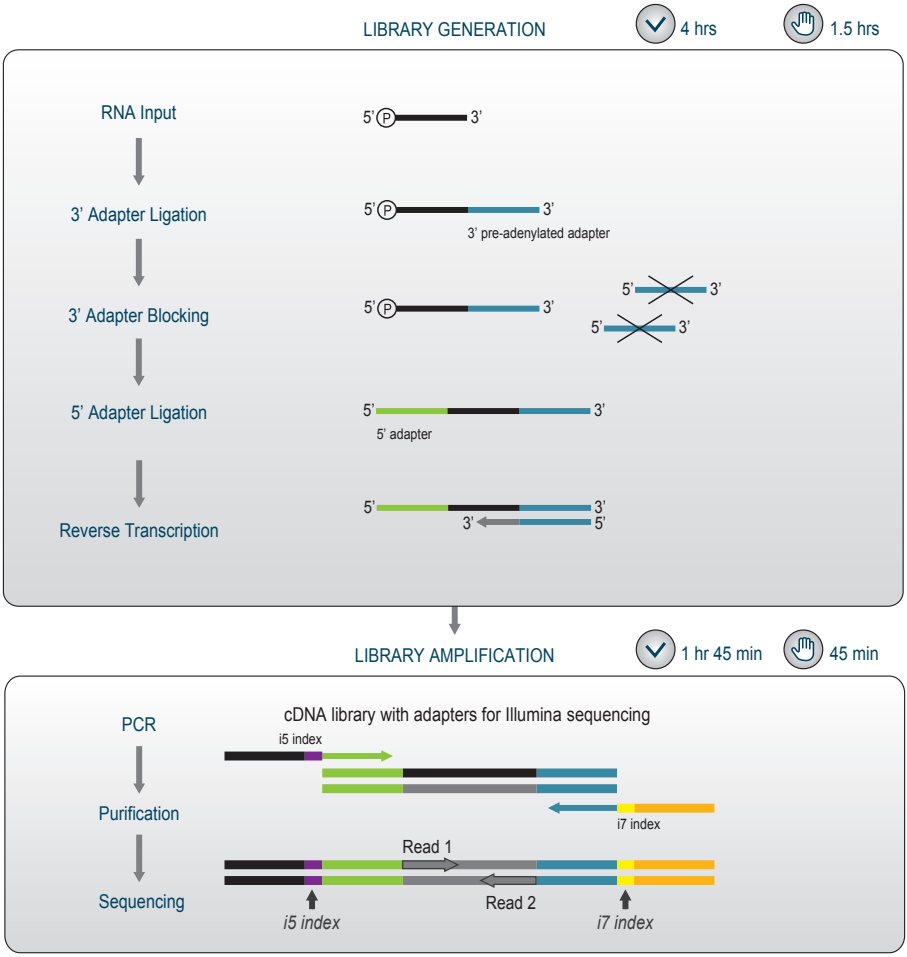

Figure 1. Schematic overview of the Small RNA-Seq Library Prep workflow.

2. Kit Components and Storage Conditions

Upon receiving the miRVEL Profiling Small RNA-Seq Kit, store the Purification Module (Cat. No. 022) containing **PB**, **PS**, and **EB** at +4 °C, and the rest of the kit in a -20 °C freezer. NOTE: Before use, check the contents of **PS**. If a precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

Kit Component	Tube / Plate Label	Volume*		Storage
		24 preps	96 preps	
RNase Inhibitor	**RI**	27 µl	106 µl	-20 °C
3' Adapter	**A3**	27 µl	106 µl	-20 °C
Ligation Mix	**LM**	264 µl	1,056 µl	-20 °C
Enzyme Mix 1	**E1**	27 µl	106 µl	-20 °C
Blocking Reagent	**BR**	27 µl	106 µl	-20 °C
5' Adapter	**A5**	27 µl	106 µl	-20 °C
ATP	**ATP**	27 µl	106 µl	-20 °C
Enzyme Mix 2	**E2**	27 µl	106 µl	-20 °C
First Strand cDNA Synthesis Mix	**FS**	80 µl	317 µl	-20 °C
DTT	**DTT**	20 µl	80 µl	-20 °C
Enzyme Mix 3	**E3**	20 µl	80 µl	-20 °C
Molecular Biology Grade Water	**H_2O**	300 µl	1,320 µl	-20 °C
Library Amplification Module				
PCR Mix	**PM**	185 µl	740 µl	-20 °C
PCR Enzyme Mix	**PE**	27 µl	106 µl	-20 °C
Lexogen small RNA UDI Set				
Lexogen small RNA UDI Set SR_UDI_0001-0024 or Lexogen small RNA UDI Set SR_UDI_0001-0096		4 µl / reaction		-20 °C
Purification Module				
Purification Beads	**PB**	264 µl	1,056 µl	+4 °C
Purification Solution	**PS**	1,782 µl	7,128 µl	+4 °C
Elution Buffer	**EB**	1,056 µl	4,224 µl	+4 °C

*including ≥10 % surplus

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.

ATTENTION: Before starting this protocol, please read the General Guidelines for Lexogen Kits, which are available online. These provide a detailed overview of RNA and kit component handling, as well as general RNA input requirements.

Reagents

- 80 % fresh ethanol (EtOH, for washing of Purification Beads, PB).
- miRVEL Profiling PCR Add-on and Reamplification Kit V2 for Illumina (Cat. No. 246), for qPCR assay.
- Recommended: SYBR Green I (Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585), 10,000x in DMSO for qPCR.

Equipment

- Magnetic rack or plate e.g., for 1.5 ml tubes: BcMag Magnetic separator-24, article# MS-03 from Bioclone; for 96-well plates: 96S Super Magnet Plate, article# A001322 from Alpaqua.
- Benchtop centrifuge (12,000 x g, rotor compatible with 1.5 ml tubes or 3,000 x g, rotor compatible with 96-well plates).
- Calibrated single-channel and multi-channel pipettes for handling 1 µl to 1,000 µl volumes.
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath, 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).
- For optional gel extraction: agarose gels, dyes (e.g., SYBR® Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Inc. #S11494), and electrophoresis rig (for optional gel extraction), small Pestil or Gel Breaker Tubes (IST Engineering #3388-100) and tubes (1.5 ml or 2 ml).

The complete set of materials, reagents, and labware necessary for quality control is not listed.

4. Detailed Protocol

4.1 Library Generation

Preparation

3' Adapter Ligation	3' Adapter Blocking	5' Adapter Ligation	Reverse Transcription of Ligated RNA
H_2O ○ – thawed at RT **A3** ● – thawed at RT **LM** ● – thawed at 30 °C **E1** ● – keep on ice or at -20 °C	**BR** ● – thawed at RT	**A5** ● – thawed at RT **ATP** ● – thawed at RT **E2** ● – keep on ice or at -20 °C	**FS** ● – thawed at RT **DTT** ● – thawed at RT **E3** ● – keep on ice or at -20 °C
70 °C, 2 min place on ice 28 °C, 60 min	75 °C, 5 min, 37 °C, 15 min 25 °C, 5 min	70 °C, 2 min place on ice 28 °C, 60 min	50 °C, 60 min

3' Adapter Ligation

RNA samples (10 ng - 1000 ng) 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.15.

ATTENTION: Please refer to Appendix B, p.17 for information on appropriate amounts of input RNA and recommended protocol adjustments for RNA input amounts lower than 100 ng.

- Before use, thaw the Ligation Mix (**LM** ●) at 30 °C and 1,250 rpm on a ThermoMixer until dissolved completely.
- **LM** and Enzyme Mix (**E2** ●) are viscous solutions! Proper mixing is essential for high yield and excellent reproducibility.

1. Dilute your input RNA (>100 ng) to a volume of 6 µl with Molecular Biology Grade Water (**H_2O** ○) 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.17.
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 10 µl Ligation Mix (**LM** ●), 1 µl of Molecular Biology Grade Water (**H_2O** ○), 1 µl RNase Inhibitor (**RI** ●), 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.

Adapter Blocking

Before proceeding to the 5′ Adapter Ligation the 3′ adapter is blocked from ligating to **A5** ●.

5 Spin down the 3′ Adapter Ligation reaction and add 1 µl of Blocking Reagent (**BR** ●). Mix well.

6 Incubate the reaction at 75 °C for 5 minutes, 37 °C for 15 minutes, and 25 °C for 5 minutes in a thermocycler.

5′ Adapter Ligation

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

7 Denature the required aliquot (1 µl per prep + 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.17.

8 Prepare Mastermix 2 containing 1 µl denatured 5′ Adapter (**A5** ●), 1 µl **ATP** ●, 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.

9 Add 3 µl of Mastermix 2 to each reaction from step 6. Mix well and spin down.

10 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.

11 Prepare Mastermix 3 containing 3 µl First Strand cDNA Synthesis Mix (**FS** ●), 1.5 µl **H_2O** ○, 0.75 µl **DTT** ●, and 0.75 µl of Enzyme Mix 3 (**E3** ●) per reaction. Mix well and spin down. **REMARK:** When preparing mastermixes always include a 10 % surplus per reaction.

12 Add 6 µl of Mastermix 3 to each reaction from step 10. Mix well and spin down.

13 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.

4.2 Library Amplification

Preparation

PCR		Purification (Cat. No. 022)*
PM ○ – thawed at RT **SR_UDI_0001-0096** – thawed at RT **PE** ○ – keep on ice or at -20 °C } **spin down before opening!**		**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **EB** – stored at +4 °C
Thermocycler	95 °C, 60 sec 95 °C, 15 sec 60 °C, 15 sec 72 °C, 60 sec } 14 - 22x Appendix B, p.17 Endpoint cycle number as determined by qPCR (Cat. No. 246). 72 °C, 6 min 10 °C, ∞	Equilibrate all reagents to room temperature for 30 minutes prior to use.

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 dual indices for multiplexing. The miRVEL Profiling Small RNA-Seq Kit contains 24 or 96 unique dual small RNA Index Primer pairs (**SR_UDI_0001-0024** or **SR_UDI_0001-0096**), respectively. There is no purification step between reverse transcription and PCR.

ATTENTION: Important notes for Library Amplification.

Perform a qPCR assay to determine the optimal PCR cycle number for endpoint PCR. The number of PCR cycles for library amplification must be adjusted according to RNA input amount, quality, and sample type. The miRVEL Profiling PCR Add-on and Reamplification Kit (Cat. No. 246) is required.

- Avoid cross contamination when using the Lexogen Small RNA UDI Indexing Sets. Spin down the Index Set before opening and visually check fill levels. Pierce or cut open the sealing foil of the wells containing the desired UDIs only. Reseal opened wells using fresh sealing foil after use to prevent cross contamination.
- Each well of the Lexogen Small RNA UDI Indexing Set is intended for single use only.

NOTE: At this point we recommend placing the Purification Module (**PB**, **PS**, and **EB**) for step 18 at room temperature to equilibrate for at least 30 minutes.

14 Prepare Mastermix 4 containing 7 µl PCR Mix (**PM** ○) and 1 µl PCR Enzyme (**PE** ○) per reaction. Mix well and spin down. **REMARK:** When preparing mastermixes always include a 10 % surplus per reaction.

15 Prealiquot 8 µl of the **PM / PE** mastermix into PCR tubes and add 23 µl of sample (from step 13).

16 Add 4 µl of the respective small RNA UDI Primer (**SR_UDI_0001-0024** or **SR_UDI_0001-0096**). Mix well and spin down. Add only one index primer pair per sample. **ATTENTION:** Spin down the SR_UDI 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 SR_UDI Plate is intended for single use only!

17 Conduct 14 - 22 cycles (see Appendix B, p.17) of PCR with the following program: Initial denaturation at 95 °C for 60 seconds, 14 - 22 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for 60 seconds, and a final extension at 72 °C for 6 minutes, hold at 10 °C. ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.
ATTENTION: For ≤100 ng RNA input, the number of PCR cycles could be increased up to 22 cycles. Please refer to Appendix B, p.17.
☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

Purification

The final library is purified to remove PCR components that can interfere with quantification. The Purification Reagents (**PB**, **PS**, and **EB**) should equilibrate for 30 minutes at room temperature before use. The Purification Beads (**PB)** must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

ATTENTION: If the libraries were stored at -20 °C, ensure that they are thawed and equilibrated to room temperature before restarting the protocol.

18 Add 10 µl of thoroughly resuspended Purification Beads (**PB**) and 37.5 µl Purification Solution (**PS**) to each reaction. Mix well and incubate 5 min at room temperature.
OPTIONAL: A mastermix of 10 µl of **PB** and 37.5 µl **PS** per reaction may be prepared for this step to minimize pipetting steps. Add 47.5 µl of thoroughly resuspended **PB**/**PS** mastermix to each reaction, mix well, and incubate 5 min at room temperature.

19 Place the plate / tube onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.

20 Remove and discard the clear supernatant without removing the plate / tube from the magnet. Do not disturb the beads!

21	Add 20 µl of Elution Buffer (**EB**), remove the plate / tube from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
22	Add 30 µl of Purification Solution (**PS**) to the **PB** / **EB** mix to reprecipitate the library. Mix thoroughly and incubate for 5 minutes at room temperature.
23	Place the plate / tube onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
24	Remove and discard the clear supernatant without removing the PCR plate / tube from the magnet. Do not disturb the beads!
25	Add 100 µl of 80 % EtOH and incubate the beads for 30 seconds. Leave the plate / tube in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant. **ATTENTION:** Remove the supernatant completely and do not let the beads dry too long! .
26	Add 20 µl of Elution Buffer (**EB**) per well, remove the plate / tube from the magnet, and resuspend the beads fully in **EB**. Incubate for 2 minutes at room temperature.
27	Place the plate / tube onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
28	Transfer 17 µl into a fresh plate / tube. At this point, the Small RNA-Seq library is finished and ready for quality control (Appendix C, p.18). ☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

5. Short Procedure

ATTENTION: All centrifugation steps are performed at 18 °C

4 hrs Library Generation

Total RNA (>100 ng)	Total RNA / Enriched Small RNA (≤100 ng)
3′ Adapter Ligation	
☐ Skip!	☐ Pre-dilute **A3** ● to 0.5x (1: 2), **or** 0.25x (1:4), **or** 0.1x (1:10) (see Appendix B, p.17).
☐ Mix 6 µl RNA with 1 µl **A3** ●.	
☐ Incubate for 2 min at 70 °C. Place on ice.	
☐ Prepare a mastermix of 10 µl **LM** ●, 1 µl **H_2O** ○, 1 µl **RI** ●, and 1 µl **E1** ● per reaction. Mix well.	
☐ Add 13 µl **LM** / **H_2O** / **RI** / **E1** mastermix per reaction. Mix well.	
☐ Incubate for 1 hr at 28 °C. ☞ Safe stopping point.	
Adapter Blocking	
☐ Add 1 µl **BR** ● per reaction. Mix well.	
☐ Incubate the reaction at 75 °C for 5 min, 37 °C for 15 min, and 25 °C for 5 min.	
5′ Adapter Ligation	
☐ Skip!	☐ Pre-dilute **A5** ● same way as used for **A3** (1:2, 1:4, or 1:10) (see Appendix B, p.17).
☐ Denature 1.1 µl **A5** ● per reaction for 2 min at 70 °C. Place on ice.	
☐ Prepare a mastermix of 1 µl denatured **A5** ●, 1 µl **ATP** ●, and 1 µl of **E2** ● per reaction. Mix well.	
☐ Add 3 µl **A5** / **ATP** / **E2** mastermix per reaction. Mix well.	
☐ Incubate for 1 hr at 28 °C.	
Reverse Transcription of Ligated RNA	
☐ Prepare a mastermix of 3 µl **FS** ●, 1.5 µl **H_2O** ○, 0.75 µl **DTT** ●, and 0.75 µl **E3** ●, per reaction. Mix well.	
☐ Add 6 µl **FS** / **H_2O** / **DTT** / **E3** mastermix per reaction. Mix well.	
☐ Incubate for 1 hr at 50 °C. ☞ Safe stopping point.	

qPCR
[Optional! Requires miRVEL Profiling PCR Add-on and Reamplification Kit (Cat. No. 246)]

Prepare a 2.5x stock of SYBR Green I nucleic acid stain (i.e., 1:4,000 dilution in DMSO; use Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585).

- [ ] Combine 2.3 µl of cDNA with: 7 µl **PM** ○, 10 µl **miRVEL Profiling qPCR Primer Mix** ●, 1 µl **PE** ○, 1.4 µl of 2.5x SYBR Green I nucleic acid stain, and 13.3 µl of **EB**, per reaction. Mix well.
- [ ] PCR: 95 °C, 60 sec

95 °C, 15 sec
60 °C, 15 sec } **35x**
72 °C, 60 sec

72 °C, 6 min

10 °C, ∞. Calculate the optimal cycle number for Endpoint PCR (please refer to 246UGxxx).

Endpoint PCR

- [ ] Prepare a mastermix with 7 µl PCR Mix (**PM** ○) and 1 µl PCR Enzyme (**PE** ○) per reaction.
- [ ] Add 8 µl of the **PM / PE** mastermix to 23 µl of the eluted library.
- [ ] Add 4 µl of one Unique Dual Index Primer pair (SR_UDI_0001-0024, or SR_UDI_0001-0096) to each sample. **ATTENTION:** Reseal opened index wells after use! Use only one UDI / sample.
- [ ] PCR: 95 °C, 60 sec

95 °C, 15 sec
60 °C, 15 sec } **14 - 22x**
72 °C, 60 sec Appendix B, p.17

72 °C, 6 min

10 °C, ∞. ☞ Safe stopping point.

Purification

- [ ] Prepare a mastermix of 10 µl **PB** and 37.5 µl **PS** per reaction, mix well.
- [ ] Add 47.5 µl well mixed **PB** / **PS** per reaction, mix well, incubate 5 min at RT.
- [ ] Place on magnet for 2 - 5 min, discard supernatant.
- [ ] Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- [ ] Add 30 µl **PS**, mix well, incubate 5 min at RT.
- [ ] Place on magnet for 2 - 5 min, discard supernatant.
- [ ] Rinse the beads once with 100 µl 80 % EtOH, 30 sec.
- [ ] Air dry beads for 5 - 10 minutes. **ATTENTION:** Do not let the beads dry too long!
- [ ] Add 20 µl **EB**, remove from magnet, mix well, incubate 2 min at RT.
- [ ] Place on magnet for 2 - 5 min, transfer 17 µl of the supernatant into a fresh PCR plate. ☞ Safe stopping point.

6. Appendix A: RNA Requirements

RNA Integrity

miRVEL Profiling 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 TraPR Small RNA Isolation Kit (Cat. No. 128) or 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 miRVEL Profiling 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.

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 minimizes co-isolation of 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 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). rRNA may also become an issue in degraded starting material. An rRNA removal step of such small rRNAs can increase the read depth for miRNA and other small RNAs. Avoid rRNA depletion kits that remove small fragments in the subsequent purification steps.

Best way to avoid rRNA contamination in small RNA library preps is to use Lexogen's TraPR Small RNA Isolation Kit (Cat. No. 128), which only isolates small RNAs from RISC complexes.

For more information please consult the online Frequently Asked Questions.

7. 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	Dilution Factors Used for A3 ● & A5 ●	Number of PCR Cycles
1,000 ng - 100 ng	1x	14 - 16
100 ng - 50 ng	0.5x (1:2 dilution)	17 - 20
50 ng - 10 ng	0.25x (1:4 dilution)	19 - 21
TraPR enriched RNA*	0.1x (1:10 dilution)	17 - 22

*TraPR enriched RNA is typically not quantifiable using capillary microfluidics devices, such as Bioanalyzer, Fragment Analyzer or ScreenTapes on TapeStation. We recommend using 3 - 6 µl of the TraPR enriched RNA fraction and directly proceed to small RNA library preparation.

For input overlaps e.g., enriched small RNA or total RNA inputs of 100 ng, it is for instance also possible to use the 0.5x 3' Adapter (**A3** ●) and 5' Adapter (**A5** ●) dilutions recommended in the table above. Dilutions may reduce the yields, but also reduce unwanted side products such as linker-linker artifacts. If possible increasing the RNA input is always the most preferred option to prevent artifact formation.

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, it is recommended to use ≥100 ng of total RNA input from cells or tissues.

RNA isolated from plasma, serum, urine, exosomes, saliva RNA, CSF, or FFPE material are often at low concentration ranges and available in limited quantities hence dilutions of **A3** ● and **A5** ● are likely required.

8. 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 mirVEL Profiling Small RNA-Seq libraries generated from 1 µg and 100 ng of Human Brain Total RNA (HBTR), small RNA isolated with TraPR (Cat. No. 128) from *Arabidopsis thaliana* leaves and human plasma RNA are shown in Figure 3 and 4.

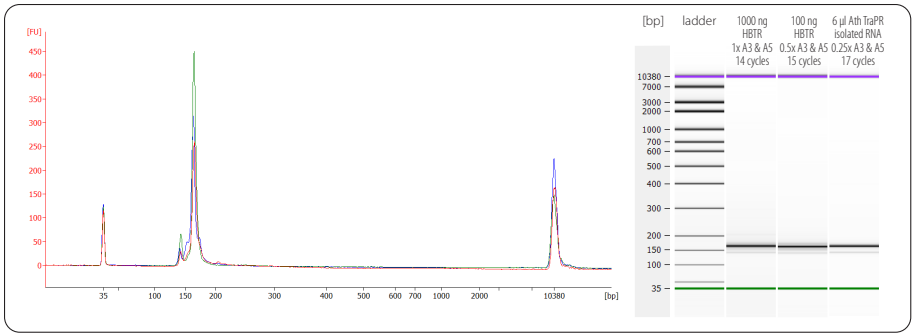

Figure 3. Bioanalyzer traces of miRVEL Profiling Small RNA-Seq libraries synthesized from 1000 ng (red trace, 14 cycles, 1x A3 and 1x A5) and 100 ng Human Brain Total RNA (HBTR) (blue trace, 15 cycles, 0.5x A3 and 0.5x A5), as well as 6 µl of TraPR purified small RNA (green trace, 17 cycles, 0.25x A3 and 0.25x A5). 1 µl of each library was loaded onto a High Sensitivity DNA chip. Peaks at 165 bp correspond to the miRNA library, a smaller peak at 143 bp are linker-linker artifacts. In this case, an additional clean-up of the lane mix with magnetic beads as described in Appendix G, p.22 is recommended. Furthermore, for libraries synthesized from total RNA a size selection using magnetic beads as described in Appendix G, p.22 or gel extraction may be performed.

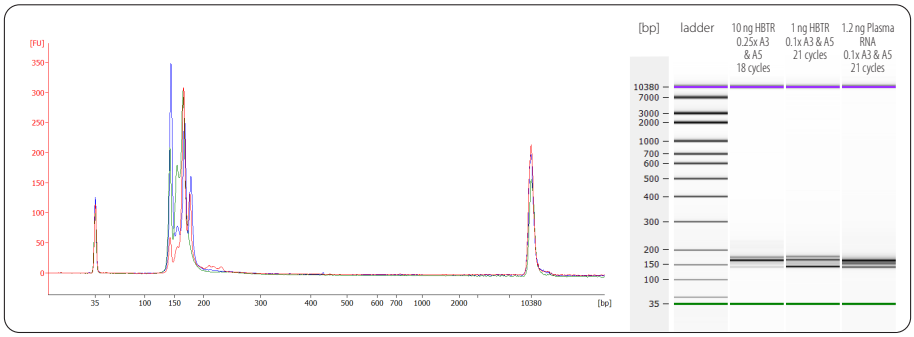

Figure 4. Bioanalyzer traces of miRVEL Profiling Small RNA-Seq libraries synthesized from 10 ng (red trace, 17 cycles, 0.25x A3 and 0.25x A5) and 1 ng (blue trace, 21 cycles, 0.1x A3 and 0.1x A5) Human Brain total RNA as well as 3 µl (~1.2 ng) of plasma RNA (green trace, 21 cycles, 0.1x A3 and 0.1x A5). 1 µl of each library was loaded onto a High Sensitivity DNA chip. Peaks at 165 bp correspond to the miRNA library. Despite Adapter dilution and adapter blocking limited input material may lead to formation of an artifact peak at 143 bp. In this case, an additional clean-up of the lane mix with magnetic beads as described in Appendix G, p.22 is recommended. In the red trace, peaks at 165 bp correspond to the miRNA library, a peak at 178 bp represents piRNA, the peak at ~232 bp corresponds to tRNA, and the peak at ~298 bp originates from snRNA.

If the small RNA-Seq libraries show linker-linker artifacts (peak at 143 bp), an additional clean-up step may be required. We recommend removing these side-products before proceeding to Next Generation Sequencing as described in Appendix G, p.22. miRVEL Profiling kits contain sufficient Purification Solutions for additional clean up of lane mixes. 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!

9. Appendix D: Multiplexing

Small RNA Unique Dual Indices (UDI) are introduced during the PCR amplification with the small RNA UDI Primers **SR UDI_0001-0096** (step 16). Each kit size contains 24, or 96 different indices, respectively, provided in a 96-well plate.

Small RNA UDIs

Small RNA UDIs allowing up to 96 samples to be sequenced per lane on an Illumina flow cell are included in the kit (96-well plate).

Small RNA Unique Dual Index sequences are available for download at www.lexogen.com. Please note that depending on which Illumina sequencing machine is used the i5 Index may need to be entered either in forward or reverse orientation, separate index sequence sheets for the 8 nt i5 and i7 indices are available for i5 FWD and REV orientation.

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 155 - 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.

For more indexing options contact Lexogen at support@lexogen.com.

10. 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 miRVEL Profiling Small RNA-Seq library adapters (Cat. No. 243) is shown below..

Small RNA-Seq Libraries with Unique Dual Indexing

i7 Indices and i5 Indices are introduced during PCR (step 16). Read 1 directly corresponds to the miRNA sequence.

```
    5'-(MiSeq: Index 1 (i5) Sequencing Primer)-3'       5'-(Read 1 Sequencing Primer)-3'
5'  AATGATACGGCGACCACCGAGATCTACAC-i5-ACACTCTTTCCCTACACGACGCTCTTCCGATCT -(Insert…
3'  TTACTATGCCGCTGGTGGCTCTAGATGTG-i5-TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGA -(Insert…
                                    3'-(NextSeq/NovaSeqIndex 1 (i5) Sequencing Primer)-5'

            5'-(Index 1 (i7) Sequencing Primer)-3'
   …Insert)- TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC-i7-ATCTCGTATGCCGTCTTCTGCTTG 3'
   …Insert)- ACCTTAAGAGCCCACGGTTCCTTGAGGTCAGTG-i7-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.

11. Appendix F: Data Analysis

Information on the data analysis pipeline for miRVEL Profiling Small RNA-Seq Library fastq sequencing files is available from Lexogen online: www.lexogen.com/mirvel-small-rna-seq-data-analysis.

12. Appendix G: Repurification of Linker-Linker Contaminated Lane Mixes

If linker-linker artifacts (at 143 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 - magnetic bead repurification (see protocol below) is recommended.
- <20% of the library - repurification is optional, 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.

13. Appendix H: Revision History

Publication No. / Revision Date	Change	Page
243UG899V0100 Oct. 1, 2025	Initial release.	

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The RNA Experts

Associated Products:

008 (SPLIT RNA Extraction Kit)
022 (Purification Module with Magnetic Beads)
128 (TraPR Small RNA Isolation Kit)
246 (PCR Add-on and Reamplification Kit for miRVEL Profiling Small RNA Kit)

miRVEL Profiling Small RNA-Seq Library Prep Kit · User Guide

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