LEXOGEN

Enabling complete transcriptome sequencing

TraPR

Functional sRNA Isolation

Small RNA Isolation Kit

User Guide

Catalog Numbers:
128 (TraPR sRNA Isolation Kit)
135 (Small RNA-Seq Library Prep Kit for Illumina with TraPR)

128UG242V0100

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1. Overview

Regulatory small RNAs (sRNAs) play essential roles in mRNA turn over, translational regulation, chromatin compaction, and many other processes and are therefore important regulators of gene expression. Small RNAs associate to specific proteins of the Argonaute family (AGOs) to form RNA-induced silencing complexes (RISCs) which guide AGO proteins to their respective targets.

Lexogen's TraPR Small RNA Isolation Kit enables the specific isolation of AGO-associated sRNAs via a fast and easy protocol from any organism, tissue, or cell type. TraPR (Trans-kingdom, rapid, affordable Purification of RISCs) is a universal, species-independent sRNA isolation method that does not require any prior knowledge of the sample. By purification of RISCs, the TraPR Small RNA Isolation Kit enriches exclusively fully functional, physiologically relevant silencing sRNAs including miRNAs, siRNAs, piRNAs, and scnRNAs. TraPR uses a simple, column-based purification procedure which enables robust and consistent isolation of sRNAs within 15 minutes requiring only a benchtop centrifuge (Fig. 1).

The TraPR Small RNA Isolation Kit allows rapid access to high-quality sRNA preparations from a variety of sources including degradation-prone biological material and limited amounts of starting material.
Using the provided TraPR Lysis Buffer, the kit is suitable for sRNA isolation from even the most notoriously recalcitrant tissues such as storage roots from plants or EDTA-treated plasma samples from mammals. After lysis and homogenization, the clarified lysate is loaded onto a TraPR column. Resin and sample are mixed thoroughly and the RISC fraction is eluted during the subsequent centrifugation steps resulting in 750 µl of highly enriched RISC-associated small RNA complexes. After isolation, sRNAs can be easily extracted via phenol / chloroform extraction resulting in pure sRNA fractions suitable for all molecular biology and Next Generation Sequencing (NGS) applications, such as RT-qPCR, low molecular weight Northern Blot and sRNA library preparation (Fig. 1).

TraPR typically achieves >90 % enrichment of the desired sRNA species over contaminating degraded RNA fragments. TraPR also eliminates the need for RNA size selection and rRNA depletion even when applied to degradation-prone samples. Contaminating RNA, such as degradation products of tRNA, rRNA and mRNA are effectively excluded from the purified RISC fraction.

The TraPR Small RNA Isolation Kit enables the universal, fast and bias-free isolation of RISCs and provides a small RNA preparation that accurately represents functional silencing RNAs, without the need of tedious and time-consuming labeling and gel extraction steps, suitable for any downstream application. For NGS library preparation we recommend using Lexogen's Small RNA-Seq Library Prep Kit for Illumina (Cat. No. 052). For convenience, a bundled version with TraPR is also available (Cat. No. 135).

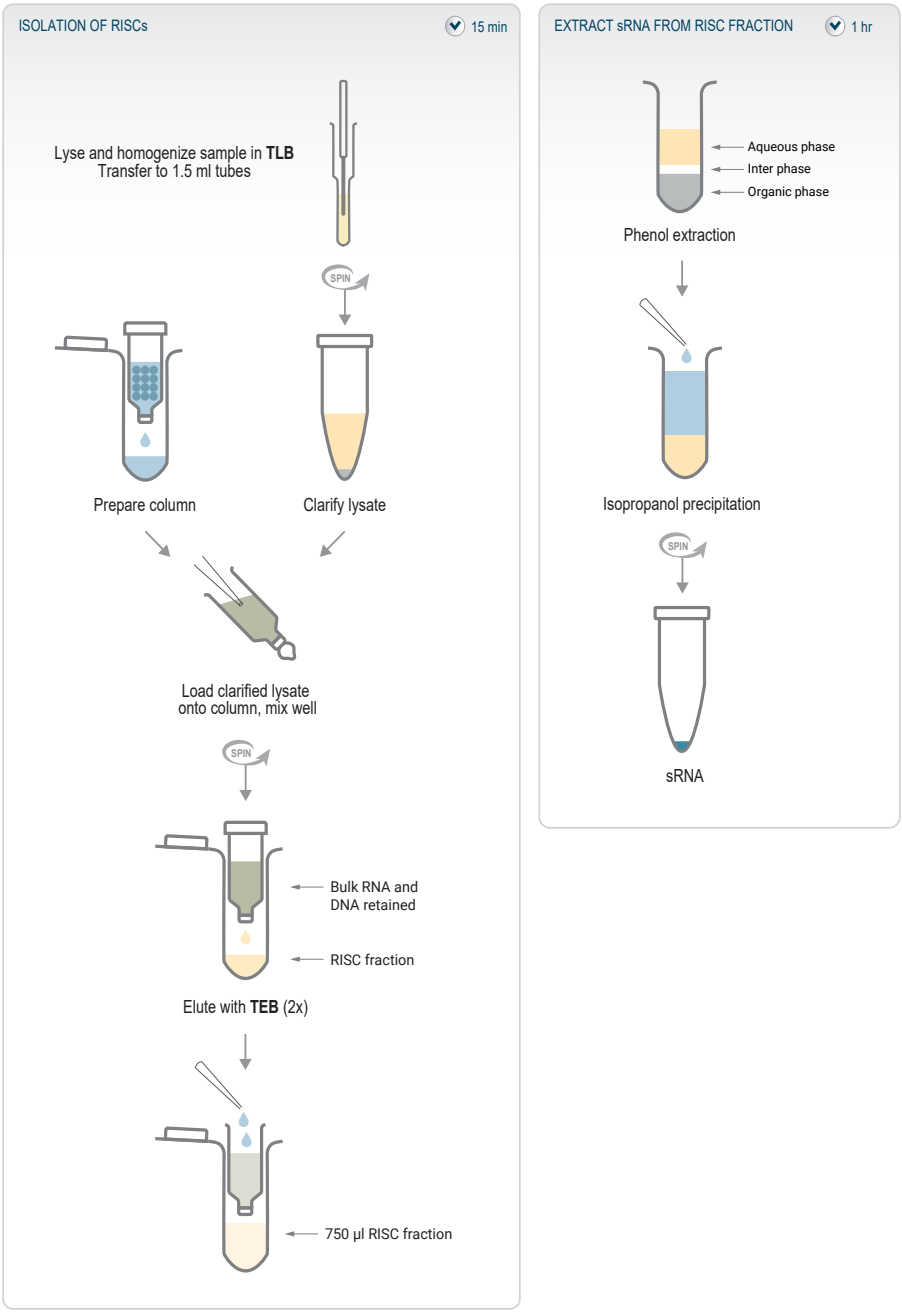

Figure 1. Schematic overview of the TraPR procedure and workflow. The proprietary TraPR technology allows the elution of RISCs and extraction of AGO-associated small RNAs.

2. Kit Components and Storage Conditions

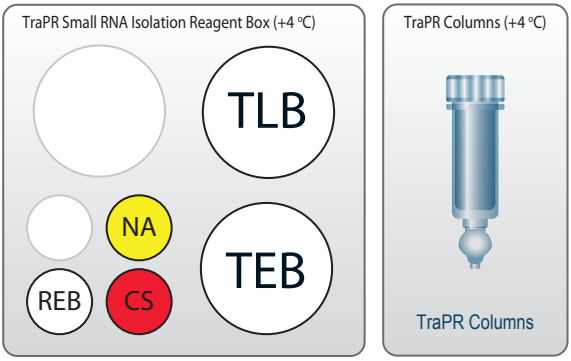

Figure 2. Location of kit components. TraPR Reagents and Columns are supplied with each TraPR Small RNA Isolation Kit.

Kit Component	Label	Volume		Storage
		8 extractions	24 extractions	
TraPR Lysis Buffer	**TLB**	2,640 µl*	7,920 µl*	+4 °C
TraPR Elution Buffer	**TEB**	4,400 µl*	13,200 µl*	+4 °C
TraPR Columns	**TraPR columns**	8	24	+4 °C
Carrier Substance	**CS** ●	8.8 µl*	26.4 µl*	+4 °C
Sodium Acetate	**NA** ●	528 µl*	1,584 µl*	+4 °C
RNA Elution Buffer	**REB** ○	176 µl*	528 µl*	+4 °C

* Including ≥10 % surplus

Upon receiving the TraPR Small RNA Isolation Kit, store all components at +4 °C.

3. User-Supplied Consumables and Equipment

Check to ensure that you have all of the necessary materials and equipment before beginning with the RNA extraction. All reagents, equipment and labware must be free of nucleases and nucleic acid contamination.

Reagents

- Phenol solution pH 4.3 e.g. Sigma-Aldrich (P4682-100ML) or VWR (Cat. No. 0981-100ML).
- Chloroform.
- Isoamylalcohol.
- Isopropanol i.e., 2-Propanol.
- Freshly prepared 80 % Ethanol (EtOH).

Equipment

- Fume hood for organic solvent handling.
- Benchtop centrifuge (12,000 x g, rotor compatible with 1.5 ml and 2.0 ml tubes).
- Calibrated single-channel pipettes for handling 10 µl to 1000 µl volumes.
- Vortex mixer.
- UV-spectrophotometer to quantify RNA.

Labware

- Suitable pipette tips (pipette tips with aerosol barriers recommended).
- 1.5 ml and 2.0 ml tubes with cap, low binding, certified ribonuclease-free.
- Benchtop cooler or ice pellets in ice box (for short-term storage of RNA).

Optional Equipment & Solutions

- 0.1 ml tissue grinder, hand-held homogenizer, Dounce homogenizer.
- Liquid nitrogen (e.g. for TraPR extraction of plant tissue).
- RNaseZap.
- RNase inhibitor.

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

4. Guidelines

Safety Information

- Liquid nitrogen or dry ice may be required for preparation of some sample types, e.g., grinding of plant tissue. Liquid nitrogen is considered hazardous and can cause cryogenic burns or injuries, especially in combination with metal. Avoid direct contact with liquid nitrogen and dry ice, and adhere to the general guidelines for working safely with liquid nitrogen.
- This kit is to be used with a phenol solution, which is toxic and corrosive and with chloroform, both of which may be health hazards if not handled properly. Phenol should not come in contact with skin, eyes, or the respiratory tract and may cause chemical burns to the exposed area. When working with the phenol solution and with chloroform, always work in a fume hood.
- 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 chemicals.
- Dispose buffers and chemicals in appropriate waste containers.
- Consult the appropriate Material Safety Data Sheets (MSDS) at www.lexogen.com, and contact your Environmental Health and Safety department for proper work and disposal guidelines.

General

- Pre-cool the necessary equipment as indicated in the protocol, e.g., bench top centrifuge set to +4 °C.
- Mixing may be performed by pipetting or vortexing. Mix until the sample is homogeneous.
- Perform all pipetting steps with calibrated pipettes and always use fresh tips.
- Equilibrate all necessary buffers at room temperature or as indicated in the preparation tables at the beginning of the protocol. Mix reagents well by vortexing or pipetting repeatedly and spin down briefly before use.
- Ensure that adequate volumes of all reagents and the necessary equipment are available and set to the proper temperatures before beginning the protocol.
- Adhere to the temperatures indicated in the protocol. Results may be negatively impacted if the protocol is performed at temperatures outside this range. While reaction set-up is often performed at RT, centrifugation or solution temperatures are explicitly defined and must be strictly adhered to.

RNA Handling

- RNases are ubiquitous, and special care should be taken throughout the procedure to avoid RNase contamination.
- Use commercial ribonuclease inhibitors (i.e., RNasin, Promega Corp.) to maintain RNA integrity when storing samples.
- Use a sterile and RNase-free workstation or laminar flow hood if available. Please note that RNases may still be present on sterile surfaces, and that autoclaving does not completely eliminate RNase contamination.
- Before starting an RNA extraction, clean your work space, pipettes, and other equipment with RNase removal spray (such as RNaseZap, Ambion Inc.) as per manufacturer's instructions. **ATTENTION:** Rinse off any RNaseZap residue with RNase-free water after use. 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.

5. Detailed Protocol

5.1 Isolation of RISCs

Preparation

Sample Lysis	Sample Loading	RISC Elution
TLB – stored at +4 °C		**TEB** – stored at +4 °C
Liquid nitrogen or dry ice, liquid nitrogen pre-cooled mortar, Douncer, or tools for sample homogenization, DNase- / RNase-free 1.5 ml and 2 ml tubes	**Centrifuge** **TraPR Column** **2 ml tube for collection**	– at +4 °C and RT (1 per sample) (2 per sample, supplied by user)

Sample Lysis and Clarification

ATTENTION: Pre-cool a centrifuge to +4 °C for clarification of the lysate.

1. Lyse fresh or flash frozen samples or tissues in 300 µl TraPR Lysis Buffer (**TLB**) using either a liquid nitrogen pre-cooled mortar, Douncer, or other tools for sample homogenization. We recommend using 5 to 10 mg of mouse organs, 1 to 10 million cells, 20 mg from plants samples, 150 µl of plasma or 25 µl of whole blood as input material.
2. Transfer the lysate into a fresh DNase- / RNase-free 1.5 ml tube.
3. Clarify the lysate by a centrifugation for 5 minutes at 10,000 x g.
4. Transfer 250 µl of the clarified lysate into a fresh DNase- / RNase-free 2 ml tube. **OPTIONAL:** 50 µl of the clarified lysate can be stored as the TraPR input fraction for immediate use or at -80 °C for later use.

TraPR Column Preparation

The TraPR columns are prepared by removing the storage buffer from the resin.

ATTENTION: Do not discard the bottom column closure! It will be needed again. DNase- / RNase-free 2 ml tubes (supplied by user) are needed. All centrifugation steps during TraPR column preparation, sample loading and elution of RISCs are performed at 18 °C or at room temperature for small bench mirco-centrifuges.

5. Re-suspend the resin in the column by vortexing for a few seconds.

6	Loosen the cap by one quarter turn and twist off the bottom closure. **ATTENTION:** Do not discard the bottom closure! It will be needed again in step 9.
7	Place the column into a 2 ml tube for collection of the storage buffer and centrifuge for 15 seconds at 1,000 x g, 18 °C.
8	Discard the collection tube and place the column inside a fresh 2 ml tube hereafter referred to as TraPR fraction collection tube.

Sample Loading

The TraPR input fraction is loaded onto columns.

ATTENTION: Avoid the formation of air bubbles while mixing.

9	Close the column with the bottom closure.
10	Open the column cap and apply 250 µl of clarified lysate to the resin.
11	Close column cap and mix sample and resin by inverting vigorously.
12	Remove the bottom closure and place the column into the 2 ml TraPR fraction collection tube again.

Elution of RISCs

Pure RISCs loaded with their cognate sRNA are eluted from the column while bulk RNA and DNA are retained on the column.

13	Centrifuge for 15 seconds at 1,000 x g, 18 °C or using a small bench mirco-centrifuge. **ATTENTION: Save the flow-through!** Do not empty the TraPR fraction collection tube.
14	Open the column cap and apply 250 µl of TraPR Elution Buffer (**TEB**) onto the column resin.
15	Close the column cap, leave the column in the 2 ml TraPR fraction collection tube which contains the flow-through from the previous step.
16	Centrifuge for 15 seconds at 1,000 x g, 18 °C or using a small bench mirco-centrifuge. **ATTENTION: Save the eluate!** Do not empty the TraPR fraction collection tube.
17	Repeat the elution with another 250 µl of TraPR Elution Buffer (**TEB**). Collect the eluate in the same TraPR fraction collection tube which should now contain ~750 µl of eluate.

18 Close the TraPR fraction collection tube (750 µl) and keep on ice for immediate use, or store at -80 °C for later use. This fraction now contains the RISC-associated AGO proteins loaded with their cognate sRNAs. ☞ Safe stopping point. The TraPR elution can be stored at -80 °C at this point.

5.2 Extraction of RISC-associated Small RNAs

Small RNA is isolated from RISCs using acidic phenol.

Preparation

			For each sample
Phenol solution pH 4.3[1]	– stored at +4 °C	provided by user	250 µl
Chloroform[1]	– stored at RT		240 µl
Isoamylalcohol	– stored at RT		10 µl
Isopropanol	**– pre-cool at - 20 °C**		600 µl
80 % EtOH	**– prepare fresh** and pre-cool at -20 °C		1,200 µl
NA	– stored at RT		60 µl
CS	– stored at RT		1 µl
REB	– stored at RT		up to 20 µl
Centrifuge	– at +4°C		
Fume hood			
Vortex mixer			

[1] **Caution:** When working with phenol or chloroform always use a fume hood and discard waste according to applicable Health and Safety regulations.

ATTENTION: Pre-cool a centrifuge to +4 °C. All centrifugation steps are performed at +4 °C.

19 Premix 250 µl acidic phenol solution, pH 4.3 with 240 µl chloroform and 10 µl isoamylalcohol per sample (see preparation table).

20 Add 500 µl acidic phenol / chloroform / isoamylalcohol to the 750 µl elution collected in the TraPR fraction collection tubes. Vortex vigorously.

21 Centrifuge for 5 minutes at 12,000 x g.

22 Transfer 600 µl of the aqueous phase into a new 1.5 ml tube. **ATTENTION:** Make sure not to disturb the inter phase. **REMARK:** Little or no aqueous phase (large inter phase) is an indication of too much input material. Add more chloroform until proper phase separation is achieved. Split in 2 tubes if the volume exceeds the capacity of one tube.

23 Add 60 µl of Sodium Acetate (**NA** ●) and 1 µl Carrier Substance (**CS** ●). Mix well.

24	Add 600 µl of cold Isopropanol. Mix well by vortexing.
25	Centrifuge for 30 minutes at 14,000 x g.
26	Remove and discard the supernatant. The small RNA is now in the pellet.
27	Wash the pellet by adding 400 µl cold 80 % Ethanol. Do not vortex or dissolve the pellet.
28	Centrifuge for 5 minutes at 14,000 x g.
29	Remove and discard the supernatant.
30	Repeat the washing step twice for a total of three washes.
31	Remove all ethanol by pipetting. **ATTENTION:** Do not disturb the pellet, pipette slowly and carefully. To remove residual ethanol (after the first removal step), a smaller pipette tip can be used.
32	Add up to 20 µl of RNA Elution buffer (**REB** ○) to the pellet and resuspend it by pipetting up and down. **OPTIONAL:** Smaller elution volumes may be used to increase the concentration of small RNAs. The sample may be heated for 1 min at 60 °C if the pellet does not dissolve well. ☞ Safe stopping point. RNA can be stored at ≤ -20 °C at this point.
33	Isolated small RNAs are now ready for downstream applications such as RT-qPCR, low molecular weight Northern Blot, and sRNA library preparation for RNA-Seq. We recommend using Lexogen's Small RNA-Seq Library Preparation Kit (Cat. No. 052) for NGS applications, refer to 052UG128 for details.

6. Short Procedure TraPR

ATTENTION: Pre-cool a centrifuge to +4 °C for clarification of the lysate. Centrifugation steps during TraPR column preparation, sample loading, and elution of RISCs are performed at 18 °C or at room temperature.

15 min Isolation of RISCs

	Sample Lysis and Clarification
☐	Homogenize flash frozen sample in 300 µl TraPR Lysis Buffer (**TLB**).
☐	Transfer lysate into a 1.5 ml tube.
☐	Centrifuge lysate for 5 min at 10,000 x g, +4 °C.
☐	Transfer clarified lysate into a 2 ml tube.
	TraPR Column Preparation
☐	Re-suspend the resin in the column by vortexing a few seconds.
☐	Loosen the cap by one quarter turn and twist off the bottom closure. **ATTENTION:** Do not discard the bottom closure!
☐	Place column into a 2 ml tube for collection.
☐	Spin 15 sec at 1,000 x g, 18 °C.
☐	Discard the collection tube, and place the column inside a fresh 2 ml tube.
	Sample Loading
☐	Close the column with the bottom closure!
☐	Open the column cap and apply 250 µl of clarified lysate to the resin.
☐	Close column cap and mix sample with resin by inverting vigorously.
☐	Remove the bottom closure and place the column into the 2 ml collection tube.
	Elution of RISCs
☐	Spin 15 sec at 1,000 x g, 18 °C. **ATTENTION: Save the flow-through!** Do not empty the collection tube.
☐	Open the column cap and apply 250 µl of TraPR Elution Buffer (**TEB**).
☐	Spin 15 sec at 1,000 x g, 18 °C. **ATTENTION: Save the eluate!** Do not empty the collection tube.
☐	Repeat elution with 250 µl **TEB** for a total of two elution steps. **ATTENTION: Save the eluate!** Do not empty the collection tube.
☐	Close the TraPR fraction collection tube containing 750 µl of collected eluate. Keep on ice for immediate use, or store at -80 °C for later use. ☞ Safe stopping point. Store samples at -80 °C.

ATTENTION: Pre-cool Isopropanol and 80 % Ethanol to -20 °C. Pre-cool a centrifuge and perform all centrifugation steps are at +4 °C.

1 hr Extraction of RISC-associated Small RNAs

	RNA Extraction
☐	Premix 250 µl acidic phenol solution, pH 4.3 with 240 µl chloroform and 10 µl isoamyl-alcohol per sample.
☐	Add 500 µl acidic phenol / chloroform / isoamylalcohol to the 750 µl TraPR elution in the 2 ml collection tube.
☐	Vortex vigorously.
☐	Centrifuge 5 min at 14,000 x g.
☐	Transfer 600 µl of the aqueous phase into a new 1.5 ml micro-centrifuge tube. **ATTENTION:** Avoid disturbing the inter phase.
☐	Add 60 µl **NA** ● and 1 µl **CS** ●. Mix well.
☐	Add 600 µl of cold Isopropanol. Mix well by vortexing.
☐	Centrifuge for 30 min at 14,000 x g.
☐	Remove and discard the supernatant.
☐	Add 400 µl cold 80 % Ethanol to the pellet. Do not vortex or dissolve the pellet.
☐	Centrifuge for 5 min at 14,000 x g. Discard the supernatant.
☐☐	Repeat the washing and centrifugation steps twice for a total of three washes.
☐	Remove all ethanol by pipetting.
☐	Add up to 20 µl **REB** ○, and resuspend the pellet by pipetting up and down. ☛ Safe stopping point. Store samples at ≤-20 °C.

7. Appendix A: Input Requirements

Input Material

TraPR input material must be in a native state. Compatible input material includes fresh material, EDTA-stored blood and plasma samples, or samples flash frozen using liquid nitrogen or dry ice. Fresh or flash frozen samples should be homogenized in TraPR Lysis Buffer (**TLB**) using either a liquid nitrogen pre-cooled mortar and pestle, Dounce homogenizer, or equivalent tools. Liquid samples can be mixed with TraPR Lysis Buffer (**TLB**), different maximum input amounts apply for blood and plasma (see below). **Tissues or samples stored in RNA*later* or denaturing agents and fixed samples are not suitable.**

TraPR was successfully tested on *Paramecium tetraurelia*, *Oryza sativa* (leaves), wheat (leaves), *Manihot esculenta* (storage roots), *Arabidopsis thaliana* (inflorescences, roots, leaves, seedlings), *Schizosaccharomyces pombe*, *Drosophila melanogaster* (ovaries), *Caenorhabditis elegans*, *Mus musculus* (cells from culture such as N2a, brain, liver, muscles, testis and plasma), human whole blood, human leukocytes, as well as human plasma.

Input Amount and Quality

We recommend using 5 to 10 mg of mouse organs, 1 to 10 million cells, 20 mg from plants samples, 150 µl of plasma or 25 µl of whole blood as input material. It is critical not to exceed the resin capacity by overloading the columns. If a larger amount of material is lysed in TraPR lysis buffer e.g., 40 mg of plant powder, use two columns for the TraPR procedure. 50 µl of whole blood was successfully used with TraPR, however during RNA extraction additional chloroform had to be added to achieve proper phase separation as described in step 22. For further information please contact support@lexogen.com.

Typical Results

Quality control of TraPR sRNA may not be possible using microcapillary electrophoresis, e.g., Bioanalyzer Small RNA chips, Agilent Technologies. For visual inspection, TraPR sRNA may be radiolabeled with T4 Polynucleotide Kinase (PNK) prior to gel electrophoresis on a 17 % denaturing polyacrylamide gel followed by autoradiography as shown in Figure 3A. Specific sRNAs can be detected by Northern Blotting using low molecular weight RNA blotting techniques and labeled probes for specific detection of known sRNAs (Fig. 3B). For comprehensive analysis of sRNAs extracted from TraPR eluates, it is recommended to proceed to small RNA library preparation, e.g., using Lexogen's Small RNA-Seq Library Prep Kit for Illumina (Cat. No. 052). Following library preparation, small RNAs can be analyzed by sequencing. This also allows detection and analysis of previously unknown small RNAs.

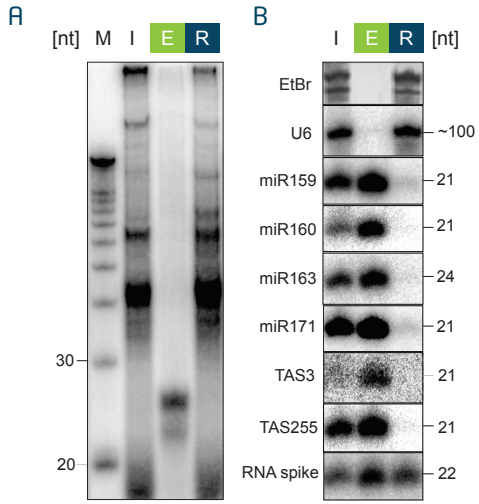

Figure 3. Results of small RNA extractions from TraPR eluates. A) Small RNAs were isolated from *Arabidopsis thaliana* (inflorescences) using TraPR, radiolabeled with T4 Polynucleotide Kinase (PNK), separated on a 17 % denaturing polyacrylamide gel, and transferred onto a nylon membrane for visualization by autoradiography alongside a low molecular weight marker (M). Input (I), TraPR elution (E) and the fraction retained on the column (R) are shown. Nucleotide [nt] lengths are given on the left. **B)** Low molecular weight RNA blot analysis of RNA extracted from TraPR fractions shown in the right panel. Again, input (I), TraPR elution (E) and retained fraction (R) are loaded. Radiolabeled oligonucleotides served as specific probes to detect known *Arabidopsis* sRNAs. Bulk RNA is retained on the column while AGO-associated small RNAs, such as microRNAs (miR) and TasiRNAs (TAS) are specifically eluted during the TraPR procedure. Nucleotide [nt] lengths of the respective RNAs are given on the right. This image is published in *Nucleic Acids Res.* (Grentzinger *et al.*, 2020) and courtesy of T. Grentzinger and co-authors.

8. Appendix B: Downstream Processing

TraPR-isolated sRNA samples are ideally suited for downstream NGS library preparation using Lexogen's Small RNA-Seq Library Prep Kit for Illumina (Cat. No. 052). Up to 6 µl of the extracted small RNA may be used as input for sRNA-Seq library preparation, please refer to 052UG128 for details. For convenience, a bundled version of the Small RNA-Seq Library Prep Kit for Illumina with TraPR (Cat. No. 135) is also available.

Depending on input material and sRNA content of the processed samples, small RNA library preps may include linker-linker artifacts. To maximize the fraction of usable reads in NGS experiments, removal of linker dimers is strongly recommended. 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. For best practice, prepare an equimolar lane mix (calculated from the Bioanalyzer quantification 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. For the user's convenience, Lexogen also offers a Gel Extraction Module (Cat. No. 054). Alternatively, Lexogen's Purification Module with Magnetic Beads (Cat. No. 022) can also be used for the removal of shorter by-products. Please refer to 052UG128 for further details.

9. Appendix C: Revision History

Publication No. / Revision Date	Change	Page
128UG242V0100 June 4, 2020	Initial Release.	

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Associated Products:

022 (Purification Module with Magnetic Beads)
052 (Small RNA-Seq Library Prep Kit for Illumina)
054 (Gel Extraction Module)

TraPR Small RNA Isolation Kit · User Guide

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