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miRVEL

Reveal sRNA Potential

miRVEL Discovery Small RNA-Seq Library Prep Kit

User Guide

Catalog Number:
242 (miRVEL Discovery Small RNA-Seq Library Prep Kit)

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1. Overview

The miRVEL Small RNA-Seq Library Prep Kit provides a highly advanced solution for small RNA discovery and expression profiling. The methodology utilizes modified oligonucleotides that effectively prevent adapter dimers in the small RNA library. The refined reaction chemistry reduces biases and background contaminants, resulting in robust, small RNA-specific libraries. The incorporation of unique molecular identifiers (UMIs) during reverse transcription allows for unbiased and precise quantification. The kit accommodates a wide range of input total RNA, from as little as 1 ng to 500 ng, and is suitable for various sample types, including cells, tissues, exosomal RNA, plasma, and other biofluids, making it a perfect choice for biomarker studies. Additionally, the kit minimizes the presence of hY4 Y RNA, which is often found at elevated levels in serum and plasma samples. Depending on the source and quantity of input RNA, some protocol adjustments may be necessary. For more information, please consult Appendix B, p.19.

The miRVEL Small RNA-Seq Library Prep Kit features a streamlined workflow that allows the entire library preparation process to be completed in under 7 hours. The standard miRVEL Discovery protocol eliminates the need for gel size selection, significantly decreasing hands-on time and shortening the overall workflow.

Initially, the input RNA (refer to Appendix A, p.17) undergoes 3’ adapter ligation using a preadenylated 3’ DNA adapter, followed by 5’ RNA adapter ligation. Mature miRNAs possess both a 3' hydroxyl group and a 5' phosphate group, which enable the specific ligation of adapters to both ends. The resulting RNA, now flanked by both adapters, is converted into cDNA with the incorporation of UMIs. During the PCR amplification step, 10 nt unique dual indices (UDIs) are introduced, utilizing a fixed combination of two unique index barcodes per sample to ensure each sample receives a distinct UDI pair. This strategy effectively reduces the risk of read misassignment due to index hopping. All purification steps are based on magnetic beads, making the protocol highly suitable for automation.

The miRVEL Discovery Small RNA-Seq libraries are compatible with single-read (SR) sequencing, requiring 72 bp read-length or 50 bp SR if UMIs are not required.

The miRVEL Discovery Small RNA-Seq Library Prep Kit provides all necessary reagents and components for constructing small RNA libraries to be used for Next Generation Sequencing on Illumina platforms.

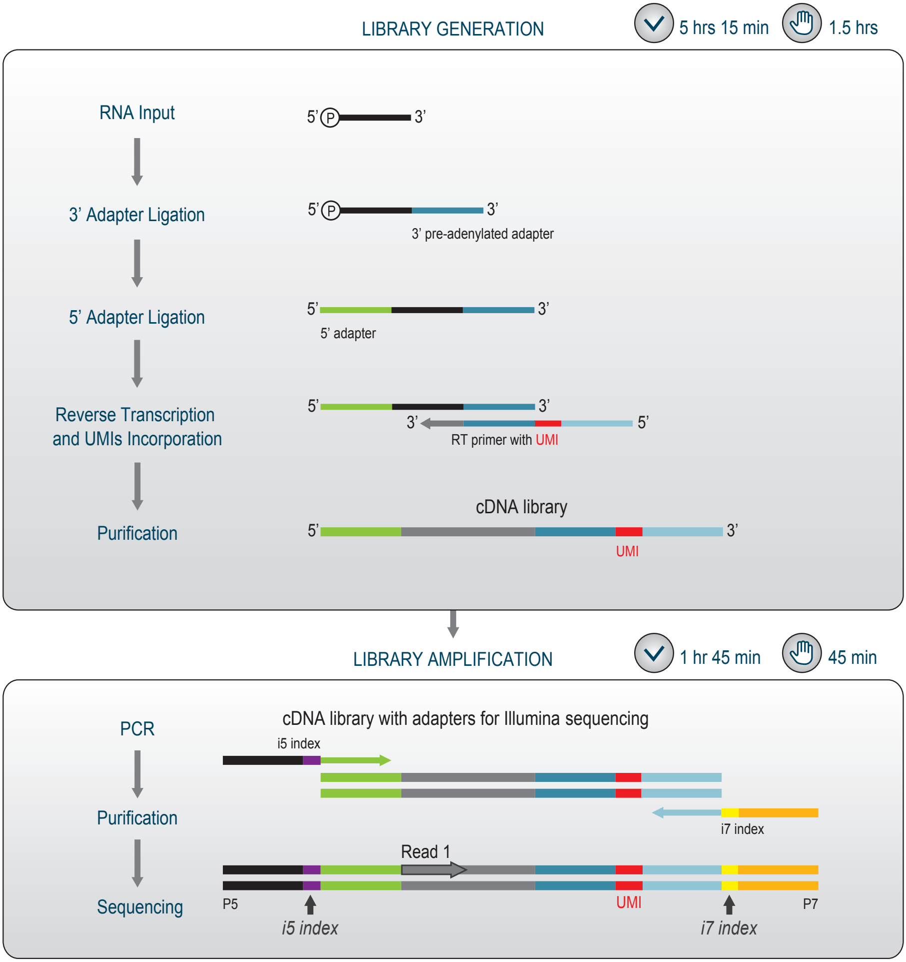


Figure 1. Schematic overview of the miRVEL Discovery Small RNA-Seq Library Prep Kit workflow.

2. Kit Components and Storage Conditions

Upon receiving the miRVEL Discovery Small RNA-Seq Library Prep Kit, store the miRVEL Discovery Small RNA-Seq Library Prep module and the Index Plate at -20 °C and the Purification Module at +4 °C. Ligation Activator (**LA** 🟣) should be stored at +4 °C or at -20 °C. The index plate is sealed with a pierceable foil cover, and each well is designed for single use only.

Kit Component	Tube / Plate Label	Volume*		Storage
		24 preps	96 preps	
miRVEL Discovery Small RNA-Seq Library Prep Kit				
3'Adapter	**A3** 🟣	24 µl	96 µl	-20 °C
Ligation Mix 1	**LM1** 🟣	48 µl	192 µl	-20 °C
Enzyme Mix 1	**E1** 🟣	24 µl	96 µl	-20 °C
RNase Inhibitor	**RI** ⚪	72 µl	288 µl	-20 °C
5' Adapter	**A5** 🟡	24 µl	96 µl	-20 °C
Ligation Mix 2	**LM2** 🟡	48 µl	192 µl	-20 °C
Enzyme Mix 2	**E2** 🟡	24 µl	96 µl	-20 °C
Reverse Transcription Initiator	**RTI** 🟢	48 µl	192 µl	-20 °C
Reverse Transcription Primer	**RTP** 🟢	48 µl	192 µl	-20 °C
Reverse Transcription Mix	**RTM** 🟢	288 µl	1152 µl	-20 °C
Enzyme Mix 3	**E3** 🟢	24 µl	96 µl	-20 °C
PCR Mix	**PM** 🔵	384 µl	1536 µl	-20 °C
PCR Enzyme Mix	**PE** 🔵	72 µl	288 µl	-20 °C
Molecular Biology Grade Water	H_2O ○	1.5 ml	1.5 ml	-20 °C
miRVEL Discovery UDI 10 nt Sets				
miRVEL UDI index plate		**miRVEL Discovery UDI Set B0 24rxn,** 9 µl / rxn	**miRVEL Discovery UDI Set A0 96 rxn,** 9 µl / rxn	-20 °C
Purification Module for miRVEL Discovery Small RNA-Seq Library Prep Kit				
Ligation Activator	**LA** 🟣	240 µl	2x 600 µl	+4 °C
Purification Beads	**PB**	20 ml	38.4 ml	+4 °C
Purification Solution	**PS**	14 ml	54 ml	+4 °C

*+ including ≥10 % surplus

3. User-Supplied Consumables and Equipment

Ensure that you have all of the necessary material and equipment before beginning the library preparation. 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 / Solutions

- 80 % fresh ethanol (EtOH, for washing of Purification Beads, **PB**).

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 pipettes for handling 1 µl to 1,000 µl volumes.
- Thermocycler.
- UV-spectrophotometer to quantify RNA.
- Ice bath or ice box, ice pellets, benchtop cooler (-20 °C for enzymes).
- Vortex mixer.

Labware

- Suitable ribonuclease-free low-binding pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 - 2 ml reaction tubes, low-binding, certified ribonuclease-free.
- 200 µl PCR tubes or 96-well plates and caps or sealing foil.

Optional Equipment

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

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

4. Detailed Protocol

4.1. Library Generation

Preparation

3' Adapter Ligation	5' Adapter Ligation	Reverse Transcription of Ligated RNA	cDNA Purification
H_2O ○ – thawed at RT **A3** ● – thawed at RT **LM1** ● – thawed at RT **E1** ● – keep on ice or at -20 °C **RI** ○ – keep on ice or at -20 °C **LA** ● – thawed at RT	**H_2O** ○ – thawed at RT **A5** ● – thawed at RT **LM2** ● – thawed at RT **E2** ● – keep on ice or at -20 °C **RI** ○ – keep on ice or at -20 °C	**RTI** ● – thawed at RT **RTP** ● – thawed at RT **RTM** ● – thawed at RT **E3** ● – keep on ice or at -20 °C **RI** ○ – keep on ice or at -20 °C	**PB** – stored at +4 °C **PS** – stored at +4 °C **80 % EtOH** – provided by user **prepare fresh!** **H_2O** ○ – thawed at RT
28 °C, 1 hour 65 °C, 20 min Hold at 4 °C (at least 5 min)	28 °C, 30 min 65 °C, 20 min Hold at 4 °C	RT Initiator incubation: 75 °C, 2 min 70 °C, 2 min 65 °C, 2 min 60 °C, 2 min 55 °C, 2 min 37 °C, 5 min 25 °C, 5 min Hold at 4 °C Reverse Transcription: 50 °C, 1 hour 70 °C, 15 min Hold at 4 °C (at least 5 min)	

3' Adapter Ligation

RNA samples are subjected to 3' ligation with a pre-adenylated DNA 3' Adapter (A3 ●). The recommended input RNA amount is 100 ng of total RNA isolated from cells or tissue samples. For more information about the RNA input, please see Appendix A, p.17 For RNA inputs <100 ng, plasma and serum samples, please consult Appendix B, p.19 for a protocol adaptation.
ATTENTION: The 3' ligation reaction is viscous. Pipette slowly to mix the reaction. Do not vortex.
REMARK: When preparing mastermixes always include a 10 % surplus per reaction.

① Mix 3' Adapter (**A3** ●), Ligation Mix 1 (**LM1** ●), and Ligation Activator (**LA** ●) by flicking the tubes, spin down briefly, and keep at room temperature.

② Set up the 3' ligation mastermix on ice by adding the components in the indicated order: 1 µl of **A3** ●, 1 µl of RNase Inhibitor (**RI** ○), 1 µl of Enzyme Mix 1 (**E1** ●), 2 µl of **LM1** ● and 10 µl of **LA** ● per reaction. Briefly spin down, then mix thoroughly by pipetting slowly up and down 15 - 20 times, and briefly spin down again.
ATTENTION: For <100 ng input RNA, plasma and serum samples, pre-dilute **A3** ● with **H_2O** ○ according to the table in Appendix B, p.19, mix thoroughly by pipetting up and down 12 times, and briefly spin down.

③ Add 15 µl of 3' ligation mastermix to 100 ng of input RNA in 5 µl **H_2O** ○, resulting in 20 µl of total volume. Briefly spin down, then mix thoroughly by pipetting slowly up and down 15 - 20 times, and briefly spin down again.

④ Incubate for 1 hour at 28°C and then for 20 minutes at 65 °C. Hold at 4°C for at least 5 minutes. **ATTENTION:** Proceed immediately to 5' ligation.

5' Adapter Ligation

During this step, the RNA 5' Adapter (**A5** ●) is ligated to the RNA.
ATTENTION: The 5' ligation reaction is viscous. Pipette slowly to mix the reaction. Do not vortex.
REMARK: When preparing mastermixes always include a 10 % surplus per reaction.

⑤ Mix 5' Adapter (**A5** ●), Ligation Mix 2 (**LM2** ●) by flicking the tubes, spin down briefly and keep at room temperature.

⑥ Set up the 5' ligation mastermix on ice by adding the components in the indicated order: 15 µl of **H_2O** ○, 2 µl or **LM2** ●, 1 µl of **RI** ○, 1 µl of Enzyme Mix 1 (**E2** ●) and 1 µl of **A5** ●. Briefly spin down, then mix thoroughly by pipetting slowly up and down 10 - 15 times, and briefly spin down again.

ATTENTION: For <100 ng input RNA, plasma and serum samples, pre-dilute **A5** ● with **H_2O** ○ according to the table in Appendix B, p.19, mix thoroughly by pipetting up and down 12 times, and briefly spin down.

⑦ Add 20 µl of the mastermix to each reaction from step ④ resulting in 40 µl of total volume. Briefly spin down, then mix thoroughly by pipetting slowly up and down 10 - 15 times, and briefly spin down again.

⑧ Incubate for 30 min at 28°C and then for 20 minutes at 65 °C. Hold at 4°C.
ATTENTION: Proceed immediately to Reverse Transcription of Ligated RNA.

Reverse Transcription of Ligated RNA

In this step, 3' and 5' adapter-ligated RNA is converted into cDNA using a Reverse Transcription Primer (**RTP** ●) that incorporates UMIs into each cDNA molecule.
REMARK: When preparing mastermixes always include a 10 % surplus per reaction.

⑨ Mix Reverse Transcription Initiator (**RTI** ●), Reverse Transcription Primer (**RTP** ●) and Reverse Transcription Mix (**RTM** ●) by flicking the tubes, spin down briefly and keep at room temperature.

⑩ Add 2 µl of **RTI** ● to each ligation reaction from step ⑧. Briefly spin down, then mix thoroughly by pipetting up and down 15 - 20 times, and briefly spin down again.

⑪ Incubate the reactions as specified below.

Time	Temperature
2 min	75 °C
2 min	70 °C
2 min	65 °C
2 min	60 °C
2 min	55 °C
5 min	37 °C
5 min	25 °C
Hold (until further processing)	4 °C

⑫ Set up a reverse transcription mastermix on ice by adding the components in the indicated order: 2 µl of **RTP** ●, 2 µl of **H_2O** ○, 12 µl of **RTM** ●, 1 µl of **RI** ○ and 1 µl of Enzyme Mix 3 **E3** ● per sample. Briefly spin down, then mix thoroughly by pipetting up and down 15 - 20 times, and briefly spin down again.

ATTENTION: For <100 ng input RNA, plasma and serum samples, pre-dilute **RTP** ● with **H_2O** ○ according to the table in Appendix B, p.19, mix thoroughly by pipetting up and down 12 times, and briefly spin down.

⑬ Add 18 µl of the mastermix to the 42 µl of reaction from step ⑪, resulting in 60 µl of total volume. Briefly spin down, then mix thoroughly by pipetting up and down 15 - 20 times, and briefly spin down again.

⑭ Incubate for 1 hour at 50 °C and then for 15 minutes at 70 °C. Hold at 4°C for at least 5 minutes.

Beads Washing

The cDNA is purified using magnetic Purification Beads (**PB**), which are prepared by rebuffering in Purification Solution (**PS**). The **PB** must be fully resuspended before use. Thorough mixing or vortexing is recommended immediately before use.

NOTE: The beads can be prepared during the incubation of the reverse transcription reactions.

NOTE: The beads up to 4 samples can be prepared at one time in a single 2 mL tube.

⑮ Homogenize the Purification Beads (**PB**) by vortexing and vortex Purification Solution (**PS**).

⑯ Carefully transfer 400 µl of **PB** to 2 ml tube. This amount of the beads is sufficient for cDNA purification and final library purification of 1 sample.

⑰ Briefly spin down and place the tube onto a magnet and let the beads migrate until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.

⑱ Remove the tube from the magnet and carefully add 150 µl of **PS** and completely resuspend the beads by vortexing. Spin down briefly and place the tube immediately back onto the magnet.

⑲ Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet. Remove as much supernatant as possible without disturbing the beads.

⑳ Remove the tube from the magnet, carefully add 400 µl of **PS** and completely resuspend the beads by vortexing.

㉑ Keep the prepared purification beads on ice or store the beads at 4 °C. The prepared beads can be stored at 4 °C for up to 1 week.

cDNA purification

The cDNA library is purified using magnetic beads to remove ligation reaction components. The Purification Beads (**PB**) must be fully resuspended before use. Thorough mixing by pipetting or vortexing is recommended.

㉒ Briefly spin down the RT reactions from step ⑭.

㉓ Homogenize the prepared **PB** from step ㉑ by vortexing immediately before use.

㉔ Add 143 µl **PB** to the RT reaction. Vortex for 3 seconds and then briefly spin down. Incubate for 5 minutes at room temperature.

㉕ Place the tubes onto the magnet and let the beads collect for ~4 minutes or until the supernatant is completely clear.
NOTE: The speed of bead collection depends on the strength of the magnet.

㉖ Remove and discard the clear supernatant without removing the tube from the magnet. Make sure that accumulated beads are not disturbed. Remove as much supernatant as possible.

㉗ Add 200 µl of 80 % EtOH and remove and discard the supernatant immediately. Leave the sample on the magnet as beads should not be resuspended during this washing step.

㉘ Repeat this washing step once for a total of two washes. Make sure the supernatant is removed completely.

㉙ Keep the sample in contact with the magnet and let the beads dry for 10 minutes at room temperature. **ATTENTION:** Extend the drying time if the ethanol has not fully evaporated, as residual ethanol negatively influences subsequent library amplification.

㉚ With the beads still on the magnet, add 17 µl of **H_2O** ○, close the tube and remove the sample from the magnet.

㉛ Carefully resuspend by pipetting up and down, spin down briefly and incubate for 2 minutes at room temperature.

㉜ Place the tube onto the magnet and let the beads collect for ~2 minutes or until the supernatant is completely clear.

㉝ Transfer 15 µl of the supernatant into a fresh tube. The cDNA library is now ready for amplification. ☞ Safe stopping point. Libraries can be stored at ≤-20 °C at this point.

4.2. Library Amplification with 10 nt Unique Dual Indices (UDIs)

Preparation

PCR		Purification
PM ● **PE** ● **H_2O** ○ **miRVEL UDI Sets (A0 or B0)**	- thawed at RT - keep on ice or at -20 °C - thawed at RT - thawed at RT	**Prepared purification Beads from step** ㉑ **80 % EtOH** – provided by user **prepare fresh!** **H_2O** ○ - thawed at RT
Thermocycler	95 °C, 15 min 95 °C, 15 sec 60 °C, 30 sec 72 °C, 15 sec } 13- 22x Consult Appendix B for endpoint cycle number 72 °C, 2 min 4 °C, ∞	

PCR

The library is amplified to add the complete adapter sequences required for cluster generation and UDIs for multiplexing, and to generate sufficient material for quality control and sequencing.

ATTENTION: Avoid cross contamination when using the miRVEL UDI Sets. Spin down the UDI plate 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 with a fresh sealing foil after use to prevent cross contamination. Each well of the miRVEL UDI Set is intended for single use only.

REMARK: When preparing mastermixes always include a 10 % surplus per reaction.

㉞ Mix PCR Mix (**PM** ●), and index plate by flicking the tube or the plate, spin down briefly and keep at room temperature.

㉟ On ice, prepare a mastermix containing 21.5 µl **H_2O** ○, 10 µl **PM** ● and 1.5 µl PCR Enzyme (**PE** ●) per reaction. Briefly spin down and then mix thoroughly by pipetting up and down 12 times, and briefly spin down again.

㊱ Add 33 µl of the **PM** / **PE** mastermix to 15 µl of the eluted cDNA library from step ㉝.

㊲ Add 2 µl of the respective Unique Dual Index Primer pair to each sample. Use only one UDI per sample! Briefly spin down and then mix thoroughly by pipetting up and down 12 times, and briefly spin down again.

㊳ Conduct 13 - 22 cycles of PCR (Appendix B, p.19) with the following program: Initial denaturation at 95 °C for 15 minutes, 13 - 22 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds and 72 °C for 15 seconds, and a final extension at 72 °C for 2 minutes, hold at 4 °C at least for 5 minutes.

Purification

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

㊴ Spin down briefly the library amplification reaction from step ㊳ and add 47 µl of thoroughly resuspended prepared **PB** from step ㉑.

㊵ Vortex for 3 seconds and spin down briefly. Incubate for 5 minutes at room temperature.

㊶ Place the tube onto the magnet and let the beads collect for ~4 minutes, or until the supernatant is completely clear. **ATTENTION:** Do not discard the supernatant. It contains the small RNA fraction!

㊷ Transfer 92 µl of the supernatant to a new tube. Discard the tube with the beads.

㊸ Add 83 µl thoroughly resuspended **PB** to the 92 µl supernatant. Vortex for 3 seconds and spin down briefly. Incubate for 5 minutes at room temperature.

㊹ Place the tube onto the magnet and let the beads collect until the supernatant is completely clear.

㊺ Remove and discard the supernatant without removing the sample from the magnet. Make sure that accumulated beads are not disturbed. Keep the beads.

㊻ Add 200 µl of 80 % EtOH and remove and discard the supernatant immediately. Leave the sample on the magnet as beads should not be resuspended during this washing step.

㊼ Repeat this washing step once for a total of two washes. Make sure the ethanol is removed completely.

㊽ Keep the tube in contact with the magnet and let the beads dry for 10 minutes at room temperature. **ATTENTION:** Extend the drying time if the ethanol has not fully evaporated.

㊾ With the beads still on the magnet, add 17 µl of **H_2O** ○, close the tube and remove the tube from the magnet.

㊿ Carefully resuspend by pipetting up and down, spin down briefly and incubate for 2 minutes at room temperature.

(51) Place the sample onto the magnet and let the beads collect for ~2 minutes or until the supernatant is completely clear.

(52) Transfer 15 µl of the supernatant into a fresh tube. 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.

5. Short Procedure

5.25 hrs Library Generation

Total RNA (100 ng)	Total RNA (<100 ng), plasma, serum samples

3’ Adapter Ligation

☐ Skip!	☐ Pre-dilute **A3** ● according to Appendix B, p.19.

☐ On ice, prepare a mastermix in the indicated order: 1 µl **A3** ●, 1 µl **RI** ○, 1 µl **E1** ●, 2 µl **LM1** ● and 10 µl **LA** ● per reaction. Mix well by pipetting up and down 15 - 20 times.

☐ Add 15 µl of the mastermix to 100 ng of input RNA in 5 µl of **H_2O** ○. Mix well by pipetting up and down 15 - 20 times.

☐ Incubate for 1 hr at 28 °C, then for 20 min at 65 °C, hold at 4 °C for 5 min.

5’ Adapter Ligation

☐ Skip!	☐ Pre-dilute **A5** ● according to Appendix B, p.19.

☐ On ice, prepare a mastermix in the indicated order: 15 µl **H_2O** ○, 2 µl **LM2** ●, 1 µl **RI** ○, 1 µl **E2** ●, 1 µl **A5** ●. Mix well by pipetting up and down 10 -15 times.

☐ Add 20 µl of the mastermix into each sample. Mix well by pipetting up and down 10 - 15 times.

☐ Incubate for 30 min at 28 °C, then for 20 min at 65 °C, hold at 4 °C.

Reverse Transcription of Ligated RNA

☐ Add 2 µl **RTI** ● to each reaction. Mix well by pipetting up and down 15 - 20 times.

Incubate:

Time	Temperature
2 min	75 °C
2 min	70 °C
2 min	65 °C
2 min	60 °C
2 min	55 °C
5 min	37 °C
5 min	25 °C
Hold	4 °C

☐ Skip!	☐ Pre-dilute **RTP** ● according to Appendix B, p.19.

☐ On ice, prepare a mastermix in the indicated order: 2 µl **RTP** ●, 2 µl **H_2O** ○, 12 µl **RTM** ●, 1 µl **RI** ○ and 1 µl **E3** ●. Mix well by pipetting up and down 15 - 20 times.

☐ Add 18 µl of the mastermix into each sample.
Mix well by pipetting up and down 15 - 20 times.

☐ Incubate for 1 hr min at 50 °C, then for 15 min at 70 °C, hold at 4 °C for 5 min.

Bead Washing

☐ Homogenize the **PB** and vortex **PS**.

☐ Transfer 400 µl of **PB** to 2 ml tube. Spin down and place on a magnet, discard supernatant.

☐ Remove the tube from the magnet, and resuspend in 150 µl of **PS**.

☐ Place on the magnet, discard the supernatant.

☐ Remove the tube from the magnet, and resuspend in 400 µl of **PS**.

☐ Keep on ice or store at 4 °C.

cDNA Purification

☐ Add 143 µl prepared **PB** into each RT reaction, vortex 3 sec, incubate 5 min at RT.

☐ Place on the magnet for ~4 min, discard the supernatant.

☐☐ Rinse the beads twice with 200 µl 80 % EtOH while on the magnet.

☐ Air dry beads for 10 min.

☐ Add 17 µl **H_2O** ○, remove from the magnet, mix well, incubate 2 min at RT.

☐ Place on the magnet for ~2 min, transfer 15 µl of the supernatant into a fresh tube.

☞ Safe stopping point.

1.75 hrs Library Amplification

PCR

☐ On ice, prepare a mastermix with 21.5 µl **H_2O** ○, 10 µl **PM** ● and 1.5 µl **PE** ● per reaction. Mix well by pipetting up and down 12 times.

☐ Add 33 µl of the **PM** / **PE** mastermix to 15 µl of the eluted cDNA library.

☐ Add 2 µl of one Unique Dual Index Primer pair (**miRVEL UDI Sets A0 or B0**) to each sample.

ATTENTION: Reseal opened index wells after use! Use only one UDI / sample.

☐ PCR: 95 °C, 15 min

$$\left.\begin{array}{l}95\,^\circ\text{C, 15 sec}\\60\,^\circ\text{C, 30 sec}\\72\,^\circ\text{C, 15 sec}\end{array}\right\}\ \begin{array}{l}\textbf{13 - 22x}\\\text{Appendix B, p.19}\end{array}$$

72 °C, 2 min

4 °C, ∞ (at least 5 min)

Purification

☐ Add 47 µl **PB** per reaction, vortex 3 sec, incubate 5 min at RT.

☐ Place on magnet for ~4 min. **ATTENTION:** Do not discard the supernatant. It contains the small RNA fraction!

☐ Transfer 92 µl supernatant to a fresh tube.

☐ Add 83 µl **PB** per reaction, vortex 3 sec, incubate 5 min at RT.

☐ Place on magnet until the supernatant is clear, discard supernatant.

☐☐ Rinse the beads twice with 200 µl 80 % EtOH while on magnet.

☐ Air dry beads for 10 min.

☐ Add 17 µl **H_2O** ○, remove from magnet, mix well, incubate 2 min at RT.

☐ Place on magnet for ~2 min, transfer 15 µl of the supernatant into a fresh tube.

☞ Safe stopping point.

6. Appendix A: RNA Input

Input Guidelines

Total RNA isolated from cells, fresh or frozen tissue, serum, plasma, blood, other biofluids, and FFPE samples can be used as input for the miRVEL Discovery Small RNA-Seq Library Prep. It is recommended to use 100 ng of total RNA as starting material for RNA derived from cells and tissue samples. However, the protocol can be used for inputs ranging from 1 ng to 500 ng of total RNA. Lower RNA inputs (<100 ng) and plasma/serum samples require protocol modifications, including adapters and RT primer dilution and adjusting the number of PCR cycles for the endpoint PCR (Appendix B, p.19).

The miRVEL Discovery Small RNA-Seq Library Prep Kit efficiently generates sequencing libraries from miRNAs and similarly sized RNA, such as piRNAs, that possess a 3' hydroxyl group and a 5' phosphate group. In essence, RNA molecules measuring 50 base pairs or shorter, which possess both a 3' hydroxyl and a 5' phosphate, will be seamlessly integrated into the library.

When isolating total RNA, ensure the extraction protocol can recover small RNAs (including miRNAs). Many commercial products use silica-based purification technology that may exclude small RNAs. Please consult the manufacturer's specifications. We highly recommend using Lexogen's SPLIT RNA Extraction Kit (Cat. No. 008), which recovers the complete RNA size range, including small RNAs (<200 nt and down to 17 nt). Additionally, large and small RNA-enriched fractions can be extracted by following a supplemental protocol. Alternatively, the TraPR Small RNA Isolation Kit (Cat. No. 128) offers a unique technology for enriching functional, physiologically relevant sRNAs that are associated with RISC complexes, even in uncharacterized samples, and thus simplifies downstream analysis and reduces sequencing costs. We recommend starting with 5 µl of RNA isolated with TraPR Small RNA Isolation Kit. Please refer to the TraPR Small RNA Isolation Kit user guide to find the recommended input amounts for your sample type. **NOTE:** TraPR isolates sRNA that is specifically loaded into AGO proteins; therefore, the sRNA isolated with TraPR is likely below the quantification limit. It is not beneficial to quantify TraPR-isolated RNA with optical or microfluidic assays.

For serum and plasma samples, we recommend starting with 5 µL of RNA eluate from 300 - 400 µl of serum or plasma and up to 50 µl blood using SPLIT RNA Extraction protocol for fluid samples. The miRVEL Discovery sRNA-Seq library Prep Kit is designed to minimize hY4 Y reads frequently found at elevated levels in the small RNA-Seq libraries from serum and plasma.

For exosome samples derived from serum and plasma choose a suitable RNA extraction kit, contact support@lexogen.com for advice.

RNA Quality

High-quality small RNA-Seq data relies on high-quality input RNA. RNA samples should be free of salts, metal ions, and organic solvents which can be carried over from RNA extraction as contaminants and may have a negative impact on the efficiency of the protocol.

It is recommended to use total RNA of high integrity with RIN ≥8. However, libraries can still be prepared from samples with RIN values below 8, including FFPE samples, that typically exhibit low RIN values. When working with low-quality RNA, the small RNA fraction is often contaminated with fragmented larger RNAs. Consequently, samples with lower integrity should be sequenced more deeply to compensate for the degraded RNA products present in the library.

DNase treatment is encouraged, particularly for low-quality/FFPE samples to prevent gDNA contamination. Any DNase treatment should be performed with extreme care to minimize the potential impact on RNA quality and yield.

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 3' Adapter (A3 ●)	Dilution Factors Used for 5' Adapter (A5 ●)	Dilution Factors Used for Reverse Transcription Primer (RTP ●)	Number of PCR Cycles
500 ng	Undiluted	Undiluted	Undiluted	13
100 ng	Undiluted	Undiluted	Undiluted	16
10 ng	Dilution 1:5	Dilution 1:2.5	Dilute 1:5	19
1 ng	Dilution 1:20	Dilution 1:10	Dilute 1:20	24
Serum/Plasma	Dilution 1:5	Dilution 1:2.5	Dilute 1:5	22

Dilute **A3** ● and **A5** ● and **RTP** ● in **H_2O** ○. Mix thoroughly by pipetting up and down 12 times, then briefly centrifuge.

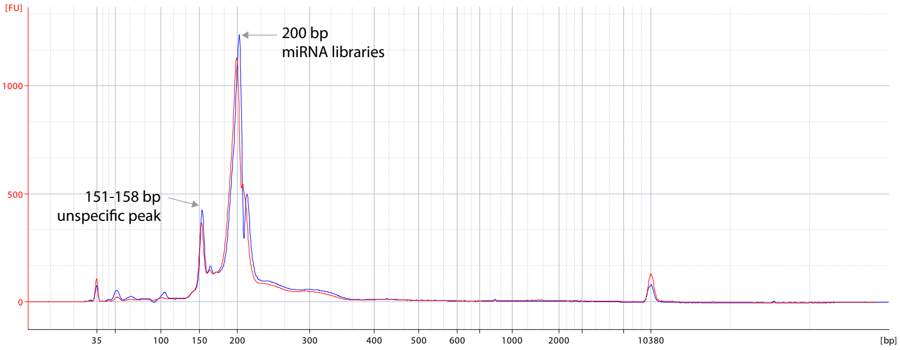
1 ng - 500 ng of total RNA can be used as input for miRVEL Discovery Small RNA-Seq Library Prep Kit. The recommended starting input is 100 ng of total high-quality RNA. Should you decide to work with small RNA-enriched samples, it is important to note that small RNA accounts for approximately 10 % of the total RNA. For instance, from 100 ng of total RNA, you can expect to extract about 10 ng of small RNA. Therefore, we recommend using the adapters and RT primer dilutions and PCR cell cycles designed for 100 ng of total RNA. For sRNA samples isolated with TraPR Small RNA Isolation Kit, use the same dilution ratio as for plasma or serum samples. Please note that further optimization may be required based on your samples and their sRNA content.

8. Appendix C: Library Quality Control

Quality control of finished miRVEL Discovery Small RNA-Seq libraries is highly recommended and should be carried out prior to pooling and sequencing. A thorough quality control procedure should include the analysis of library concentration and size distribution (i.e., library shape).

Quality Control Methods

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.). For high throughput applications instruments such as the Fragment Analyzer or 2200 TapeStation (Agilent Technologies, Inc.), or LabChip GX II (Perkin Elmer) are recommended. Typically, 1 µl of the miRVEL Discovery Small RNA-Seq library is sufficient for analysis. However, for quantification using HS chips libraries may need to be diluted.
Peak of approximately 200 bp corresponds to the miRNA library, a peak at 208 bp represents piRNA, and the peak at ~185 -192 bp corresponds to other small RNAs and RNA fragments - this peak can be particularly prominent in biofluids total RNA samples. Even with a peak detected at 151-158 bp, it is still probable that miRNA is present in the sample. RNA molecules measuring 50 base pairs or shorter, which possess both a 3' hydroxyl and a 5' phosphate, will be integrated into the miRVEL Discovery Small RNA libraries. Peaks at 178 bp are adapter dimers, which can occur at lower RNA input amounts. If there are prominent adapter dimers peak (greater than 25% of the height of the miRNA peak) or other unwanted peaks in the library traces, we recommend performing gel size selection (Appendix G, p.24). To avoid adapter dimers, use at least 1 ng of total RNA and make sure to add all reaction components in the specified order.


200 bp
miRNA libraries

151-158 bp
unspecific peak

Figure 2. Bioanalyzer traces of miRVEL Discovery Small RNA-Seq libraries prepared from 50 ng of Human Brain Reference total RNA. Purified libraries were quality controlled using a Bioanalyzer High Sensitivity DNA assay. Libraries were amplified with 17 PCR cycles.

Library Quantification Methods

More accurate library quantification can be achieved with custom or commercially available qPCR assays. With these assays, the relative or absolute abundance of amplifiable fragments contained in a finished miRVEL Discovery Small RNA-Seq library is calculated by comparing Cq values to a set of known standards. While delivering a more accurate quantification, these assays do not supply the user with information regarding library size distribution. Unwanted side-products such as adapter dimers are not discernible from the actual library in the qPCR assay as both will be amplified. Hence it is highly recommended to combine such an assay for quantification with microcapillary electrophoresis analysis for library size distribution. If microcapillary electrophoresis platforms and qPCR machines are not available, basic quality control can also be performed by separating a small aliquot of the library on a polyacrylamide or agarose gel. Library quantification can also be performed with an inexpensive benchtop fluorometer using one of several commercially available assays, e.g., Qubit dsDNA HS assay. Most UV-Vis spectrophotometers (e.g., NanoDrop, Thermo Fisher Scientific Inc.), are not sensitive enough to accurately quantify NGS libraries at these concentrations and should be avoided.

9. Appendix D: Multiplexing

i5 and i7 Indices are introduced during the PCR amplification with miRVEL UDI primers. miRVEL UDIs are provided in two distinct, non-overlapping sets: Set A0 includes 96 unique UDIs, while Set B0 comprises 24 UDIs, enabling the multiplexing of up to 120 samples.

miRVEL UDI index sequences are available for download at www.lexogen.com.

Lane Mix Preparation

Libraries should ideally be pooled in an equimolar ratio for multiplexed sequencing. It is important to ensure accurate quantification of the individual libraries prior to pooling, as well as for the library pool (lane mix). To quantify your libraries:

① Measure the concentration of each library, using either qPCR or fluorescence-based assays (e.g., Qubit, Thermo Fisher Scientific Inc.).

② Determine the average library size, using microcapillary electrophoresis analysis (e.g., Bioanalyzer, Agilent Technologies, Inc.). Set the range to 180 - 600 bp to include the whole library size distribution, and to exclude any linker-linker artifacts (178 bp).

The molarity (nM) of each sample is calculated using the equation:

$$(X\ \mathrm{ng}/\mu\mathrm{L})(10^6)/(130000) = Y\ \mathrm{nM}$$

The equation is for a 200 bp library, utilizing an average weight of 650 g/mol for each DNA base pair.

After pooling the libraries, the prepared lane mix and any dilutions made for denaturing (4 nM, except for the NovaSeq (10 nM), NextSeq 2000 (2 nM), or when an instrument requires a different dilution), should be reanalyzed to ensure the accuracy of the concentration. This can be performed according to steps ① and ② as above.

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 for how much to load. For information on machine-specific loading amounts please see the online Frequently Asked Questions available at www.lexogen.com.

The required sequencing depth per sample may vary depending on the intended application and sample type. A general recommendation is 5 - 10 million reads per sample.

For low input RNA amounts or libraries with lower small RNA content, a higher number of reads per sample may be required. For small RNA-Seq libraries short read lengths are sufficient. We recommend a 72 bp single-read protocol with 10 bp dual indexing. A 50 bp single-read protocol is adequate if unique molecular identifiers (UMIs) are not required.

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 miRVEL Small RNA-Seq Library Prep Kit (or any other small RNA library prep kit) should not be sequenced together with QuantSeq, QuantSeq-Flex, LUTHOR, 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).

11. Appendix F: Data Analysis

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

12. Appendix G: Size Selection of NGS Libraries Using Gel Purification

Standard polyacrylamide (PAA) and agarose gel purification, or Pippin Prep can be performed to remove adapter dimer artifacts and to select for small RNA-Seq libraries. For best practice, prepare an equimolar lane mix (calculated based on the Bioanalyzer traces) 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.

12.1 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 (12.2, p.24). For agarose gel purification (12.3, p.24), 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.

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_2O** ○	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** ● at -20 °C. The rest of the kit components (**CB**, **CW**, columns) should be stored at room temperature (RT) and protected from light. Before use, check the contents of **CB** and **CW**. If a white precipitate is visible, incubate at 37 °C until buffer components dissolve completely.

ATTENTION: Before use, add 42 ml absolute ethanol to the **CW** bottle and shake to combine. Additional absolute ethanol (100 % EtOH) is also required for the column purification.

12.2 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 adapter dimer 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 (52) of the miRVEL Discovery Small RNA-Seq Library Prep protocol.

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 runs at approximately 193 bp, and a piRNA containing libraries at approximately 201 bp. Avoid recovering shorter 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 (**H_2O** ○) 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 ~14,000 x g (~12,000 rpm) for 2 minutes. Discard the Gel Filtration Column and retain the eluate in the 1.5 ml tube.

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

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

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

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

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

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

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

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

㉒ At this point, the libraries are finished and ready for quality control and cluster generation.
☞ Safe stopping point. Libraries can be stored at -20 °C at this point.

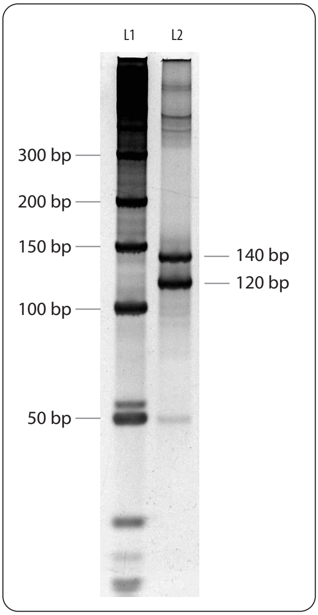


Figure 3. A non-denaturing polyacrylamide gel (6%) displaying the band size distribution of L1 and L2 ladders. Lane 1 contains the L1 ladder and lane 2 contains the L2 ladder.

12.3 Detailed Protocol: Agarose Gel Purification

Size selection or adapter dimers 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 exraction.

1. Prepare a 3% non-denaturing TBE agarose gel (with EtBr in the gel and running buffer).
2. Prepare an equimolar lane mix (calculated based on the Bioanalyzer traces).
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 shorter adapter dimer artifacts that contain no insert. The adapter-ligated library fragments containing miRNA runs at approximately 193 bp, and a piRNA containing libraries at approximately 201 bp.
   REMARK: For final gel extraction, any commercially available gel extraction kit that will recover double-stranded DNA of ≥180 bp in size can be used.

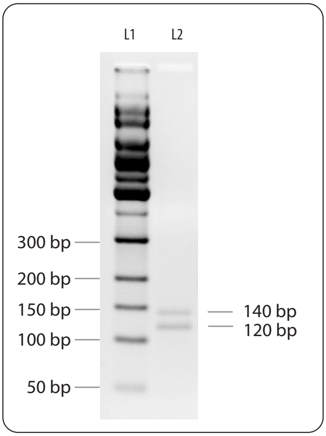


Figure 4. 3% non-denaturing TBE agarose gel (with EtBr in the gel and running buffer) of L1 and L2 ladder. Lane 1 contains the L1 ladder and lane 2 contains the L2 ladder.

13. Appendix H: Revision History

Publication No. / Revision Date	Change	Page
242UG885V0100 May 20, 2025	Initial release.	

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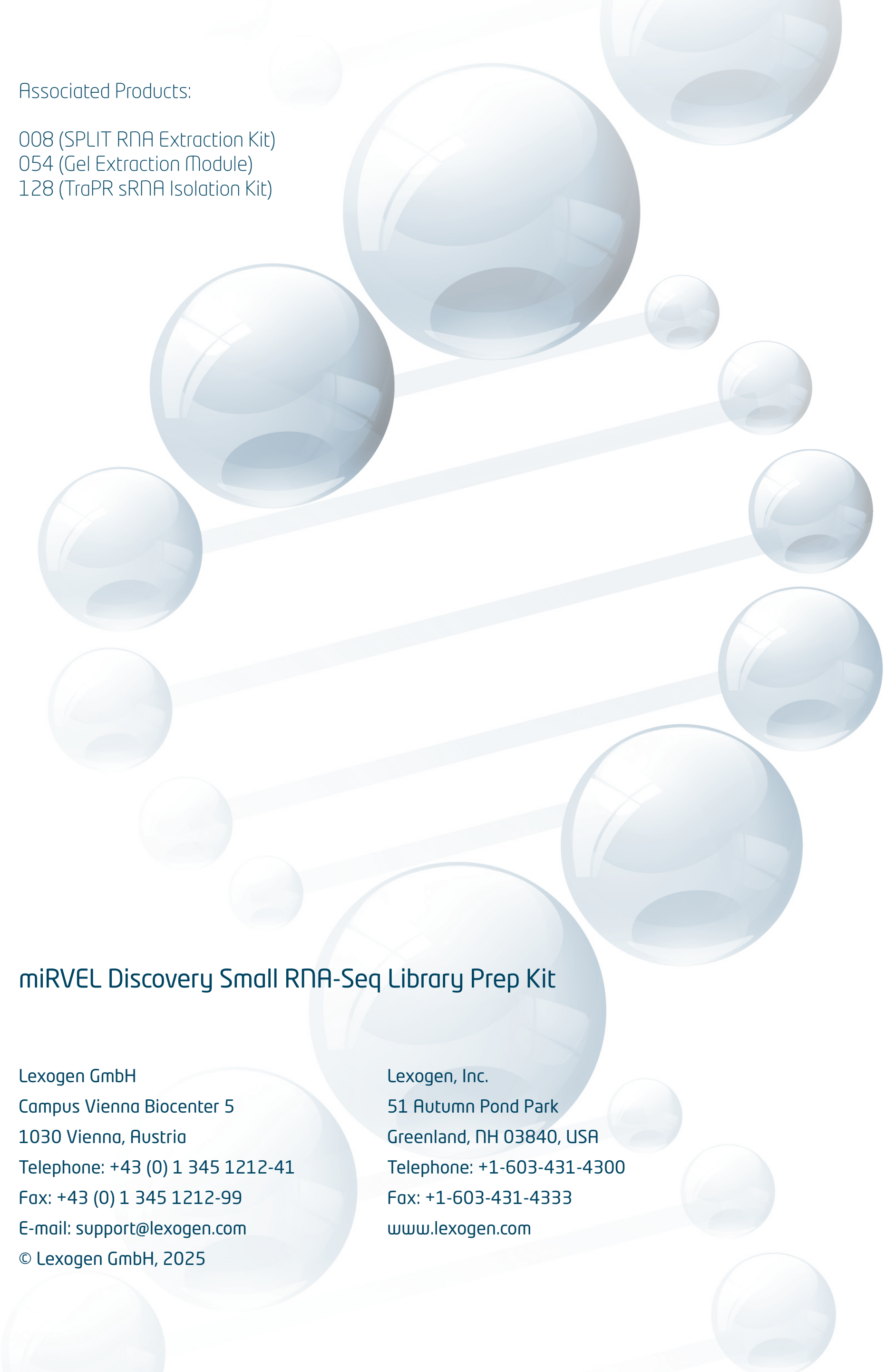
Associated Products:

008 (SPLIT RNA Extraction Kit)
054 (Gel Extraction Module)
128 (TraPR sRNA Isolation Kit)



miRVEL Discovery Small RNA-Seq Library Prep Kit

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