Takara Bio USA

SMARTer® microRNA-Seq Kit User Manual

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I. Introduction

A. Overview

The SMARTer microRNA-Seq Kit is used to construct high-quality microRNA libraries for sequencing on Illumina® platforms. This kit uses 100 ng to 1 µg of total RNA or 2 ng to 200 ng of enriched small RNA (including microRNA) as input material, allowing reliable results from even challenging samples. The kit is powered by the innovative MAGIC (**M**ono-**A**dapter li**G**ation and **I**ntramolecular **C**ircularization) technology which minimizes ligation-induce bias in library preparation. This technology enables the SMARTer microRNA-Seq Kit to capture small RNAs and microRNAs with high accuracy, sensitivity, and reproducibility, ensuring consistency and validity of research findings. The kit also features a convenient single-day, single-tube workflow for successful generation of libraries every time. Amplified microRNA libraries can be generated in about six hours using the streamlined protocol, providing ease of use and reducing risk of mix up and contamination.

The SMARTer microRNA-Seq Kit includes indexes for multiplexing up to 48 samples. Once amplified, the resulting libraries are purified, size selected, and quantified. These libraries are then ready for Illumina NGS using standard Illumina sequencing reagents and protocols. The kit's proprietary technology minimizes ligation-induced bias and ensures the most accurate measurement of small RNA expression levels can be obtained.

B. Principle

The SMARTer microRNA-Seq Kit allows users to analyze diverse small RNA species from inputs of total RNA or enriched small RNA. The kit is powered by a novel proprietary chemistry relying on the 5'-phosphate and 3'-hydroxyl modifications present in small RNAs. The chemistry is highly sensitive, can use as little as 10 ng of total RNA, and minimizes bias induced by the library preparation process. As a result, small RNA-seq libraries generated using the kit provide a very accurate representation of small RNA expression levels.

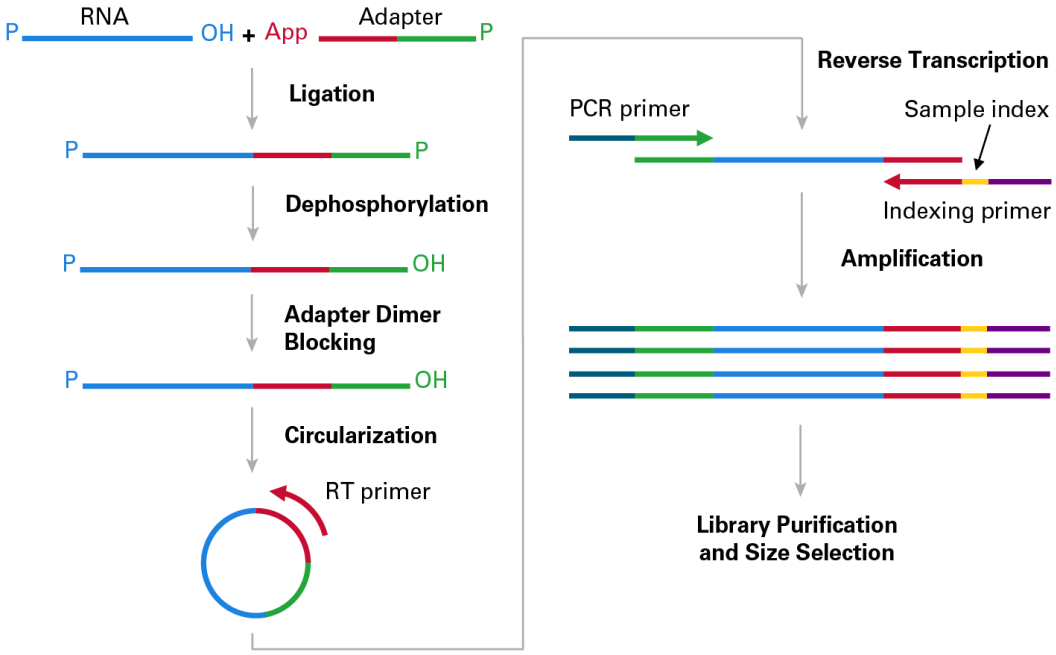

Figure 1. Schematic of technology used by SMARTer microRNA-Seq Kit. The SMARTer microRNA-Seq Kit is powered by a novel technology called Mono-Adapter Ligation and Intramolecular Circularization (MAGIC). miRNA molecules containing 5'-phosphate and 3'-hydroxyl groups are first ligated to an adenylated adapter that is blocked at the 3'-end by a phosphate group. Ligated molecules are then dephosphorylated, and adapter dimer formation is blocked prior to circularizing all molecules. Once circularized, the ligated miRNA molecules are reverse transcribed, followed by amplification of the product in a PCR reaction. At this last amplification step, the sample is indexed, and the Illumina library structure is completed, resulting in sequencing-ready library molecules. The amplified library is finally purified and size-selected, and the resulting library is ready for sequencing on an Illumina NGS platform.

C. SMARTer microRNA-Seq Kit Workflow

The SMARTer microRNA-Seq Kit workflow consists of the following steps:

- Ligation
- Dephosphorylation
- Adapter Dimer Blocking
- Circularization
- Reverse Transcription
- Amplification
- Library Purification and Size Selection

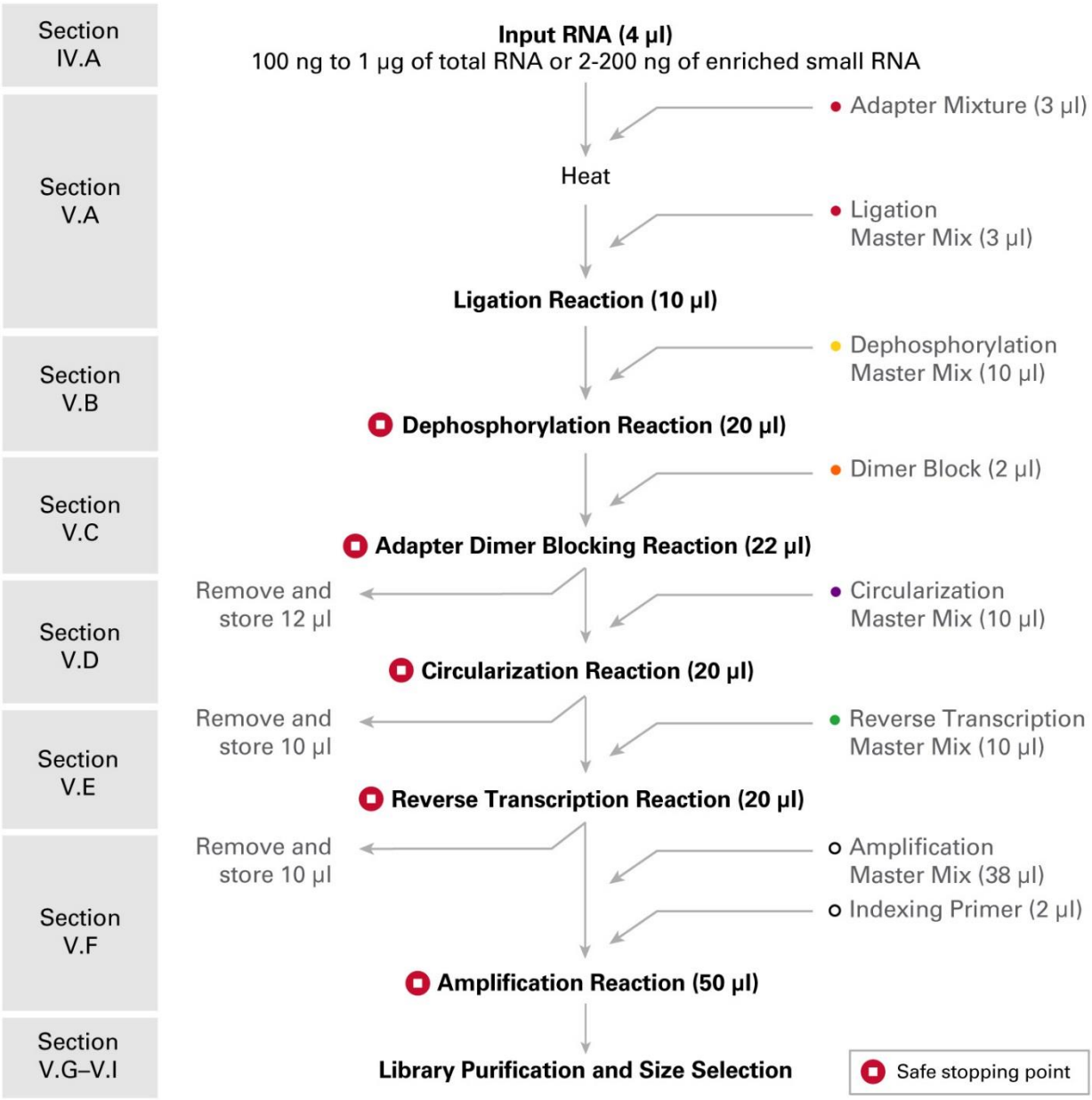

Figure 2. Library preparation workflow for SMARTer microRNA-Seq Kit. This kit employs a single-tube workflow, which can be completed in a single day or about six hours to generate amplified libraries from input RNA.

II. List of Components

A. Components

The SMARTer microRNA-Seq Kit contains sufficient reagents to prepare up to the specified number of reactions. **These components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions.** The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results.

SMARTer microRNA-Seq Kit[1]	Cap color	R500653 (16 rxns) 8 indexes	R500654 (48 rxns) 24 indexes	R500655 (96 rxns) 48 indexes
Package 1 (Store at –70°C).				
Adapter[2]	Red	16 µl	48 µl	96 µl
Control microRNA[3] (10 ng/µl)	Orange	5 µl	5 µl	5 µl
Package 2 (Store at –20°C).				
Reaction Enhancer (RE)	Pink	100 µl	300 µl	600 µl
Ligation Buffer (LB)	Red	16 µl	48 µl	96 µl
RNase Inhibitor (RI; 40 U/µl)	Pink	32 µl	96 µl	192 µl
Ligation Enzyme Mix (LEM)	Red	16 µl	48 µl	96 µl
Dephosphorylation Buffer (DB)	Yellow	16 µl	48 µl	96 µl
Dephosphorylation Enzyme Mix (DEM)	Yellow	8 µl	24 µl	48 µl
Adapter Dimer Block (ADB)	Orange	32 µl	96 µl	192 µl
Circularization Buffer 1 (CB1)	Purple	32 µl	96 µl	192 µl
Circularization Buffer 2 (CB2)	Purple	8 µl	24 µl	48 µl
Circularization Enzyme Mix (CEM)	Purple	20 µl	60 µl	120 µl
RT Buffer (RTB; 5X)	Green	64 µl	192 µl	384 µl
SMARTScribe™ Reverse Transcriptase (100 U/µl)	Green	16 µl	48 µl	96 µl
dNTP Mix (10 mM)	Green	16 µl	48 µl	96 µl
Amplification Buffer (AB; 2X)	White	400 µl	1.2 ml	2 x 1.2 ml
Universal Primer (UP; 10 µM)	White	32 µl	96 µl	192 µl
SeqAmp™ DNA Polymerase[4] (SeqAmp; 1.25 U/µl)	White	16 µl	48 µl	96 µl
Nuclease-Free Water	Pink	500 µl	1.5 ml	2 x 1.5 ml

[1] Indexing primers provided with this kit are listed on Pages 6 and 7.

[2] To prevent multiple freeze-thaw cycles, the Adapter stock or dilutions (Section V.A) can be aliquoted into microcentrifuge tubes and stored –70°C. The individual tubes can then be removed for each future experiment.

[3] Control Total RNA is derived from the human let-7d-3p microRNA.

[4] SeqAmp DNA Polymerase is a hot-start enzyme.

WARNING: Do not freeze/thaw the reagents more than **four** times! In the event, that the entire workflow will not be completed in a single day, users should only thaw the reagents for the steps that will be completed in the available time. Consult Figure 2 (above) or Section V (below) to identify the safe stopping points.

Indexing Primers

SMARTer microRNA-Seq Kit	Cap color	R500653 (16 rxns) 8 indexes	R500654 (48 rxns) 24 indexes	R500655 (96 rxns) 48 indexes
Package 3 (Store at –20°C).				
Index Primer 1 (10 µM)	White	8 µl	8 µl	8 µl
Index Primer 2 (10 µM)	White	8 µl	8 µl	8 µl
Index Primer 3 (10 µM)	White	8 µl	8 µl	8 µl
Index Primer 4 (10 µM)	White	8 µl	8 µl	8 µl
Index Primer 5 (10 µM)	White	8 µl	8 µl	8 µl
Index Primer 6 (10 µM)	White	8 µl	8 µl	8 µl
Index Primer 7 (10 µM)	White	8 µl	8 µl	8 µl
Index Primer 8 (10 µM)	White	8 µl	8 µl	8 µl
Index Primer 9 (10 µM)	White	-	8 µl	8 µl
Index Primer 10 (10 µM)	White	-	8 µl	8 µl
Index Primer 11 (10 µM)	White	-	8 µl	8 µl
Index Primer 12 (10 µM)	White	-	8 µl	8 µl
Index Primer 13 (10 µM)	White	-	8 µl	8 µl
Index Primer 14 (10 µM)	White	-	8 µl	8 µl
Index Primer 15 (10 µM)	White	-	8 µl	8 µl
Index Primer 16 (10 µM)	White	-	8 µl	8 µl
Index Primer 17 (10 µM)	White	-	8 µl	8 µl
Index Primer 18 (10 µM)	White	-	8 µl	8 µl
Index Primer 19 (10 µM)	White	-	8 µl	8 µl
Index Primer 20 (10 µM)	White	-	8 µl	8 µl
Index Primer 21 (10 µM)	White	-	8 µl	8 µl
Index Primer 22 (10 µM)	White	-	8 µl	8 µl
Index Primer 23 (10 µM)	White	-	8 µl	8 µl
Index Primer 24 (10 µM)	White	-	8 µl	8 µl

Package 4 contents are listed on Page 7

SMARTer microRNA-Seq Kit	Cap color	R500653 (16 rxns) 8 indexes	R500654 (48 rxns) 24 indexes	R500655 (96 rxns) 48 indexes
Package 4 (Store at –20°C).				
Index Primer 25 (10 µM)	White	-	-	8 µl
Index Primer 26 (10 µM)	White	-	-	8 µl
Index Primer 27 (10 µM)	White	-	-	8 µl
Index Primer 28 (10 µM)	White	-	-	8 µl
Index Primer 29 (10 µM)	White	-	-	8 µl
Index Primer 30 (10 µM)	White	-	-	8 µl
Index Primer 31 (10 µM)	White	-	-	8 µl
Index Primer 32 (10 µM)	White	-	-	8 µl
Index Primer 33 (10 µM)	White	-	-	8 µl
Index Primer 34 (10 µM)	White	-	-	8 µl
Index Primer 35 (10 µM)	White	-	-	8 µl
Index Primer 36 (10 µM)	White	-	-	8 µl
Index Primer 37 (10 µM)	White	-	-	8 µl
Index Primer 38 (10 µM)	White	-	-	8 µl
Index Primer 39 (10 µM)	White	-	-	8 µl
Index Primer 40 (10 µM)	White	-	-	8 µl
Index Primer 41 (10 µM)	White	-	-	8 µl
Index Primer 42 (10 µM)	White	-	-	8 µl
Index Primer 43 (10 µM)	White	-	-	8 µl
Index Primer 44 (10 µM)	White	-	-	8 µl
Index Primer 45 (10 µM)	White	-	-	8 µl
Index Primer 46 (10 µM)	White	-	-	8 µl
Index Primer 47 (10 µM)	White	-	-	8 µl
Index Primer 48 (10 µM)	White	-	-	8 µl

B. Shipping and Storage Conditions

The SMARTer microRNA-Seq Kit is shipped on dry ice. The contents of Package 1 should be stored at –70°C upon arrival, while the remaining components should be stored at –20°C.

III. Additional Materials Required

The following reagents and materials are required but not supplied. They have been validated to work with this protocol. For size selection, either the Gel Size Selection protocol or the Pippin Prep protocol can be used, as available to the user.

- Single-channel pipettes: 10 µl, 20 µl, and 200 µl
- Eight-channel pipettes (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, and 200 µl
- Microcentrifuge for 1.5-ml tubes
- Microcentrifuge for 0.2-ml tubes or strips
- Centrifuge for 96-well plates

For Library Purification

- AMPure XP beads (Beckman Coulter, Cat No. A63881)

  NOTE: SPRI beads need to come to room temperature before the container is opened. Therefore, we recommend aliquoting the beads into 1.5-ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 minutes). This will also decrease the chances of bead contamination. Mix well to disperse the beads before adding them to your reactions. The beads are viscous, so pipette slowly.

- 100% ethanol (molecular biology-grade)
- Magnetic separation device for small volumes—used to purify amplified libraries
  - For 1.5-ml tubes: DynaMag-2 Magnet (Thermo Fisher, Cat. No.12321D)
  - For 8-tube strips: Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)
  - For 96-well plates: Magnetic Stand-96 (Thermo Fisher, Cat. No. AM10027)
- Optional, depending on the choice of magnetic separation device (96-well format):
  - 96-well V-bottom Plate (500 µl; VWR, Cat. No. 47743-996)
  - MicroAmp Clean Adhesive Seal (Thermo Fisher, Cat. No. 4306311)
  - Low-speed benchtop centrifuge for a 96-well plate

For Gel Size Selection

- Novex TBE Gels, 8%, 10 well (Thermo Fisher Scientific, Cat. No. EC6215BOX)
- Novex Hi-Density TBE Sample Buffer (5X; Thermo Fisher Scientific, Cat. No. LC6678)
- Novex Chamber for XCell SureLock Mini-Cells (Thermo Fisher Scientific, Cat. No. EI0013)
- 10X TBE Buffer (Thermo Fisher Scientific, Cat. No. 15581044)
- 50 bp DNA Ladder or 10 bp DNA Ladder (Thermo Fisher Scientific, Cat. No. 10416014 or 10821015, respectively)
- Nuclease-free water (Thermo Fisher Scientific, Cat. No. 10977-023)
- SYBR® Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Cat. No. EI0013)
- GelDoc-It^2 Imager (UVP, Cat. No. 97-0138-04)
- GeneCatcher Disposable Gel Excision Tips (Gel Company, Cat. No. PKB6.5)

For Gel Elution and DNA Precipitation

- 1.5-ml microcentrifuge tubes (USA Scientific, Cat. No. 1615-5500)
- RNase-free pellet pestle grinder (VWR, Cat. No. KT749521-1590)
- Ammonium acetate solution, 7.5 M (Sigma, Cat. No. A2706)
- Corning Costar Spin-X Centrifuge Tube Filters (Sigma, Cat. No. CLS8162-96EA)
- UltraPure Glycogen (Thermo Fisher Scientific, Cat. No. 10814010)
- Ethanol (100%, 200 proof) for molecular biology (Sigma, Cat. No. E7023-500 ml)

For Pippin Prep Size Selection

- Pippin Prep DNA Size Selection System (Sage Science, Cat. No. PIP0001)
- Pippin Prep 3% Agarose Gel, dye-free, w/internal standards (100–200 bp) (Sage Science, Cat. No. CDP3010)

IV. General Considerations

A. Sample Requirements

1. Starting Material

Sample requirements	
Type	Total RNA or enriched RNA
Quality	RIN ≥8
Input amount	100 ng to 1 µg (total RNA) or 2 ng to 200 ng (enriched small RNA)
Input volume	4 µl
Input buffer	Nuclease-free water

Sample Type

The SMARTer microRNA-Seq Kit is designed to generate high-quality Illumina NGS libraries from inputs consisting of either total RNA or enriched small RNA.

Sample Quality

The use of high-quality total RNA (RIN ≥8) is recommended. The kit may also be used with partially degraded samples; however, it must be noted that performance is dependent on the quality of the starting RNA material.

Input RNA Amount

The recommended input amount is 100 ng to 1 µg of total RNA or 2 ng to 200 ng enriched small RNA.

Input Volume

The maximum sample volume input is 4 µl. Bring the volume of each sample to 4 µl with nuclease-free water if starting from <4 µl of sample.

Input Buffer

Purified input RNA should be resuspended in nuclease-free water, not in TE or other buffers containing EDTA. Samples should also be free of DNA contaminants, which could be amplified and incorporated in final sequencing libraries.

2. Positive and Negative Controls

If you are using this protocol for the first time, we strongly recommend that you perform negative and positive control reactions.

The positive control RNA (Control miRNA, orange cap) is provided at 10 ng/µl. The Control miRNA is an equimolar pool of 4,096 synthetic microRNAs. The sequences of the synthetic microRNAs are derived from the human let-7d-3p microRNA, but with randomized ends:

5' Phos-NNNUACGACCUGCUGCCUUNNN 3'

For library preparation of the positive control, use 1 µl of the Control miRNA plus 3 µl of nuclease-free water. Perform 13 cycles of PCR. The resulting library serves as an excellent size marker for gel size selection (see Figure 5 in Section V.A.8). The yield is typically around 1.2–3.0 ng/µl after gel size selection as determined by Agilent Bioanalyzer analysis. In addition, sequencing data obtained from the control RNA can be used to determine bias induced by library preparation.

Include a negative control (No Template Control, NTC) using nuclease-free water.

3. Preparation of Master Mixes

A master mix with appropriate buffers and enzymes must be prepared just in time at each workflow step based on the number of reactions to be performed. Prepare 5-10% excess of each master mix to allow for pipetting losses.

4. Indexing Primers

Indexing Primers are predispensed in individual tubes with white caps. Each tube contains sufficient volume for up to four uses. No more than four freeze/thaw cycles are recommended for the Indexing Primers.

Before starting the protocol (Section V), refer to the planning steps in the TruSeq® Library Prep Pooling Guide (Illumina, Document # 15042173 v01, November 2015) when preparing libraries for Illumina sequencing systems that require balanced sample index combinations.

B. Safety Guidelines

Follow standard laboratory safety procedures and wear a suitable lab coat, protective goggles, and disposable gloves to ensure personal safety as well as to limit potential cross contaminations during the sample preparation and subsequent amplification reactions. For more information, please refer to the appropriate Material Safety Data Sheets (MSDS) available online at http://www.takarabio.com/SMARTer_microRNA_Seq_Kit.

V. Procedure

NOTES:
- Thaw all reagents and RNA samples on ice.
- We recommend making an additional 5-10% of the total reaction mix volumes for all of the mixtures and master mixes described in the protocols below.

A. Protocol: Ligation

1. Prepare 100 µl of **Adapter** as per the sample amounts in the table below. Mix well by pipetting, when necessary.

Sample Type (Amount)	Adapter Preparation
500 ng to 1 µg total RNA	Take 10 µl of the Adapter and dilute in 90 µl of nuclease-free water
100 ng to 400 ng total RNA	Take 1 µl of the Adapter and dilute in 99 µl of nuclease-free water
2 to 200 ng enriched small RNA	No extra preparation

NOTE: To prevent multiple freeze-thaw cycles, the Adapter stock or dilutions can be aliquoted into microcentrifuge tubes and stored at –70°C. The individual tubes can then be removed for each future experiment.

2. Prepare **Adapter Mixture** as described in the table below. Mix thoroughly by pipetting or quick vortexing and spin down to collect the contents at the bottom of the tube.

Adapter Mixture		
Component	**Cap Color**	**Volume/Reaction**
Adapter (diluted or not)	Red/NA*	1 µl
Input RNA sample	-	4 µl

*If your sample type required dilution of the Adapter, then the Adapter will come from the Eppendorf tube in which your dilution was prepared.

3. Add 2 µl of **Reaction Enhancer** (pink cap), to 5 µl of the **Adapter Mixture** (which should be individually dispensed in tubes). This is now your **Sample Mixture** as shown in the table below. Mix thoroughly by vortexing and spin down to collect the volume at the bottom of the tube.

Sample Mixture		
Component	**Cap Color**	**Volume/Reaction**
Adapter Mixture	-	5 µl
Reaction Enhancer	Pink	2 µl

NOTES:
- The Reaction Enhancer and resulting mixture are very viscous and require extra attention and care during pipetting to ensure thorough mixing.
- We recommend using the provided Control microRNA (orange cap) as a positive control. A no-template negative control may also be included in each experiment.

4. Heat thermal cycler to 70°C and incubate the **Sample Mixture** at this temperature for 2 minutes. Immediately after, put the Sample Mixture on ice and leave it there untouched for at least 30 seconds.
5. Prepare the **Ligation Master Mix** as described in the table below for the desired number of reactions. Mix thoroughly.

Ligation Master Mix		
Component	**Cap Color**	**Volume/Reaction**
Ligation Buffer (LB)	Red	1 µl
RNase Inhibitor (RI)	Pink	1 µl
Ligation Enzyme Mix (LEM)	Red	1 µl

6. Assemble the **Ligation Reaction Mixture** as shown in the table below. To each 7 µl of the **Sample Mixture**, add 3 µl of the **Ligation Master Mix**. Mix thoroughly by quick vortexing and spin down to collect volume at the bottom of the tube.

Ligation Reaction Mixture	
Component	**Volume/Reaction**
Sample Mixture	7 µl
Ligation Master Mix	3 µl
Total Volume	10 µl

7. Perform the **Ligation Reaction** in a thermal cycler using the conditions outlined in the table below:

Template Preparation Reaction	
Temperature	**Time**
25°C	70 min
65°C	20 min
4°C	Hold

8. After the reaction is done, centrifuge briefly to collect all liquid at the bottom of each well or tube.
9. Proceed to **Dephosphorylation** (Section V.A.2) in the same plate or tube(s).

B. Protocol: Dephosphorylation

1. Prepare the **Dephosphorylation Master Mix** as described in the table below for the desired number of reactions. Mix thoroughly.

Dephosphorylation Master Mix		
Component	**Cap Color**	**Volume/Reaction**
Dephosphorylation Buffer (DB)	Yellow	1.0 µl
Reaction Enhancer	Pink	1.0 µl
Nuclease-Free Water	Pink	7.5 µl
Dephosphorylation Enzyme Mix (DEM)	Yellow	0.5 µl

2. Assemble the **Dephosphorylation Reaction Mixture** as shown in the table below. To each 10 µl of the **Ligation Reaction Product**, add 10 µl of the **Dephosphorylation Master Mix**. Mix thoroughly by quick vortexing and spin down to collect volume at the bottom of the tube.

Dephosphorylation Reaction Mixture	
Component	**Volume/Reaction**
Ligation Reaction Product	10 µl
Dephosphorylation Master Mix	10 µl
Total Volume	20 µl

3. Perform the **Dephosphorylation Reaction** in a thermal cycler using the conditions outlined in the table below:

Dephosphorylation Reaction	
Temperature	**Time**
37°C	15 min
65°C	20 min
4°C	Hold

4. After the reaction is done, centrifuge briefly to collect all liquid at the bottom of each well or tube.
5. Continue to the **Adapter Dimer Blocking** step (Section V.A.3) in the same plate or tube(s).

STOPPING POINT: The tubes can be stored at –20°C overnight.

C. Protocol: Adapter Dimer Blocking

1. To each 20 µl of the **Dephosphorylation** reactions add 2 µl of **Adapter Dimer Block** (ADB, orange cap). Spin down to collect samples at the bottom of the tubes or wells.
2. Incubate the samples at the following temperatures.

Adapter Dimer Block Reaction	
Temperature	**Time**
65°C	5 min
4°C	Hold

3. After the reaction is done, centrifuge briefly to collect all liquid at the bottom of each well or tube.
4. Continue to the **Circularization** step (Section V.A.4) in the same plate or tube(s).

STOPPING POINT: The tubes can be stored at –20°C overnight.

D. Protocol: Circularization

NOTE: Only 10 µl of the 22 µl Adapter Dimer Blocked Reaction Product is required for the Circularization reaction. Remove 12 µl from each sample and store material at –20°C.

1. Prepare the **Circularization Master Mix** as described in the table below. Mix thoroughly.

Circularization Master Mix		
Component	**Cap Color**	**Volume/Reaction**
Circularization Buffer 1 (CB1)	Purple	2.0 µl
Circularization Buffer 2 (CB2)	Purple	0.5 µl
Nuclease-Free Water	Pink	6.25 µl
Circularization Enzyme Mix (CEM)	Purple	1.25 µl

2. Assemble the **Circularization Reaction Mixture** as described in the table below. To each 10 µl of the **Adapter Dimer Blocked Reaction Product**, add 10 µl of the **Circularization Master Mix**. Mix thoroughly by quick vortexing and spin down to collect volume at the bottom of the tube.

Circularization Reaction Mixture	
Component	**Volume/Reaction**
Dephosphorylation Reaction Product	10 µl
Circularization Master Mix	10 µl
Total Volume	20 µl

3. Perform the **Circularization Reaction** in a thermal cycler using the conditions outlined in the table below:

Circularization Reaction	
Temperature	**Time**
25°C	90 min
65°C	10 min
4°C	Hold

4. After the reaction is done, centrifuge briefly to collect all liquid at the bottom of each well or tube.
5. Continue to the **Reverse Transcription** step (Section V.A.5) in the same plate or tube(s).

STOPPING POINT: The tubes can be stored at –20°C overnight.

E. Protocol: Reverse Transcription

NOTE: Only 10 µl of the 20 µl **Circularization Reaction Product** is required for the Reverse Transcription reaction. Remove 10 µl from each sample and store material at –20°C.

1. Assemble the **Reverse Transcription Master Mix** as described in the table below. To each 10 µl of the **Circularization Reaction Product**, add 10 µl of **Reverse Transcription Master Mix**. Mix thoroughly by quick vortexing and spin down to collect volume at the bottom of the tube.

Reverse Transcription Master Mix		
Component	**Cap Color**	**Volume/Reaction**
dNTP Mix (10 mM)	Green	1 µl
RT Buffer (RTB)	Green	4 µl
Nuclease-free Water	Pink	3 µl
RNase Inhibitor (RI; 40 U/µl)	Pink	1 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Green	1 µl

2. Perform **Reverse Transcription Reaction** in a thermal cycler using the conditions outlined in the table below:

Reverse Transcription Reaction	
Temperature	**Time**
42°C	30 min
4°C	Hold

3. After the reaction is done, centrifuge briefly to collect all liquid at the bottom of each well or tube.
4. Continue to the **Amplification** step (Section V.A.6) in the same plate or tube(s).

STOPPING POINT: Perform an optional heat inactivation of the **Reverse Transcription Reaction Product** by heating to 70°C for 15 minutes. Heat-inactivated samples can be stored overnight at −20°C.

F. Protocol: Amplification

NOTE: Only 10 µl of the 20 µl **Reverse Transcription Reaction Product** is used. Remove 10 µl from each sample and store material at –20°C.

1. Prepare the **Amplification Master Mix** as described in the table below. Mix thoroughly.

Amplification Master Mix		
Component	**Cap Color**	**Volume/Reaction**
Amplification Buffer (AB)	White	25 µl
Universal Primer	White	2 µl
Nuclease-Free Water	Pink	10 µl
SeqAmp Polymerase	White	1 µl

2. Assemble the **Amplification Reaction Mixture** as described in the table below. To each 10 µl of the **Reverse Transcription Reaction Product**, add 38 µl of the **Amplification Master Mix**. Mix thoroughly by quick vortexing and spin down to collect volume at the bottom of the tube.

Amplification Reaction Mixture	
Component	**Volume/Reaction**
Amplification Reaction Product	10 µl
Amplification Master Mix	38 µl
Indexing Primer	2 µl
Total Volume	50 µl

3. Perform the **Amplification Reaction** in a thermal cycler using the conditions outlined in the table below:

Amplification Reaction			
Stage	**Temperature**	**Time**	**# Cycles**
1	94°C	1 min	1
2	94°C 62°C 70°C	30 sec 30 sec 15 sec	13–17*
3	65°C	10 min	1
4	4°C	Hold	1

NOTE: Perform 13 cycles of PCR when using 10 ng of enriched small RNA, 15 cycles of PCR when using 1 µg of total RNA and 17 cycles of PCR when using 100 ng of total RNA. Additional cycles are required for lower input amounts. Also, perform 13 cycles of PCR when using the included Control miRNA as positive control (see Section IV.A.2).

4. After the reaction is done, centrifuge briefly to collect all liquid at the bottom of each well or tube.
5. Continue to the Library Purification (Section V.A.7) in the same plate or tube(s).

STOPPING POINT: The tubes can be stored at –20°C overnight.

G. Protocol: Library Purification

Users will purify their library and have the choice of performing size selection with either the "Gel Size Selection" protocol outlined below, or the "Pippin Prep Size Selection" protocol.

Library Purification by AMPure XP

1. To 50 µl of PCR product, add 90 µl of AMPure beads.
2. Incubate at room temperature for 5–8 minutes. Afterwards, bind samples to a magnet for 2–5 minutes or until the solution is clear.
3. Gently remove the supernatant and add 200 µl of fresh 80% EtOH, taking care not to disrupt the beads. Incubate for 30 seconds at room temperature, and then gently remove the EtOH.
4. Repeat the previous step one additional time.
5. After removing the EtOH from the last wash, quickly spin down the tubes and bind to the magnet again. Remove any leftover EtOH with care.
6. Dry the beads for 3 minutes.
7. Elute the samples in 20 µl of TE or water, of which you can transfer 18 µl to a new tube.

After library purification, the user can run the samples on a Bioanalyzer High Sensitivity chip to determine if the library preparation worked prior to performing size-selection. Below are examples of traces.

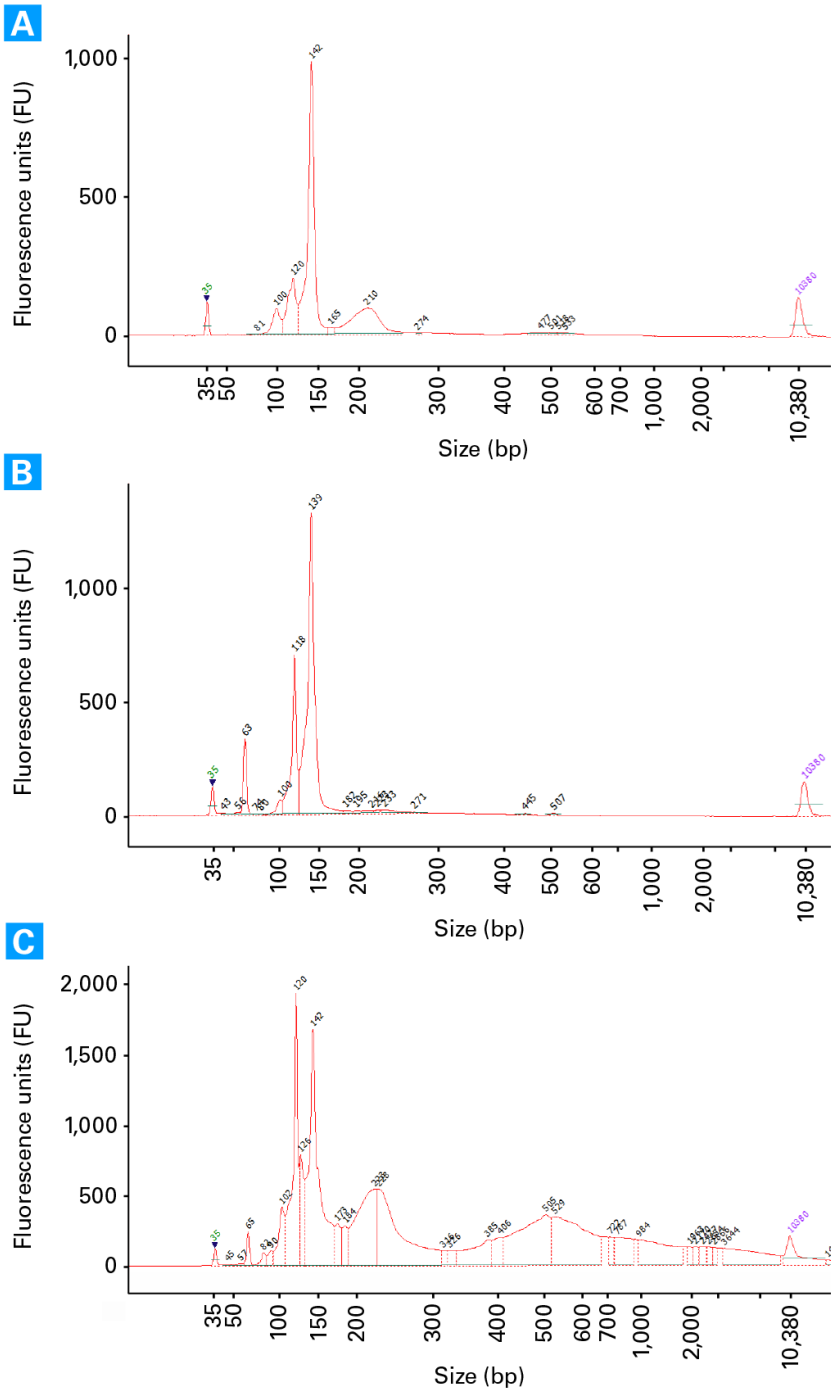

Figure 3. Example of electropherogram traces of libraries prior to size selection, generated with 10 ng of a synthetic miRNA pool. The adapter dimer band (115–125 bp) can be very small (**Panel A**), around half the size of the library peak at 140–150 bp (**Panel B**) or slightly larger than the library peak at 140–150 bp (**Panel C**). You can also see that sometimes there will be a smear with bumps all the way to 2,000 bp. All these traces are fine and show that the ligation and the adapter dimer minimization steps worked. If a single peak at 115–125 bp is present, the adapter dimer minimization step did not work.

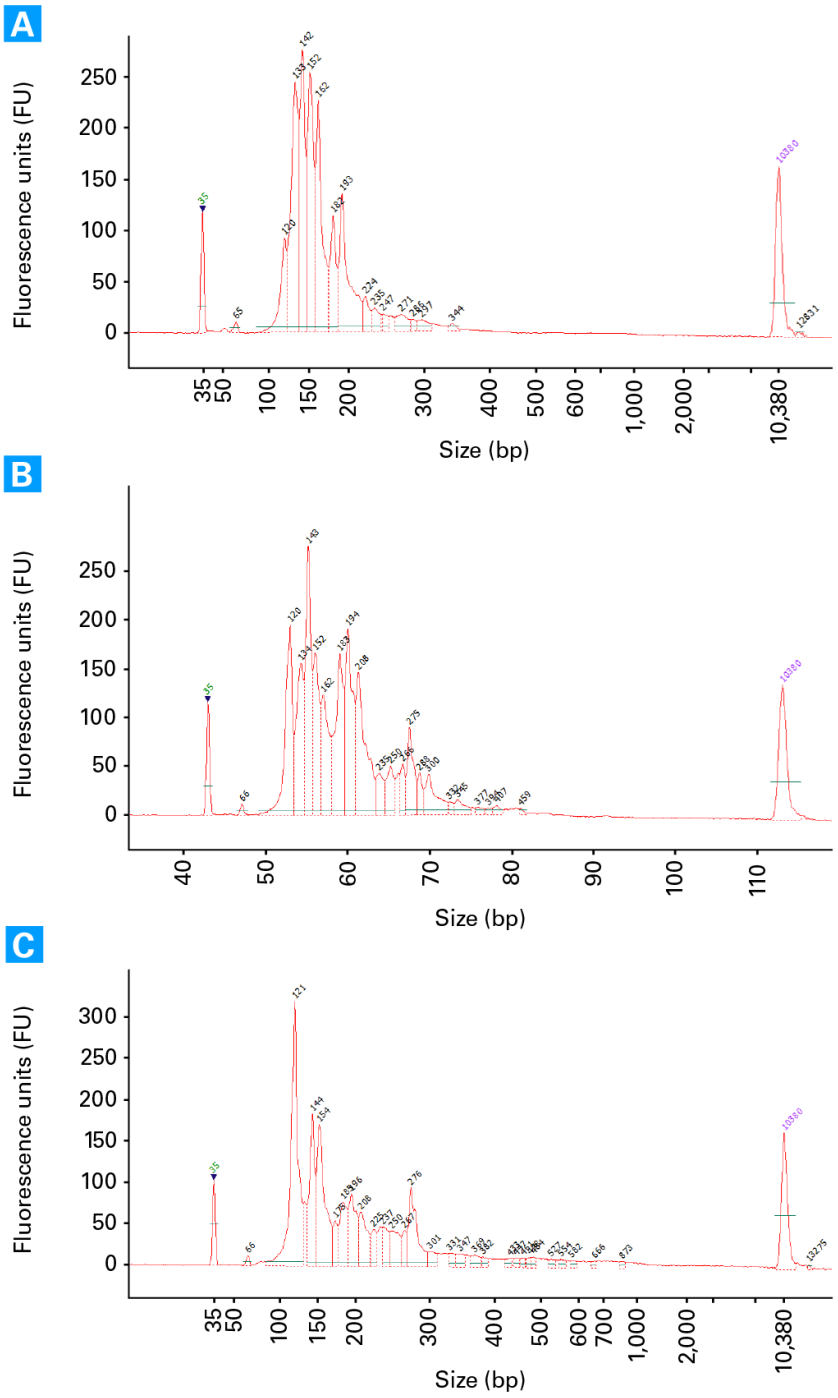

Figure 4. Example of electropherogram traces of libraries prior to size selection, generated with 100 ng Human Brain Total RNA. The adapter dimer band (115–125 bp) can be around half the size of the library peak at 140–150 bp (**Panel A**) or slightly larger (**Panels B, C**). You can also see that sometimes there will be a smear with bumps all the way to 1,000-2,000 bp. All these traces are fine and show that the ligation and the adapter dimer minimization steps worked. If a single peak at 115–125 bp is present, the adapter dimer minimization step did not work.

H. Protocol: Size Selection, Option 1: Gel

Users have the choice of performing size selection with either the "Gel Size Selection" protocol outlined below or the "Pippin Prep Size Selection" protocol.

1. Run Gel

Size-select the purified DNA product using an 8% PAGE gel.

- **PAGE gel:** Novex TBE Gels, 8%, 10 well (Thermo Fisher Scientific, Cat. No. EC6215BOX)
- **Sample buffer:** Novex Hi-Density TBE Sample Buffer (5X) (Thermo Fisher Scientific, Cat. No. LC6678)
- **Ladder:** 50 bp or 10 bp DNA Ladder (Thermo Fisher Scientific, Cat. No. 10416014 or 10821015)

1. Prepare **Sample Mixture** by mixing 5 µl of purified DNA product with 5 µl of sample buffer.
2. Prepare **Ladder Mixture** by mixing 0.5 µl of ladder with 5 µl of sample buffer and 4 µl of nuclease-free water.
3. Rinse the wells of the gel prior to loading samples.
4. Load 10 µl of the **Ladder Mixture** in one well of the 8% PAGE 10-well gel.
5. For each sample, load one well with 10 µl of the **Sample Mixture**.
6. Load 5 µl of sample buffer in unused wells.
7. Run the gel for 1 hour 10 min at 145 V or until the bottom blue dye exits the gel.
8. Remove the gel from the apparatus and stain with 1X SYBR Gold in a clean container for 2–10 minutes. View the gel on a UV transilluminator.
9. The 140-nt and 150-nt bands correspond to adapter-ligated constructs. The ~120-nt band corresponds to adapter-dimers (Figure 5).
10. Using a gel-extraction tool, isolate the bands or smear corresponding to the 140–150 nt region of the gel. The negative control (no-template control) should guide as to which bands are the adapter-dimer only. DO NOT cut out the adapter-dimer bands.

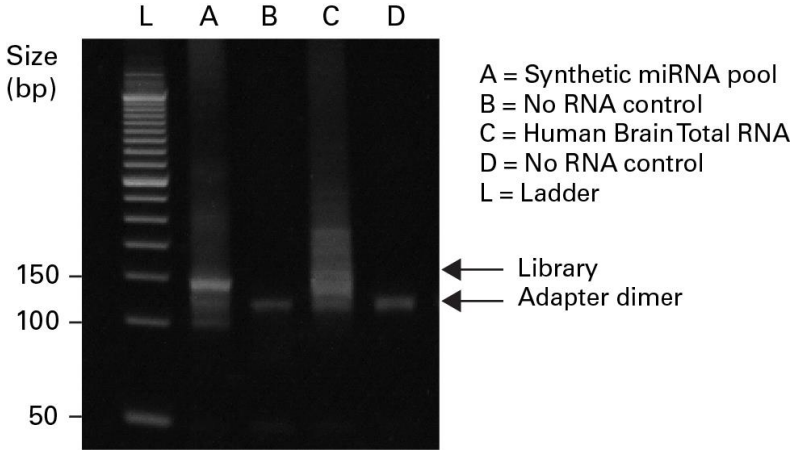

Figure 5. Representative gel image of SMARTer microRNA-Seq Kit libraries. Libraries were generated, and size selected on a PAGE gel. A 50-bp ladder was used to aid visualization. The image shows typical results from 10 ng of a synthetic microRNA pool (Lane A) followed by its barcoded negative control (Lane B), and 100 ng of Human Brain Total RNA (Lane C) also followed as well by its barcoded negative control (Lane D).

2. Gel Elution and DNA Precipitation

11. Place each gel slice in one 1.5-ml tube and crush them into a fine slurry with RNase-free disposable pellet pestles. Add 200 µl of nuclease-free water.
12. Vortex horizontally for 3 hours at room temperature.
13. Collect the sample and gel pieces and pass them through a Costar Spin-X Column, spinning at 14,000*g* for 2 minutes. Keep the flow-through and discard the column.
14. Add 1 µl of 20 µg/µl Ultrapure Glycogen, 100 µl of 7.5 M ammonium acetate solution, and 750 µl of cold 100% Ethanol. Mix well.
15. Precipitate samples at –80 °C for at least 40 min.

STOPPING POINT: Samples can be stored at –20°C overnight.

16. After precipitation, spin samples at 4°C for 30 min at 16,000*g*.
17. Remove the supernatant with care without disturbing the pellet, and wash with 500 µl of freshly-made cold 70% Ethanol.
18. Spin again for 10 minutes at 4°C at 16,000*g*.
19. Remove as much of the remaining ethanol as possible. Do not disturb the pellet and dry the pellet at room temperature for 10 minutes.
20. Resuspend the pellet in 12 µl of TE buffer or nuclease-free water by pipetting up and down until fully respuspended (~20 times).

3. Quality Control

Check the size, purity, and concentration of the samples by running them in a High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer. Load 1 µl of library into a well of a chip and run according to the manufacturer's instructions.

Electropherogram traces should show a single thin or slightly broad peak around 147–150 nt (see Figure 6 below).

Use the "Region" function to calculate the concentration of the library. A typical yield from 1 µg of total RNA input should be around 0.8 to 1.8 µg/µl.

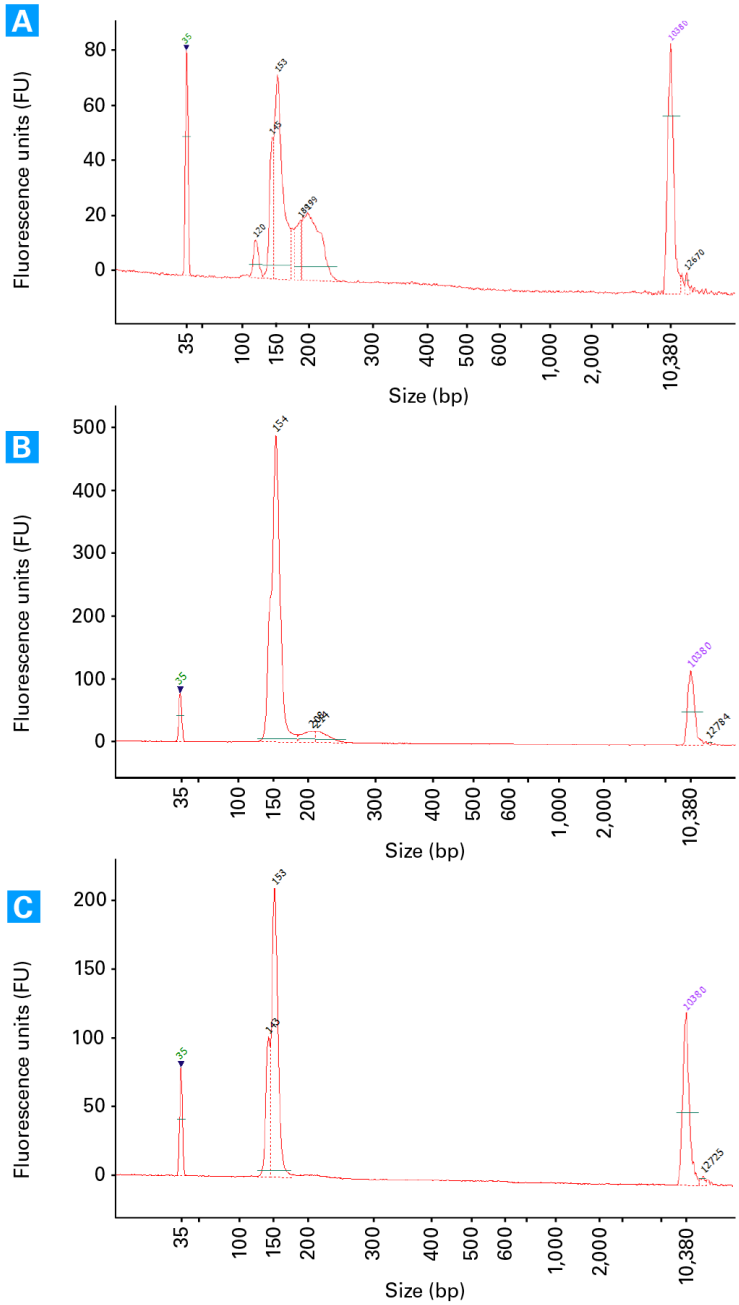

Figure 6. Representative electropherogram results for PAGE size-selected SMARTer microRNA-Seq Kit libraries prepared from Human Brain Total RNA. Libraries were generated from 100 ng of Human Brain Total RNA. **Panel A–B.** Typical electropherogram traces of a good library after gel size-selection prepared by two different Operators. A small adapter dimer peak around 120 bp is acceptable, and its presence depends on the accuracy of size selection. Small bumps around 200 bp in size are also acceptable and do not affect results. **Panel C.** Additional electropherogram profile exhibiting two peaks of the desired size fused together. When using enriched small RNA, the peaks should look very similar, if not identical to those seen here.

I. Protocol: Size Selection, Option 2: Pippin Prep

This method allows for a more stringent size selection of library molecules within user-specified size ranges. Please refer to the Pippin Prep Size Selection user manual for detailed instructions. For size selection of libraries generated from total RNA, recovery using this protocol is typically 3–15% of input library DNA. When using enriched small RNA, recovery can be as high as 25% of input library DNA.

NOTES:

- The guidelines below were validated on a Pippin Prep system. Adjustment of the protocol may be necessary if using different equipment. It is also particularly important that libraries are purified prior to running in this system, which requires that input DNA be in a low ionic buffer free or with minimal amount of detergent.
- To save time and resources when working with many samples, libraries that have been differentially-indexed and have similar size profiles may be pooled in equal amounts (we routinely pool up to 6 libraries together per lane).
- The protocol requires 3% Dye-free agarose gels (Catalog No. CDP3010).

When Using Cat. No. CDP3010 with Maker P

1. In the Pippin Prep software, go to the Protocol Editor Tab, click in the "Cassette" folder, and select "3% DP Marker P".

   NOTE: You may need to perform a firmware update on the Pippin in order to be able to use Marker P.

2. In the collection mode setting, make sure "Range" is selected and enter the following parameters: BP start (128) and BP end (160). The base pair range flag should indicate "broad". This should select for 144–150 bp size fragments.
3. Click the "Use of Internal Standards" button, and make sure the "Ref Lane" values match each of the lane numbers.
4. Name your samples, press "Save As" and name and save the protocol.

Prepare the Sample for Size Selection as Follows:

1. Bring the buffer and marker loading solutions to room temperature.
2. For each individual sample, combine 30 µl sample with 10 µl of DNA marker P.

   NOTE: If pooling libraries, do so in equal amounts up to a 30 µl total volume, then add 10 µl of marker to the pool. We routinely pool up to six libraries.

3. Mix samples thoroughly (vortex mixer) and briefly centrifuge to collect.
4. Load the entire 40 µl (DNA plus marker) on one well of the 3% agarose cassette and run the program with the settings indicated above.
5. After the sample has been eluted, collect 40 µl from the elution well. Run 1 µl in a Bioanalzyer using the high sensitivity chip.

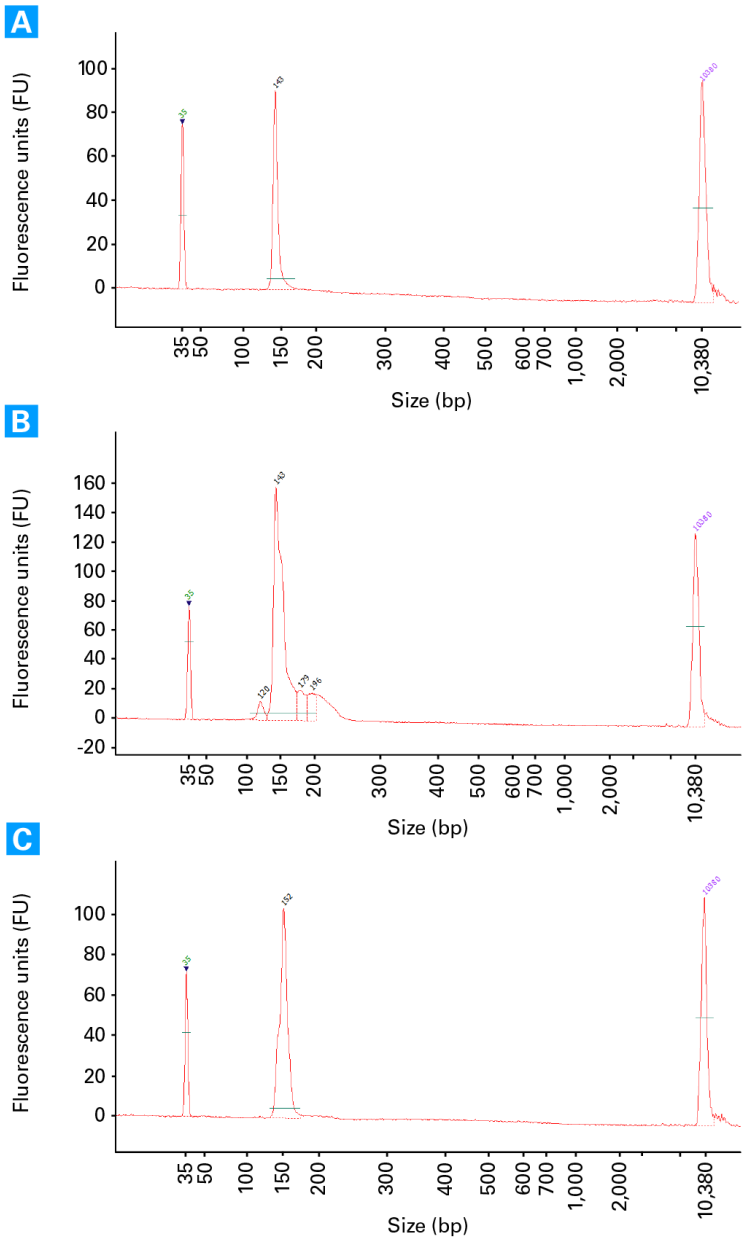

Figure 7. Representative electropherogram results for Pippin Prep size-selected SMARTer microRNA-Seq Kit libraries prepared with miRNA and Agilent Universal Human Reference Total RNA. Libraries were generated from 10 ng of a synthetic pool of miRNAs (**Panel A)** or 1 µg of Agilent Universal miRNA Human reference Total RNA (**Panels B and C**). The traces in Panels B and C represent different size-selections to illustrate how traces may look.

Appendix A. Indexing Primers

A. Overview

The SMARTer microRNA-Seq Kit provides Indexing Primers for multiplexing up to 48 samples. Table I. below summarizes the characteristics of the included Illumina-compatible Indexing Primers. Indexing Reagents should be stored at –20°C and should not be subjected to more than four freeze/thaw cycles.

NOTE: Indexing Primers provided with SMARTer microRNA-Seq Kit cannot be substituted with indexing reagents from any other sources.

Table I. SMARTer microRNA-Seq Kit Indexing Primers

Name	R500653 (16 rxns)	R500654 (48 rxns)	R500655 (96 rxns)
Number of Reactions	16	48	96
Number of Indexes	8	24	48
Index Type	Single	Single	Single
Length of Indexes	6 nt	6 nt	6 nt
Format	8 Tubes	24 Tubes	48 Tubes
Number of Uses	Up to 4	Up to 4	Up to 4
Ilumina Experiment Manager Kit Selection	TruSeq Small RNA	TruSeq Small RNA	TruSeq Small RNA

B. Index Sequences

Small RNA-seq indexes are 6 nt long and identical to the Illumina TruSeq Small RNA indexes. Each Indexing Primer tube contains a unique index sequence.

Table II. SMARTer microRNA-Seq Index. Each Indexing Primer Tube contains a unique Illumina-compatible 6-nt index sequence. The 48 indexes are identical to the Illumina TruSeq Small RNA indexes RPI1 through RPI48.

Tube	Index	Sequence	Tube	Index	Sequence
1	RPI1	ATCACG	**13**	RPI13	AGTCAA
2	RPI2	CGATGT	**14**	RPI14	AGTTCC
3	RPI3	TTAGGC	**15**	RPI15	ATGTCA
4	RPI4	TGACCA	**16**	RPI16	CCGTCC
5	RPI5	ACAGTG	**17**	RPI17	GTAGAG
6	RPI6	GCCAAT	**18**	RPI18	GTCCGC
7	RPI7	CAGATC	**19**	RPI19	GTGAAA
8	RPI8	ACTTGA	**20**	RPI20	GTGGCC
9	RPI9	GATCAG	**21**	RPI21	GTTTCG
10	RPI10	TAGCTT	**22**	RPI22	CGTACG
11	RPI11	GGCTAC	**23**	RPI23	GAGTGG
12	RPI12	CTTGTA	**24**	RPI24	GGTAGC
Tube	**Index**	**Sequence**	Tube	**Index**	**Sequence**
25	RPI25	ATCAGT	37	RPI37	ATTCCG
26	RPI26	GCTCAT	38	RPI38	AGCTAG
27	RPI27	AGGAAT	39	RPI39	GTATAG
28	RPI28	CTTTTG	40	RPI40	TCTGAG
29	RPI29	TAGTTG	41	RPI41	GTCGTC
30	RPI30	CCGGTG	42	RPI42	CGATTA
31	RPI31	ATCGTG	43	RPI43	GCTGTA
32	RPI32	TGAGTG	44	RPI44	ATTATA
33	RPI33	CGCCTG	45	RPI45	GAATGA
34	RPI34	GCCATG	46	RPI46	TCGGGA
35	RPI35	AAAATG	47	RPI47	CTTCGA
36	RPI36	TGTTGG	48	RPI48	TGCCGA

C. Mutliplexing and Index Pooling

If libraries will be pooled for sequencing, use the Illumina Experiment Manager (IEM) or BaseSpace to record information about your samples before beginning the Small RNA-seq Library Preparation Protocol.

Refer to Illumina's TruSeq Library Prep Pooling Guide (Illumina, Document # 15042173 v01, November 2015) for Illumina sequencing systems that require balanced index combinations. For these Illumina sequencing systems, at each sequencing cycle of the index read, at least one of the two nucleotides for each colored laser should be present to ensure proper image registration and accurate demultiplexing of the pooled samples. Therefore, when libraries with less than the full set of small RNA-seq indexes will be prepared and pooled, it is critical to follow the following steps before library preparation to ensure compatible index combinations are used:

1. Determine the number of libraries that will be pooled for sequencing.
2. Select the appropriate index combinations to use based on Illumina's multiplexing and index pooling guidelines. The IEM can be used to validate the compatibility of the chosen index combinations.

Appendix B. Troubleshooting Guide

Table III. Troubleshooting Guide for the SMARTer microRNA-Seq Kit.

Problem	Potential Cause	Suggested Solutions
Concentration of libraries after PAGE size-selection too low for sequencing.	Loss of pellet during EtOH precipitation.	Use leftover RT reaction and repeat PCR step, purification and size-selection.
Adapter dimers not completely removed after size-selection during PAGE.	Gel was overloaded, or operator cut out wrong band.	Repeat size selection.
Mapping of total human RNA samples to reference human genome too low.	1. Sample contamination with foreign DNA 2. Operator used CB1 instead of DB during Dephosphorylation step	1. Repeat library preparation with RNAse/DNAse free conditions, after performing decontamination of all surfaces and materials. 2. Repeat library preparation with correct buffers at designated steps.

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