Takara Bio USA, Inc.

SMARTer® smRNA-Seq Kit for Illumina® User Manual

Cat. Nos. 635029, 635030, 635031
(121219)

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I. Introduction

smRNA-seq using SMART® Technology

The **SMARTer smRNA-Seq Kit for Illumina** (Cat. Nos. 635029, 635030, 635031) is designed to generate high-quality smRNA-seq libraries for sequencing on Illumina platforms. This kit was developed to work directly with total RNA or enriched small RNA inputs ranging from 1 ng–2 µg. By incorporating features including Takara Bio's proprietary SMART (**S**witching **M**echanism **a**t the 5' end of **R**NA **T**emplate) technology and locked nucleic acids (LNAs), this kit allows users to analyze a wide range of smRNA species and generate sequencing libraries of considerable complexity from as little as 1 ng of input material. Illumina adapter and index sequences are incorporated in a ligation-free manner during library amplification (Figure 1), ensuring that diverse smRNA species are represented with minimal bias.

This kit:

- Enables users to analyze diverse RNA species, including miRNA, piRNA, snoRNA, and snRNA, from inputs of total RNA or enriched smRNA
- Generates sequencing libraries using a method that includes polyadenylation, cDNA synthesis, and PCR amplification steps, with optional protocols for library purification and size selection
- Avoids biases associated with adapter ligation through 3' polyadenylation of input RNA and template switching during cDNA synthesis
- Incorporates Illumina TruSeq® HT (currently known as TruSeq CD) index sequences during library amplification, allowing for multiplexing of sequencing libraries on a single flow cell lane
- Includes the Macherey-Nagel NucleoSpin Gel and PCR Clean-Up kit for easy library purification following PCR amplification
- Employs a quick, single-tube workflow (Figure 2) which can be performed within ~3 hours (not including validation and post-PCR size-selection steps)

Following PCR amplification, purification, and validation, sequencing libraries may require size selection depending on the input material and experimental objectives. Size selection can be performed using either SPRI (Solid Phase Reversible Immobilization) beads or the BluePippin system, and is particularly beneficial when processing libraries derived from total RNA inputs. In contrast, libraries generated from PAGE-purified smRNA fractions may not require post-PCR size selection. Whereas a gel-free, bead-based double size selection broadly retains library molecules containing inserts in the 15 bp–150 bp size range, the BluePippin system allows for more stringent selection of library molecules containing inserts of specific sizes.

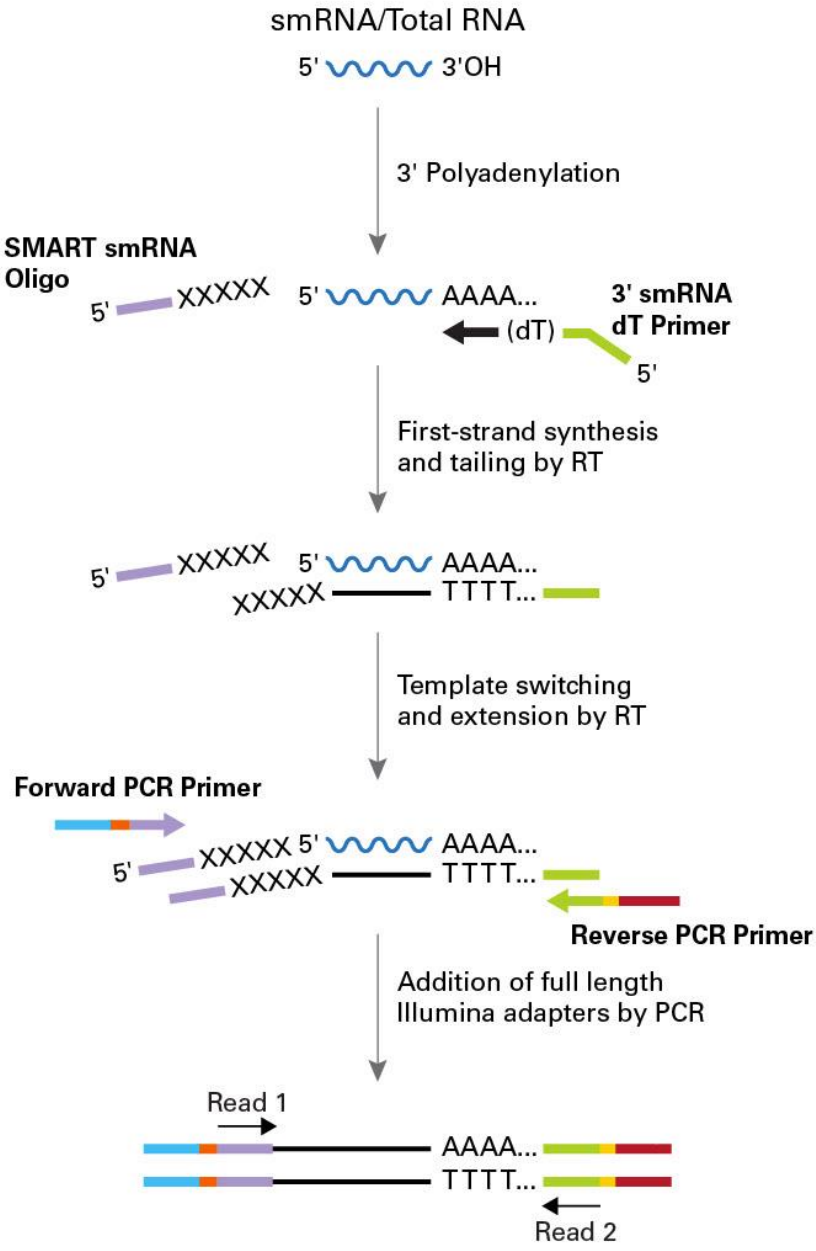

Figure 1. Schematic of technology used by the SMARTer smRNA-Seq Kit for Illumina. SMART technology is used in a ligation-free workflow to generate sequencing libraries for Illumina platforms. Input RNA is first polyadenylated in order to provide a priming sequence for an oligo(dT) primer. cDNA synthesis is primed by the 3' smRNA dT Primer, which incorporates an adapter sequence (green) at the 5' end of each first-strand cDNA molecule. When the MMLV-derived PrimeScript™ Reverse Transcriptase (RT) reaches the 5' end of each RNA template, it adds non-templated nucleotides which are bound by the SMART smRNA Oligo—enhanced with locked nucleic acid (LNA) technology for greater sensitivity. In the template-switching step, PrimeScript RT uses the SMART smRNA Oligo as a template for the addition of a second adapter sequence (purple) to the 3'end of each first-strand cDNA molecule. In the next step, full-length Illumina adapters (including index sequences for sample multiplexing) are added during PCR amplification. The Forward PCR Primer binds to the sequence added by the SMART smRNA Oligo, while the Reverse PCR Primer binds to the sequence added by the 3' smRNA dT Primer. Resulting library cDNA molecules include sequences required for clustering on an Illumina flow cell (P5 shown in light blue, P7 shown in red), Illumina TruSeq HT (currently known as TruSeq CD) index sequences (Index 2 [i5] shown in orange, Index 1 [i7] shown in yellow), and sequences bound by the Read Primer 1 or Read Primer 2 sequencing primers (shown in purple and green, respectively). Note that adapter sequences included in the final library add 153 bp to the size of RNA-derived insert sequences.

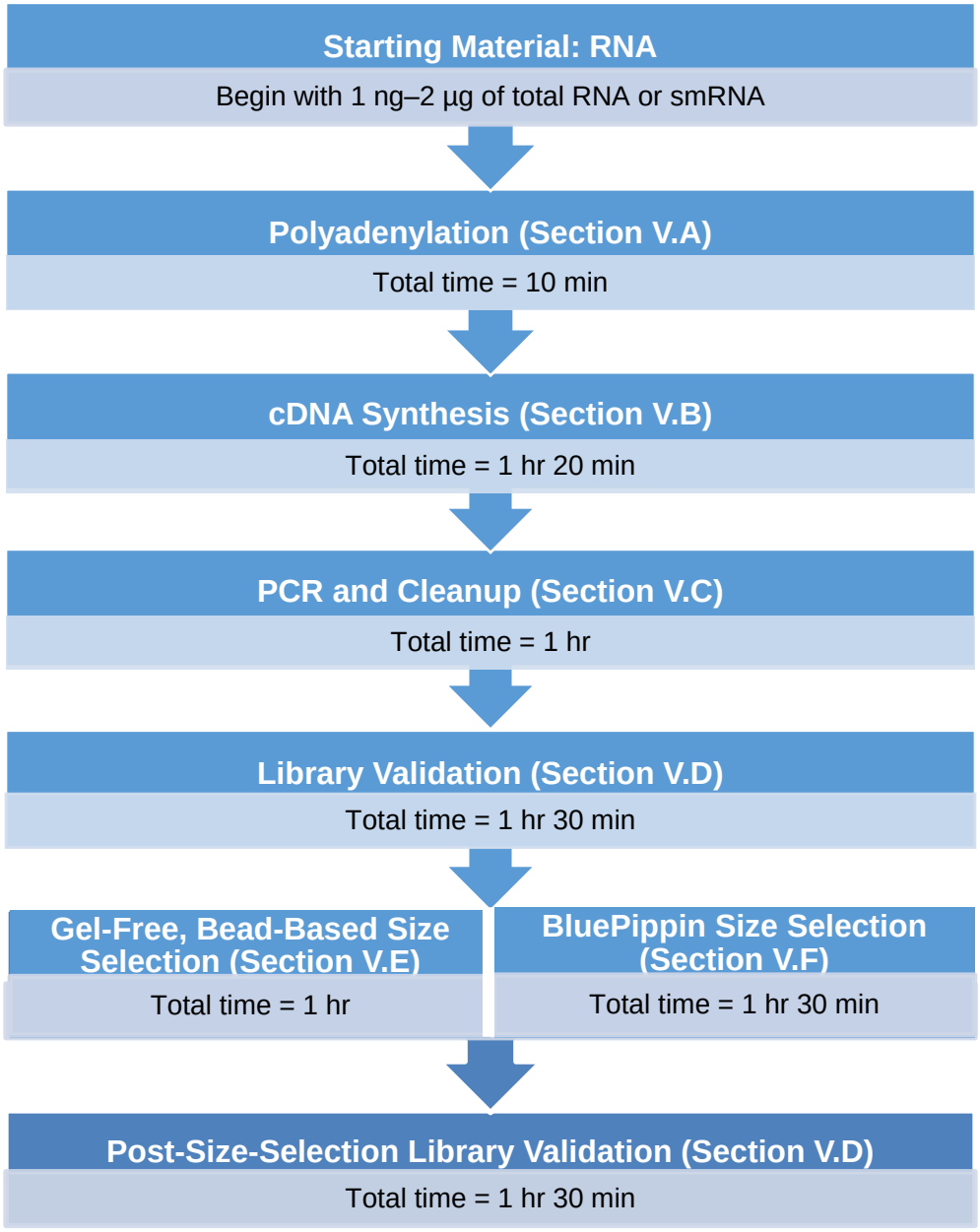

Figure 2. Library preparation workflow for the SMARTer smRNA-Seq Kit for Illumina. Size selection (highly recommended) is performed after library production, purification, and validation using either SPRI beads (Section V.E) or the BluePippin system (Section V.F), with the latter method affording greater user control over final library insert sizes. Total time for each section will vary depending on the number of samples being processed.

I. List of Components

The SMARTer smRNA-Seq Kit for Illumina consists of the SMARTer smRNA-Seq Kit Components (not sold separately), the Indexing Primer Set HT for Illumina (not sold separately), the NucleoSpin Gel and PCR Clean-Up kit, and SeqAmp™ DNA Polymerase. **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.** Please make sure to spin down tubes to collect all the liquid at the bottom before first use.

SMARTer smRNA-Seq Kit for Illumina	Cap color	635029 (12 rxns)	635030 (48 rxns)	635031 (96 rxns)
SeqAmp DNA Polymerase (Store at –20°C.)				
SeqAmp DNA Polymerase[a]	-	50 µl	200 µl	200 µl
SeqAmp PCR Buffer (2X)	-	1.25 ml	4 x 1.25 ml	4 x 1.25 ml
SMARTer smRNA-Seq Kit for Illumina Components (Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)				
Package 1 (Store at –70°C.)				
Control RNA - miR163s[b] (10 ng/µl)	Red	5 µl	5 µl	5 µl
smRNA Mix 2[c]	Pink	80 µl	320 µl	640 µl
Package 2 (Store at –20°C.)				
smRNA Mix 1	Blue	30 µl	120 µl	240 µl
ATP	Blue	12 µl	48 µl	96 µl
RNase Inhibitor (40 U/µl)	White	12 µl	48 µl	96 µl
Poly(A) Polymerase (2 U/µl)	Blue	10 µl	24 µl	48 µl
3’ smRNA dT Primer	Pink	12 µl	48 µl	96 µl
PrimeScript RT (200 U/µl)	White	24 µl	96 µl	192 µl
Nuclease-Free Water	-	1.25 ml	2 x 1.25 ml	4 x 1.25 ml
Tris Buffer (5 mM)	Orange	1.25 ml	3 x 1.25 ml	5 x 1.25 ml
NucleoSpin Gel and PCR Clean-Up (Store at room temperature)				
Binding Buffer NT1	-	40 ml	40 ml	200 ml
Washing Buffer NT3 (Concentrate)	-	25 ml	25 ml	2 x 50 ml
Elution Buffer NE (10 mM Tris-Cl, pH 8.5)	-	13 ml	13 ml	30 ml
NucleoSpin Gel and PCR Clean-Up Columns	-	50	50	250
Collection Tubes (2 ml)	-	50	50	250

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

[b] Control RNA - miR163s is derived from plant miR163. See “Using Control RNA - miR163” in Section IV.C for sequence information.

[c] smRNA Mix 2 contains the template-switching oligo with LNA technology.

Indexing Primer Sets (Not sold separately. Store at –70°C.)

Indexing Primer Set version		HT for Illumina - 12	HT for Illumina - 48 A	HT for Illumina
Cat. No.		635029	635030	635031
Size		12 rxns	48 rxns	96 rxns
Forward Primers	F1[b]		2 x 15 µl	2 x 15 µl
12 µM	F2	2 x 15 µl	2 x 15 µl	2 x 15 µl
Full names of	F3		2 x 15 µl	2 x 15 µl
primers have	F4		2 x 15 µl	2 x 15 µl
been shortened[a]	F5			2 x 15 µl
	F6			2 x 15 µl
	F7			2 x 15 µl
	F8			2 x 15 µl
Reverse Primers	R1	12 µl	12 µl	2 x 12 µl
12 µM	R2	12 µl	12 µl	2 x 12 µl
Full names of	R3	12 µl	12 µl	2 x 12 µl
primers have	R4	12 µl	12 µl	2 x 12 µl
been shortened[a]	R5	12 µl	12 µl	2 x 12 µl
	R6	12 µl	12 µl	2 x 12 µl
	R7	12 µl	12 µl	2 x 12 µl
	R8	12 µl	12 µl	2 x 12 µl
	R9	12 µl	12 µl	2 x 12 µl
	R10	12 µl	12 µl	2 x 12 µl
	R11	12 µl	12 µl	2 x 12 µl
	R12	12 µl	12 µl	2 x 12 µl

[a] Full names of primers have been shortened: for example, Forward PCR Primer HT Index 2 has been shortened to F2 and Reverse PCR Primer HT Index 1 has been shortened to R1.

[b] F1–F8 indexes correspond to Illumina TruSeq HT (currently known as TruSeq CD) indexes D501–D508; R1–R12 indexes correspond to Illumina TruSeq HT (currently known as TruSeq CD) indexes D701–D712.

Indexing Primer Set HT for Illumina barcode sequences:

i5 Index (Tube Label)	Barcode Sequence	i7 Index (Tube Label)	Barcode Sequence
F1	TATAGCCT	R1	ATTACTCG
F2	ATAGAGGC	R2	TCCGGAGA
F3	CCTATCCT	R3	CGCTCATT
F4	GGCTCTGA	R4	GAGATTCC
F5	AGGCGAAG	R5	ATTCAGAA
F6	TAATCTTA	R6	GAATTCGT
F7	CAGGACGT	R7	CTGAAGCT
F8	GTACTGAC	R8	TAATGCGC
		R9	CGGCTATG
		R10	TCCGCGAA
		R11	TCTCGCGC
		R12	AGCGATAG

Storage Conditions

- Store Control RNA - miR163s, smRNA Mix 2, and Indexing Primer Set HT for Illumina at –70°C.
- Store NucleoSpin Gel and PCR Clean-Up reagents at room temperature.
- Store all other reagents at –20°C.

II. Additional Materials Required

The following reagents and materials are required but not supplied. They have been validated to work with this protocol. Please do not make any substitutions because you may not obtain the expected results:

- Single-channel pipettes: 10 µl, 20 µl, and 200 µl, two each (one set for pre-PCR amplification steps and one set dedicated for PCR amplification)
- Eight-channel pipettes (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, and 200 µl
- Vortex mixer
- Hot-lid PCR thermal cyclers: two (one dedicated for pre-PCR amplification steps and one dedicated for PCR amplification).
  NOTE: The PCR thermal cycler intended for PCR amplification must be compatible with 100 µl of PCR reaction mix per reaction.
- Microcentrifuge for 1.5-ml tubes
- Minicentrifuge for 0.2-ml tubes or strips
- 96–100% ethanol (molecular biology grade)
- 96-well PCR chiller rack: IsoFreeze PCR Rack (MIDSCI, Cat. No. 5640-T4) or 96-Well Aluminum Block (Light Labs, Cat. No. A-7079)
  NOTE: A PCR chiller rack is essential to keep samples cold during several steps of the protocol. Be sure to decontaminate the ice bucket and the PCR chiller rack before each use.

For PCR Amplification & Validation:

- Agilent Bioanalyzer and Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)
  NOTE: Library validation can also be performed using an Advanced Analytical Fragment Analyzer with the High Sensitivity Small Fragment Kit (Advanced Analytical, Cat. No. DNF-477)
- Qubit Fluorometer (Thermo Fisher Scientific); Qubit dsDNA HS Assay Kit and 500-µl Assay Tubes (Thermo Fisher Scientific; Q32851 and Q32856, respectively)
- Nuclease-free thin-wall PCR tubes or strips (0.2-ml PCR 8-tube strip; USA Scientific, Item No.1402-4700)
- Nuclease-free low-adhesion 1.5-ml tubes (USA Scientific, Item No. 1415-2600) or LoBind tubes (Eppendorf, Cat. No. 022431021)

For Gel-Free Size Selection Using SPRI (Solid Phase Reversible Immobilization) Beads (Protocol V.E):

- Agencourt AMPure XP PCR purification kit
  (5-ml size: Beckman Coulter Item No. A63880; 60-ml size: Beckman Coulter Item No. A63881)
  NOTE: In order to decrease the chances of bead contamination and to ensure that beads are uniformly distributed, we strongly recommend aliquoting the beads into 1.5-ml tubes upon receipt, and then refrigerating the aliquots. Individual aliquots can be removed for each experiment, allowing them to come to room temperature more quickly (~30 minutes). Mix well to disperse the beads before adding them to your reactions. The beads are viscous, so pipette slowly.

- 80% ethanol (molecular biology grade): freshly made for each experiment
- Magnetic separation device for small volumes (Magnetic Separator - PCR Strip; Takara Bio, Cat. No. 635011)

For Size Selection Using the BluePippin System (Protocol V.F):

- BluePippin Size Selection System (Sage Science, BLU0001)
- BluePippin 3% Agarose Gel Cassettes—for targets between 100 and 200 bp (Sage Science, Cat. No. BDQ3010)

III. General Considerations

A. Recommendations for Preventing Contamination

1. **Before you set up the experiment, it is advisable to have three physically separated work stations:**
    - **A PCR-clean work station** for all pre-PCR experiments that require clean room conditions such as first-strand cDNA synthesis (Section V.B).
    - **A second work station located in the general laboratory** where you will perform PCR (Section V.C).
    - **A third work station located in the general laboratory** where you will purify (Section V.C), quantify, and validate the library (Section V.D).

      IMPORTANT: We recommend three separate work areas in order to avoid contaminating samples with PCR products from previous reactions. Since the PCR primers recognize sequences common to all libraries, setting up new reactions in the same area where the final library cleanup occurs increases the risk of contamination. The PCR-clean work station must be located in a room with positive air flow, as contamination may occur very easily. Once contamination occurs, it can be difficult to remove. While the use of three separate work areas is not an absolute requirement, it can greatly minimize contamination and ensure the preparation of great-quality libraries every time.

2. **Guidelines for PCR-clean work station operation:**
    - Only move materials/supplies from the PCR-clean work station to the general lab, NOT the other way around. Do not share any equipment/reagents between the PCR-clean work station and the general lab work stations.
    - Use a separate PCR thermal cycler (dedicated to first-strand cDNA synthesis) inside the PCR-clean work station for first-strand cDNA synthesis.
    - Wear gloves and sleeve covers throughout the procedure to protect your RNA samples from degradation by contaminants and nucleases. Be sure to change gloves and sleeve covers between each section of the protocol.

B. General Requirements

- **The success of your experiment depends on the purity of your input RNA and the retention of smRNA during RNA extraction. Prior to using this kit, please make sure that your RNA is free of contaminants and has been extracted using a method that preserves smRNA fractions.**
- The assay is very sensitive to variations in pipette volume, etc. Please make sure that all pipettes are calibrated for reliable reagent delivery and that nothing adheres to the outside of the tips when dispensing liquids.

- All lab supplies related to cDNA synthesis need to be stored in a DNA-free, closed cabinet. Ideally, reagents for cDNA synthesis should be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Add enzymes to reaction mixtures last, and thoroughly incorporate them by gently pipetting the reaction mixtures up and down.
- Do not change the amount or concentration of any of the components in the reactions; they have been carefully optimized for the SMARTer reagents and protocol.
- If you are using this protocol for the first time, we **strongly recommend** that you perform negative (without RNA) and positive (with provided Control RNA - miR163s) control reactions.

C. Sample Recommendations

Input RNA Quality

- This kit is designed to generate high-quality sequencing libraries from inputs consisting of either total RNA or enriched (e.g. PAGE- or column-purified) smRNA. For column purification of smRNA species <200 nt, Takara Bio offers the Macherey-Nagel NucleoSpin miRNA kit (Cat. No. 740971).
- For inputs consisting of total RNA, performance of the SMARTer smRNA-Seq Kit for Illumina is dependent on the quality of the RNA starting material. While the use of high-quality total RNA (RIN >8) is recommended, the kit may also perform well with partially degraded samples. Please be aware, though, that sample degradation may result in underrepresentation of smRNAs in final sequencing libraries. After RNA extraction, purification, and size selection (optional), we recommend evaluating total RNA quality using the Agilent RNA 6000 Pico Kit (Agilent, Cat. No. 5067-1513). Refer to the manufacturer's instructions about how to use the Agilent RNA 6000 Pico Kit.
- The initial polyadenylation step in the kit workflow (Section V.A) requires the presence of an -OH group at RNA 3' ends, and RNA templates obtained from methods such as ribosome profiling or CLIP may require dephosphorylation prior to library preparation in order to provide a suitable substrate for Poly(A) Polymerase.

Input RNA Purity and Quantity

- **Purity of input RNA:** Input RNA should be free from poly(A) carrier RNA and contaminants that would interfere with oligo(dT)-primed cDNA synthesis, and (ideally) dissolved in water. Samples should also be free of DNA contaminants, which could be amplified and incorporated in final sequencing libraries.

  IMPORTANT: Purified total RNA should be resuspended in Nuclease-Free Water (included), **not in TE or other buffers containing EDTA**. Chelation of divalent cations by EDTA will interfere with the efficiency of reverse transcription.

- **Volume and amount of input RNA:** The kit protocol has been optimized for cDNA synthesis with 1 ng–2 µg inputs of total RNA or enriched smRNA. Starting volumes of dissolved RNA should generally be ≤7 µl, or ≤6 µl for higher input amounts. Please refer to Section V.A for additional guidelines regarding suitable amounts and volumes of RNA inputs.

  NOTE: Inputs in the range of 1 ng to 2 µg of total RNA are recommended. Inputs higher than 2 µg have not been validated. It is strongly recommended that working conditions for your samples be established before trying inputs outside the recommended range. For RNA inputs <1 ng, the yield

may be low and is highly dependent on the RNA source (e.g., the smRNA content in a particular sample/cell type/tissue), but may be sufficient for sequencing. It is recommended that similar library preparation conditions (i.e. input amount, number of PCR cycles, size-selection protocol, etc.) are used for all samples included in a given analysis.

Using Control RNA - miR163s

The positive control RNA (Control RNA - miR163s*) is provided at a concentration of 10 ng/µl, and should be diluted in nuclease-free water and aliquoted prior to first use. For best performance, freeze-thaw cycling of the control RNA should be avoided, especially once it has been diluted.

*The Control RNA - miR163s sequence is derived from plant miR163, but truncated by two nucleotides: 5' Phos-GAAGAGGACUUGGAACUCGAUC

1. Dilute Control RNA - miR163s (10 ng/µl) to 1 ng/µl by mixing 18 µl of Nuclease-Free Water with 2 µl of Control RNA - miR163s in a sterile microcentrifuge tube.
2. Make single-use aliquots of the 1 ng/µl dilution, and store them at –70ºC until needed.
3. For library preparation, use 1 µl of the 1 ng/µl dilution plus 6 µl of nuclease-free water in the polyadenylation step, and perform 12 cycles of PCR. The yield is typically 10–20 ng/µl.

IV. Protocols

NOTE: Please read the entire protocol before starting. This protocol has been optimized for generation of sequencing-ready libraries from 1 ng–2 µg of input RNA. Due to the sensitivity of the protocol, the input material needs to be prepared under clean-room conditions to avoid contamination. Polyadenylation and cDNA synthesis steps should be carried out in a PCR Clean Work Station under clean-room conditions.

A. Protocol: Polyadenylation

To facilitate oligo(dT)-primed cDNA synthesis, an artificial poly(A) tail is added to input RNA using Poly(A) Polymerase.

For this protocol you will need the following components:

Control RNA - miR163s (red cap), Nuclease-Free Water, Poly(A) Polymerase (blue cap), RNase Inhibitor (white cap), smRNA Mix 1 (blue cap), ATP (blue cap; optional)

NOTE: We recommend analyzing the provided Control RNA - miR163s* as a positive control (consult the previous page for preparation guidelines). A no-RNA negative control should be included in each experiment, and is especially important when performing a high number of PCR cycles during library production.

1. Thaw all RNA samples and reagents for Polyadenylation (this section) and cDNA Synthesis (Section V.B) on ice. Keep all samples and reagents on ice.
2. Precool the thermal cycler to 16°C.
3. Place a 0.2-ml PCR tube for each RNA sample to be analyzed in a pre-chilled PCR chiller rack.
4. Distribute ≤7 µl of each RNA sample into a pre-chilled 0.2-ml PCR tube. Bring the volume of each sample to 7 µl with nuclease-free water.

   NOTE: Starting sample volumes for inputs consisting of >25 ng of total RNA or enriched smRNA, or >10 ng of PAGE-purified miRNA must not exceed 6 µl, because 1 µl of ATP per reaction will be added to the Polyadenylation Master Mix.
5. OPTIONAL (perform this step if you are only doing 1–3 reactions): Prepare a premix with enough Poly(A) Polymerase and RNase Inhibitor for four reactions as shown below:

Volume		
One reaction	Four reactions	
0.25 µl	1 µl	Poly(A) Polymerase (2 U/µl)
0.25 µl	1 µl	RNase Inhibitor (40 U/µl)
0.5 µl	2 µl	Total volume

NOTES:

- Due to the viscosity of the Poly(A) Polymerase, at least 1 µl should be pipetted. The Poly(A) Polymerase and RNase Inhibitor have been provided in excess to account for the possibility that users may perform only 1–3 reactions at a time.
- Include 0.5 µl of this Poly(A) Polymerase and RNase Inhibitor mixture *per reaction* (plus 10%) in the Polyadenylation Master Mix generated in Step 6 (below).

6. Place a 1.5-ml tube on ice. Prepare enough Polyadenylation Master Mix for all reactions, plus 10%, by combining the following reagents in the 1.5-ml tube in the order shown. Mix the contents of the tube by slowly pipetting up and down 6 times. Spin down briefly if necessary.

0.25 µl	Poly(A) Polymerase (2 U/µl)
0.25 µl	RNase Inhibitor (40 U/µl)
2.5 µl	smRNA Mix 1
(1 µl)	ATP (Optional*)
3 µl (4 µl)	Total volume added per reaction

*If using >25 ng of total RNA or enriched smRNA, include the optional ATP in the Polyadenylation Master Mix, as it will improve the efficiency of the polyadenylation reaction. Do not include the ATP for lower input amounts.

NOTES:
- Make sure to keep the Polyadenylation Master Mix cold/on ice. Do not prepare the Polyadenylation Master Mix before distributing the RNA samples into the tubes (Step 4), as it should be made fresh immediately before it is added to the RNA.
- If using a sample other than total RNA or enriched smRNA, you may need to determine empirically whether the addition of extra ATP is beneficial or not.

7. Add 3 µl (4 µl if ATP was included) of Polyadenylation Master Mix to each sample from Step 4. Keep samples in the chiller rack during distribution of the Polyadenylation Master Mix.
8. Mix briefly by flicking the tubes with fingers a few times (enough for droplets to form on the side of each tube). Spin briefly to collect the contents at the bottom of each tube. Alternatively, mixing can be done by pipetting up and down 5 times.

   NOTE: If mixing by pipetting, make sure to minimize the amount of liquid left behind in the pipette tips. Regardless of how samples are mixed, great care should be taken to keep the samples ice cold.
9. Place each tube in the thermal cycler precooled to 16°C. Incubate for 5 minutes, then immediately transfer each tube to the PCR chiller rack and proceed to Section V.B (cDNA Synthesis).

   NOTE: Proceed to Section V.B no later than 5 minutes after completion of Step 9.

B. Protocol: cDNA Synthesis

cDNA synthesis is performed by PrimeScript RT, and primed by the 3' smRNA dT Primer, which anneals to the poly(A) tail added to RNA in the previous section (Section V.A). Non-templated nucleotides added to cDNA 3' ends are bound by an oligo included in smRNA Mix 2, allowing for template switching by the RT. These processes result in the addition of adapters to 5' and 3' ends of first-strand cDNA.

For this protocol you will need the following components:
smRNA Mix 2 (pink cap), RNase Inhibitor (white cap), PrimeScript RT (200 U/µl; white cap), 3' smRNA dT Primer (pink cap)

1. Preheat the thermal cycler to 72°C.

   NOTE: Necessary reagents should already be thawed on ice (Section V.A., Step 1).
2. Allow samples from Section V.A to cool to 4°C on the PCR chiller rack for 1 minute (**and no more than 5 minutes**), then add 1 µl of 3' smRNA dT Primer to each tube.
3. Mix briefly by flicking the tubes with fingers a few times (enough for droplets to form on the side of each tube). Spin briefly to collect the contents at the bottom of each tube. Alternatively, mixing can be done by pipetting up and down 5 times.

   NOTE: If mixing by pipetting, make sure to minimize the amount of liquid left behind in the pipette tips. Regardless of how samples are mixed, great care should be taken to keep the samples ice cold.
4. Place each tube in the thermal cycler preheated to 72°C. Incubate for 3 minutes, then immediately transfer each tube to the PCR chiller rack and allow samples to cool to 4°C for 2 minutes.

NOTES:
- After transferring the samples to the PCR chiller rack, set the thermal cycler to 42°C in preparation for Step 7.
- The next reaction steps (Steps 5–7) are critical for first-strand cDNA synthesis and should not be delayed after Step 4. Start Step 5, preparing the Reverse Transcription Master Mix, while your tubes are incubating (Step 4), or have it almost ready before starting Step 4.

5. While samples are incubating, prepare enough Reverse Transcription Master Mix for all the reactions, plus 10% of the total reaction mix volume, by adding the following reagents to a 1.5-ml tube on ice in the order shown:

   Per reaction:

6.5 µl	smRNA Mix 2
0.5 µl	RNase Inhibitor
2 µl	PrimeScript RT (200 U/µl)
9 µl	Total volume added per reaction

6. While keeping samples on the PCR chiller rack, add 9 µl of the Reverse Transcription Master Mix to each tube from Step 4. Simultaneously rinse the pipet tip and mix by pipetting up and down 6 times. The total reaction volume is now 20 µl.
7. Place the tubes in the thermal cycler preheated to 42°C. Run the following program:

42°C	60 min
70°C	10 min
4°C	hold

STOPPING POINT: For convenience, samples can be left overnight in the thermal cycler at 4°C, or frozen at –80 °C for several weeks.

C. Protocol: PCR and Cleanup

cDNA is amplified and full-length Illumina adapters are added via PCR. PCR products are purified using the NucleoSpin Gel and PCR Clean-Up kit.

For this protocol you will need the following components:
Nuclease-Free Water, SeqAmp PCR Buffer (2X), SeqAmp DNA Polymerase, Indexing Primer Set HT for Illumina, NucleoSpin Gel and PCR Clean-Up kit.

IMPORTANT: Transfer the samples from the PCR Clean Work Station to the general lab. All downstream processes should be performed in the general lab.

1. Thaw all the reagents needed for PCR (except the enzyme) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. Preheat the thermal cycler to 98°C.
3. Prepare enough PCR Master Mix for all the reactions, plus 10% of the total reaction mix volume. Combine the following reagents (per reaction) in the order shown:

24 µl	Nuclease-Free Water
50 µl	2X SeqAmp PCR Buffer
2 µl	SeqAmp DNA Polymerase
76 µl	Total volume added per reaction

NOTE: Mix the Master Mix well by vortexing gently and spin the tube briefly to collect the contents at the bottom of the tube. DO NOT reduce the reaction volume. The 100-µl final volume is important for yield. If your thermal cycler cannot accommodate 100-µl sample volumes, it is important to equally divide each sample into two tubes (containing ~50 µl each) after the PCR Master Mix has been added, mixed, and spun down.

4. Add 76 µl of PCR Master Mix to each sample from the previous section (Section V.B), then add 2 µl of each Forward and Reverse primer to each sample. Mix well by gentle vortexing or tapping and briefly spin to collect the contents at the bottom of the tube(s).

NOTE: If a single Forward primer is to be used for all samples, it can be included in the PCR Master Mix (2 µl per reaction, plus 10%).

5. Place the tube(s) in a preheated thermal cycler with a heated lid and run the following program:

98°C	1 min
7–17 cycles*:	
98°C	10 sec
60°C	5 sec
68°C	10 sec
4°C	forever

* Consult Table 1 (below) for PCR cycle number guidelines. For Control RNA – miR163s, we recommend using 11–12 cycles for an input of 1 ng. The no-RNA negative control should be amplified with the same number of cycles as the sample inputs. For smRNA enriched by PAGE or other means, it is suggested that users perform a pilot experiment using the number of cycles recommended for an equivalent input amount of total RNA.

Table I. Cycling guidelines based on type and amount of starting material.

Input Type	Input Amount	Number of Cycles
RNA <200 nt*	1 ng	11–12
RNA <200 nt*	50 ng	7–8
Total RNA	1 ng	16–17
Total RNA	50 ng	13–14
Total RNA	500 ng	11–12
Total RNA	2 µg	7–8
Total RNA from plasma	1 ng	16–17

*Enriched smRNA species <200 nt, isolated through column purification using the Macherey-Nagel NucleoSpin miRNA kit (Cat. No. 740971)

NOTES:

- For inputs consisting of total RNA, an adequate number of PCR cycles should yield library concentrations of >10 ng/µl following purification of PCR products. Such yields are necessary because only 10–30% of library will be retained following broad, bead-based size selection (Section V.E), and only 3–10% of library will be retained following narrow, BluePippin size selection (Section V.F). PCR cycling parameters that yield library concentrations >20 ng/µl should be avoided. We recommend performing a pilot experiment and reducing or increasing the number of PCR cycles, as necessary, to achieve adequate library yields.
- For convenience, samples can be left overnight in the thermal cycler at 4°C, or frozen at –20°C for several weeks.

6. Use the NucleoSpin Gel and PCR Clean-Up kit to purify the entire 100-µl PCR reaction for each sample. Follow the standard protocol in Section 5.1 of the "PCR clean-up Gel extraction User manual NucleoSpin Gel and PCR Clean-up" (available for download at takarabio.com; search for Cat. No. 740609.50 or 740609.250). This protocol involves mixing 1 volume of sample with 2 volumes of 100% Binding Buffer NTI (e.g., add 200 µl of undiluted Binding Buffer NTI to 100 µl PCR reaction mix.) Elute each sample in 30 µl of provided Elution Buffer NE. Consult the user manual for detailed instructions.

STOPPING POINT: For convenience, purified libraries can be stored at –20°C. Alternatively, users may proceed to Section V.D (Library Validation).

D. Protocol: Library Validation

Validation is performed to determine if library production was successful.

1. Quantify purified libraries on a Qubit Fluorometer using the Qubit dsDNA HS Assay Kit. Yields ≥10 ng/µl are desirable for libraries that will undergo size selection. Yields for libraries generated from Control RNA - miR163s using 12 cycles of PCR should be ≥14 ng/µl. The negative control may yield ~1–2 ng/µl of adapter dimers.

   NOTE: Do not use a NanoDrop for library quantification; it lacks the required sensitivity.
2. Evaluate the size distribution of each library by running samples on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. Aliquots of libraries should be diluted to ~1.5 ng/µl prior to analysis on the Bioanalyzer, while aliquots of libraries generated from the positive control (Control RNA - miR163s) and no-RNA negative control should be diluted to 0.5 ng/µl.
3. Use the library profile results to determine whether each sample is suitable for further processing. Figure 3 (next page) includes example library profiles for positive and negative controls, and inputs consisting of Total RNA and enriched smRNA, respectively. Successful library production should yield a major peak at ~175 bp for Control RNA - miR163s, and a major peak at ~147 bp for the negative control. Small peaks observed in the ~140–153 bp size range are the result of adapter dimers. **When calculating expected library molecule sizes for a particular RNA input, use the following formula:**

   153 + input RNA size (nt) = expected size of library molecules (bp)
4. Following validation of sequencing libraries, proceed with either of the size selection protocols (Section V.E. or Section V.F) or directly to Illumina sequencing.

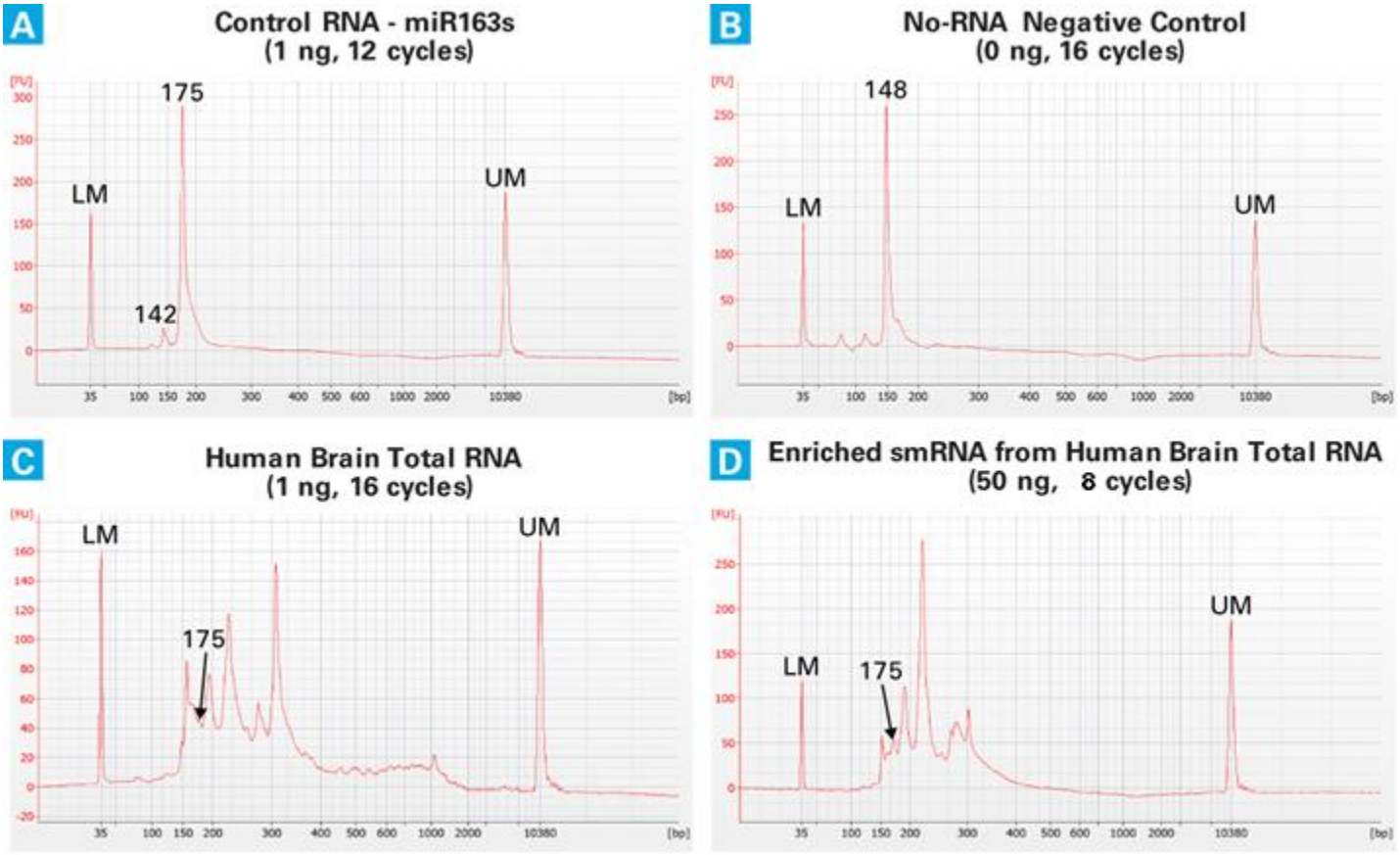

Figure 3. Example electropherogram results for smRNA-seq libraries. Libraries were generated using the SMARTer smRNA-Seq Kit for Illumina with the indicated inputs and cycling parameters. Libraries were analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis. **Panel A** shows a typical result for Control RNA - miR163s. The peak at 175 bp corresponds with the combined size of miR163s plus adapters, while the peak at 142 bp corresponds with the size of primer dimers. **Panel B** shows a typical result for a no-RNA negative control. The peak at 148 bp corresponds with the size of adapter dimers. **Panel C** shows an example profile for an input consisting of 1 ng of total RNA. The peak at 175 bp corresponds with the combined size of processed miRNAs plus adapters. **Panel D** shows an example profile for an input consisting of 50 ng of enriched smRNA species (<200 nt; isolated through column purification using the Macherey-Nagel NucleoSpin miRNA kit [Cat. No. 740971]). The peak at 175 bp corresponds with the combined size of processed miRNAs plus adapters.

E. Protocol: Size Selection Using Agencourt AMPure XP Beads

In order to enrich for inserts <150 bp, smRNA sequencing libraries are size selected using AMPure beads.

For this step you will need the following components:

Agencourt AMPure XP PCR Purification beads, 80% ethanol (made fresh), Nuclease-Free Water, Tris Buffer (5 mM; orange cap), a magnetic separation device.

NOTES:

- Aliquot AMPure XP beads into 1.5-ml tubes upon receipt in the laboratory.
- Before each use, bring bead aliquots to room temperature for at least 30 minutes and mix well to disperse.
- Prepare fresh 80% ethanol for each experiment. You will need 800 µl per sample (for two rounds of double size selection).
- You will need a magnetic separation device for 0.2-ml tubes.

1. Transfer each purified library (from Section V.C) to a clean 0.2-ml tube, and add Nuclease-Free Water to bring the total volume to 50 µl.
2. Add 40 µl of AMPure XP beads to each sample and mix well by vortexing for 5 sec or by pipetting the entire volume up and down at least 10 times.

   NOTE: The beads are viscous; pipette the entire volume, and push it out slowly.
3. Incubate at room temperature for 8 minutes to let the DNA bind to the beads.
4. Place the tubes on the magnetic separation device for 5–10 minutes or until the solution is completely clear. The time for the solution to clear will depend on the strength of the magnet.

   NOTE: Ensure that the solution is completely clear, as any bead carryover will decrease the efficiency of size selection. There is no disadvantage in separating the samples for longer than 10 minutes.
5. While the tubes are sitting on the magnetic separation device, use a pipette to transfer ~85 µl of the supernatant (which contains library molecules in the desired size range) to clean 0.2-ml tubes.

   NOTE: Be careful not to pick up any beads from the pellet during the transfer, as these beads are bound to library molecules that are outside of the desired size range.
6. Add 60 µl of AMPure XP beads to each supernatant collected in Step 5 and mix well by vortexing for 5 sec or by pipetting the entire volume up and down at least 10 times.

   NOTE: The beads are viscous; pipette the entire volume, and push it out slowly.
7. Incubate at room temperature for 8 minutes to let the DNA bind to the beads.
8. Place the tubes on the magnetic separation device for 5–10 minutes or until the solution is completely clear. The time for the solution to clear will depend on the strength of the magnet.

   NOTE: Ensure that the solution is completely clear, as any bead carryover will decrease the efficiency of size selection. There is no disadvantage in separating the samples for longer than 10 minutes.
9. While the tubes are sitting on the magnetic separation device, remove the supernatant with a pipette and discard it (library molecules in the desired size range are now bound to the beads).
10. Keep the tubes on the magnetic separation device. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads, to wash away contaminants. Wait for 30 seconds and use a pipette to carefully remove the supernatant containing contaminants. The library will remain bound to the beads during the washing process.
11. Repeat Step 10 once.
12. OPTIONAL (extra step helps accelerate the drying of the beads): Briefly spin the tubes (~2,000 *g*) to collect the remaining liquid at the bottom of each tube. Place the tubes on the magnetic separation device for 30 seconds, then remove all remaining liquid with a pipette.

13. Let the sample tubes rest open on the magnetic separation device at room temperature for ~3–5 minutes until the pellet appears dry and is no longer shiny.
    NOTE: You may see a tiny crack in the pellet. Do not overdry. Visit https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips to view examples of moist, dry, and overly dry pellets.
14. Once the bead pellet has dried, remove the tubes from the magnetic separation device and add 52 µl of Tris Buffer to cover the pellet. Mix thoroughly by pipetting up and down to ensure complete bead dispersion.
15. Incubate at room temperature for at least 5 minutes to rehydrate.
16. Place the sample tubes on the magnetic separation device for 2 minutes or longer, until the solution is completely clear.
17. Transfer 50 µl of the clear supernatant from each tube to a clean 0.2-µl tube.
18. Repeat Steps 2–13, then proceed to Step 19.
19. Once the bead pellet has dried, remove the tubes from the magnetic separation device and add 22 µl of Tris Buffer to cover the pellet. Mix thoroughly by pipetting up and down to ensure complete bead dispersion.
20. Incubate at room temperature for at least 5 minutes to rehydrate.
21. Place the sample tubes on the magnetic separation device for 2 minutes or longer, until the solution is completely clear.
22. Transfer clear supernatant containing purified smRNA library from each tube to a nuclease-free, low-adhesion tube.
23. Quantify and validate each library as described in Section V.D. See Figure 4 (on the next page) for examples of electropherogram results obtained both before and after bead-based double size selection.
    STOPPING POINT: Store each library at –20°C until ready for sequencing.

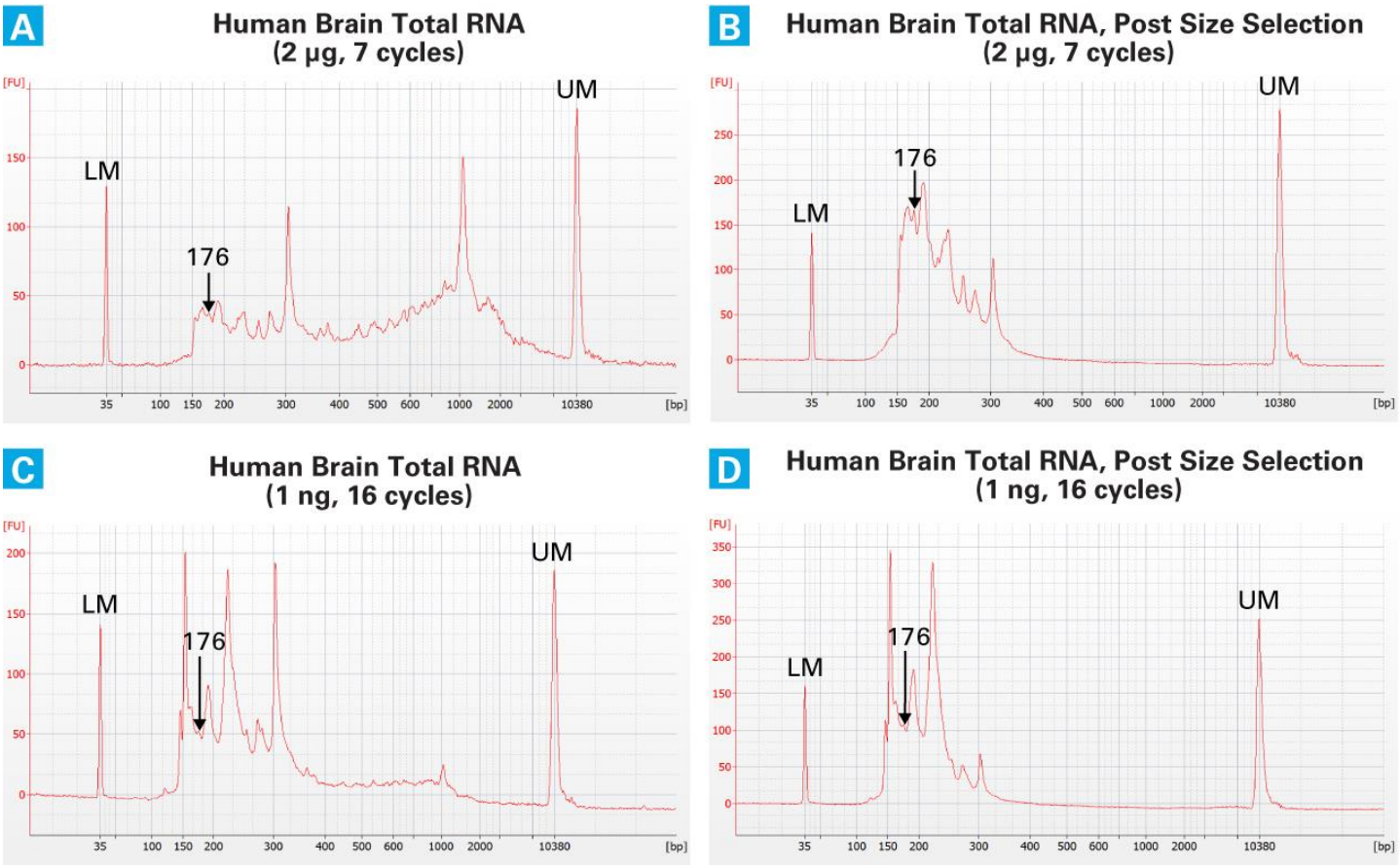

Figure 4. Example electropherogram results for smRNA-seq libraries before and after bead-based size selection. Libraries were generated using the SMARTer smRNA-Seq Kit for Illumina with the indicated inputs and cycling parameters, and were analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit, both before and after double size selection. Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis. **Panel A** shows a profile of a library generated from 2 µg of Human Brain Total RNA, prior to size selection. The peak at 176 bp corresponds with the combined size of processed miRNAs plus adapters. **Panel B** shows a profile of the library from Panel A following two rounds of bead-based size selection. The peak at 176 bp corresponds with the combined size of processed miRNAs plus adapters. **Panel C** shows a profile of a library generated from 1 ng of Human Brain Total RNA, prior to size selection. The peak at 176 bp corresponds with the combined size of processed miRNAs plus adapters. **Panel D** shows a profile of the library from Panel C following bead-based size selection. The peak at 176 bp corresponds with the combined size of processed miRNAs plus adapters.

F. Protocol: Size Selection Using the BluePippin System

Size selection is performed using the BluePippin Size Selection System, which allows for more stringent selection of library molecules within user-specified size ranges relative to bead-based selection. Please refer to the BluePippin Size Selection System user manual for detailed instructions.

For this step you will need the following components:

BluePippin Size Selection System, 3% Agarose Gel Cassettes—for targets between 100 and 200 bp; Nuclease-Free Water.

NOTES:

- The guidelines below were validated on a BluePippin Size Selection System (Sage Science, Cat. No. BLU0001) using 3% Agarose Gel Cassettes (Sage Science, Cat. No. BDQ3010). Adjustment of the protocol may be necessary if you are using different equipment.
- It is particularly important that libraries are purified with the NucleoSpin Gel and PCR Clean-Up kit prior to size selection with the BluePippin system, which requires that input DNA is in a low ionic buffer with a minimal amount of detergent.

- To save time and resources when working with many samples, differentially-indexed sequencing libraries with similar size profiles can be pooled in equal amounts prior to BluePippin selection.
- For size selection of libraries generated from total RNA, this protocol typically recovers 3–10% of input library DNA. Output library DNA may need to be concentrated prior to sequencing.

1. Bring the DNA marker solution (labelled "Q3") and electrophoresis buffer to room temperature.
2. Program the size-selection protocol on the BluePippin instrument as follows (please refer to the manufacturer's user manual for complete instructions):
    - In the "Protocol Editor" tab of the software, click the "Cassette" folder and select "3% DF Marker Q3".
        - Select "Range" as the collection mode and specify the following parameters: **BP start = 158**, **BP end = 195**. The BP Range Flag should indicate "broad".
    - Click the "use of Internal Standards" button
    - Double-check that the "Ref Lane" values match the lane numbers
    - Use "Save As" to name and save the protocol
3. Add Nuclease-Free Water to bring the total volume of each sample to 30 µl. Alternatively, pool several samples in equimolar amounts to be run together in the same lane, making sure that the final total volume of pooled samples is 30 µl.

    NOTES:
    - We routinely pool up to 5 samples per lane—larger pools may be used but have not been tested.
    - It is best to load as much DNA as possible per sample, as only 3–10% of starting material will be recovered.
4. Add 10 µl of the Q3 DNA marker to each sample or pool. Mix thoroughly by vortexing and spin down briefly.
5. Load each 40-µl mixture of sample and marker solution on a lane of a 3% agarose gel cassette, prepared as described in the BluePippin user manual.
6. Run the program from Step 2.
7. After samples have been eluted, collect 40–45 µl from each elution chamber (containing the size-selected library) into a low-adhesion, nuclease-free tube.
8. Use 5 µl of the size-selected library for quantification on a Qubit Fluorometer with the Qubit dsDNA HS Assay Kit. Do not use less than 5 µl, as the library concentration can be very low depending on the input.
9. Use 1 µl of the size-selected library for profile evaluation on an Agilent 2100 Bioanalyzer with the Agilent High Sensitivity DNA Kit. See Figure 5 (on the next page) for examples of electropherogram results obtained following size selection with the BluePippin system. For examples of electropherogram results using Advanced Analytical's Fragment Analyzer and High Sensitivity Small Fragment Analysis Kit, see Figure 6.

STOPPING POINT: Store each library at –20°C until ready for sequencing.

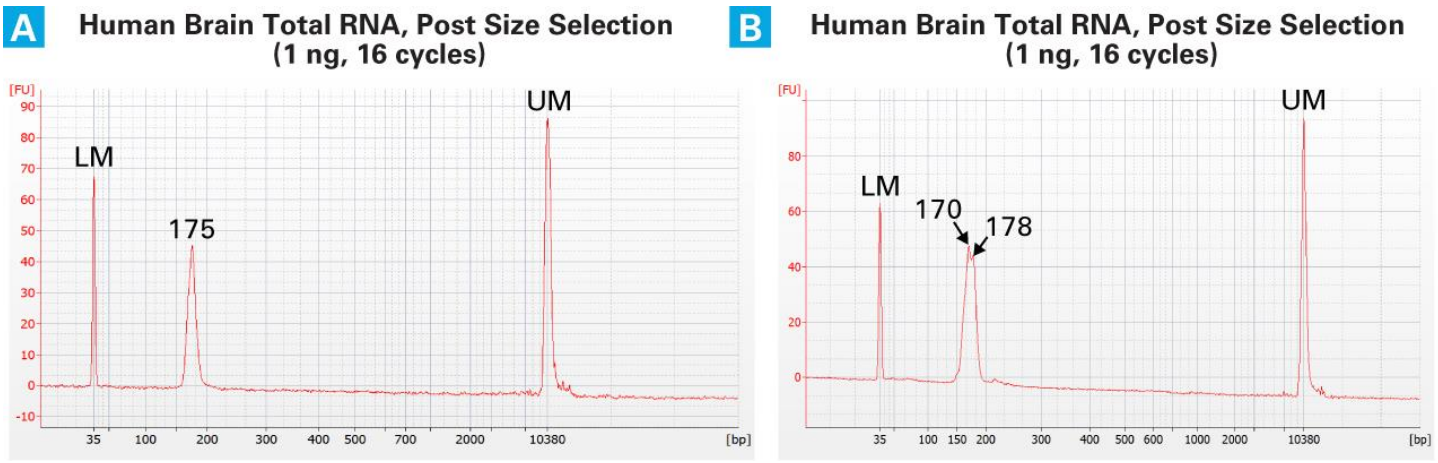

Figure 5. Example electropherogram results for smRNA-seq libraries after size selection with the BluePippin system. Libraries were generated using the SMARTer smRNA-Seq Kit for Illumina with the indicated inputs and cycling parameters, and following size selection with the BluePippin system, were analyzed on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit. Peaks labeled "LM" and "UM" correspond to DNA reference markers included in each analysis. When libraries are selected using the specified range (148–185 bp), profiles exhibit a narrow peak extending from 150 bp to 200 bp, and either a single summit (**Panel A**), or a double summit (**Panel B**) at ~175 bp due to minor variation in size-selection efficiency.

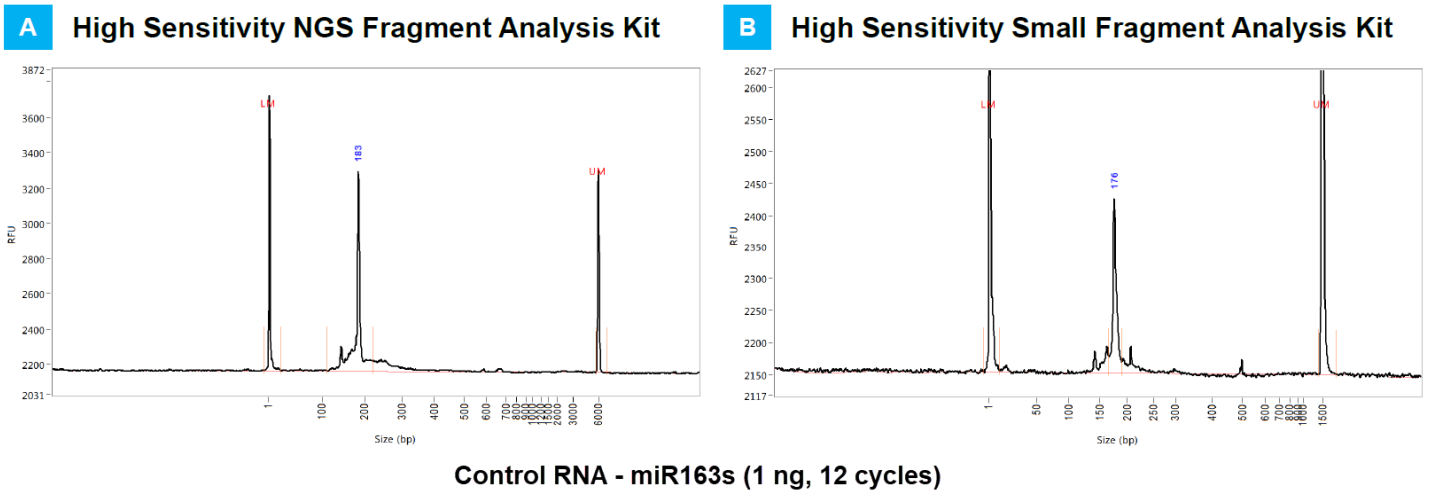

Figure 6. Example electropherogram results for smRNA-seq libraries profiled on a fragment analyzer. For profiling sequencing libraries on the Fragment Analyzer (Advanced Analytical), we only recommend the High Sensitivity Small Fragment Analysis Kit (Advanced Analytical, Cat. No. DNF-477), as it provides the most accurate size information for libraries generated with the SMARTer smRNA-Seq Kit for Illumina. **Panel A.** Typical result for library generated from Control RNA - miR163s, analyzed with the High Sensitivity NGS Fragment Analysis Kit. While the expected size of miR163s plus adapters is 175 bp, analysis with this kit typically yields a major peak that is ~10 bp larger than expected (in this example the peak is at 183 bp). **Panel B.** Analysis of the library from Panel A using the High Sensitivity Small Fragment Analysis Kit yields a major peak at 176 bp, which is much closer to the expected size of miR163s plus adapters (175 bp).

Appendix A: Sequencing Guidelines

A. General Recommendations

- Libraries generated with this kit are stranded, with Read 1 corresponding to the sense strand of the input RNA.
- Determine the SMARTer smRNA cDNA library concentration and average size by Bioanalyzer/Fragment Analyzer. If determining the concentration by Qubit Fluorometer or Qubit dsDNA HS Assay Kit (ng/µl), convert the value into the molar concentration (nM) based on the size of the library determined by the Bioanalyzer/ Fragment Analyzer profile. The following web tool is convenient for the conversion: http://www.molbiol.edu.ru/eng/scripts/01_07.html. Most Illumina sequencing library preparation protocols require cDNA libraries with a final concentration of 2 nM or 4 nM, depending on the sequencing platform. Lower concentrations can also be accommodated, depending on the instrument.
- When setting up the sample sheet for sequencing, treat the libraries as TruSeq HT libraries. **Do not select TruSeq Small RNA indexes!**
- If using NextSeq®/MiniSeq™, turn off the default automatic adapter trimming. The default automatic adapter trimming can cause your reads to change from the original sequence to a poly(N) sequence because of the default mask setting in BaseSpace. The minimum mask length is 35 cycles, and any trimmed reads shorter than 35 bases will become poly(N) reads. More information can be found on the Illumina website.
- Due to the low diversity A /T region (see additional details below), we do not recommend paired-end reads unless you use a Custom Read2 Primer
  - 5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTTTTTTTTTTTTT 3'
  You may still read As in the beginning of Read2. Follow Illumina recommendations for the use of a custom primer with your specific instrument. Some instruments, including NextSeq and MiniSeq, will also require the use of a Custom Index1 Primer when using a Custom Read2 Primer. However, the regular Index1 Primer included in Illumina sequencing cartridges is in fact compatible with the SMARTer smRNA-Seq Kit for Illumina. Consult Illumina for how to address this issue.
- Consult Table II below for SMARTer smRNA-seq library loading recommendations.

Table II. Recommended SMARTer smRNA-seq library loading concentrations

Instrument	MiSeq®	NextSeq/MiniSeq
Loading concentration	12–15 pM	1.6 pM (AMPure*) or 1.8–2.0 pM (BluePippin)

*Load 1.6 pM of library for AMPure bead-based size-selected smRNA libraries and 1.8–2.0 pM for BluePippin size-selected smRNA libraries.

- To alleviate the low diversity of the SMARTer smRNA-seq libraries, we suggest a PhiX spike-in, with amounts that vary depending on platform and size-selection method used. Consult Table III below for library and PhiX loading recommendations. For an explanation of the PhiX recommendations, please see Section B.2, below.

Table III. Recommended PhiX spike-in amounts

Instrument	MiSeq/HiSeq® 2500*	NextSeq/MiniSeq†	HiSeq 3000/4000, NovaSeq™
% PhiX spike-in	5% (MiSeq) or 10% (HiSeq)	30% (AMPure) or 20% (BluePippin)	Not recommended‡

*Please note that if using HiSeq 2500 High Output single-read/v4 chemistry, i7 D703 index "CGCTCATT" may present challenges during the multiplexing.

†Use 30% PhiX spike-in for AMPure bead-based size-selected smRNA libraries and 20% PhiX spike-in for BluePippin size-selected smRNA libraries.

‡ PhiX spike-in is not recommended due to a very low tolerance to low diversity, unless the libraries represent only a small fraction (5–10%) of the samples included in the sequencing lane.

B. Expected Performance During Sequencing

1. Size selection

In general, BluePippin size-selected SMARTer smRNA-seq kit libraries, or any libraries that show a very narrow library size, perform better during sequencing than libraries selected with the AMPure bead-based size-selection protocol.

2. Library diversity

SMARTer smRNA-seq kit libraries include low-diversity regions: overrepresentation of As at the 3′-end and GC-rich nucleotides (originating from the SMART smRNA oligo) at the 5′ end.

Due to the disproportionate amount of As introduced during library preparation, the expected Passing Filter (PF) rate for SMARTer smRNA-seq libraries sequenced on a MiSeq instrument is typically between 70% and 80% (up to 85% has been observed). On a MiSeq instrument we have found that inclusion of a PhiX spike-in at concentrations of 10–20% does not improve sequencing performance. However, it is still good practice to include ~5% PhiX in each sequencing run. We have found that aiming for a lower than recommended clustering does not improve the PF rate. In our hands, a slightly higher than recommended clustering (e.g. ~1,000–1,100 k/mm^2 on a MiSeq instrument (using v2 chemistry) typically yields the expected 12–15 million reads PF. The elevated frequency of As may also affect the error rate, sometimes leading to a lower percent of Q30 scores than are typically obtained with well-balanced libraries.

For a well-defined and high-quality artificial RNA sample like the miRXplore library (Figure 7), all RNA molecules are similarly sized (~21–23 nt), resulting in an overrepresentation of As starting around cycle 24. However, real-world samples (Figures 8 and 9) typically contain RNA molecules of diverse sizes, including some <15 nt, which results in overrepresentation of As starting around cycles 8–10.

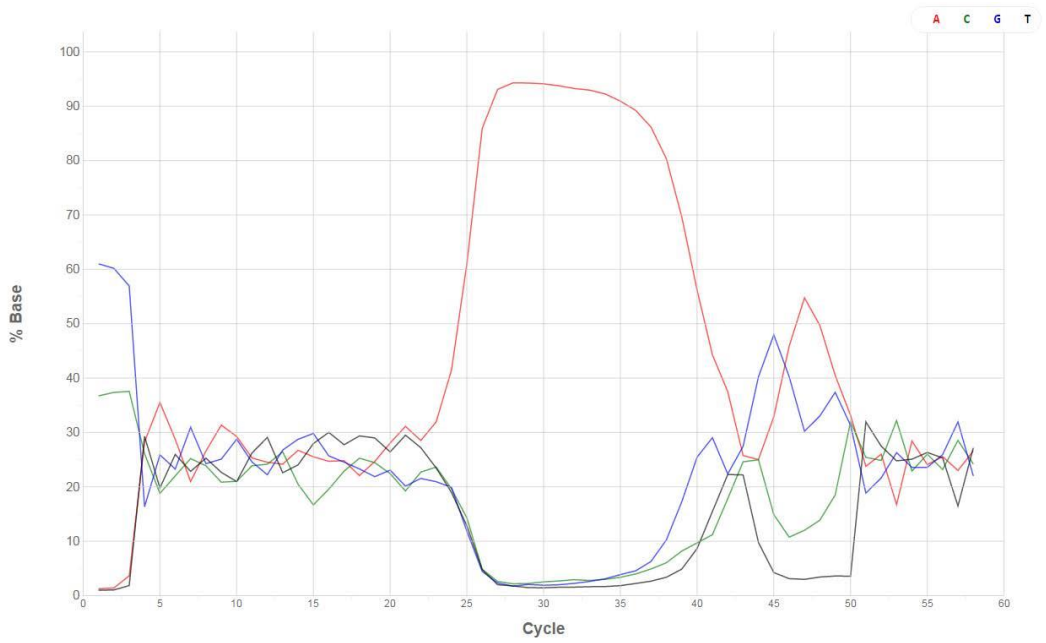

Figure 7. Percentage base calling from a typical MiSeq run performed on libraries generated from the miRXplore Universal Reference (pool of artificial miRNAs). Libraries were prepared without size selection. The proportions of A:T:G:C for each cycle are presented in graph form using software included on Illumina's BaseSpace platform. Cycles 1–50 correspond to Read1, while cycles 51–58 correspond to the library barcode. Note that overrepresentation of As begins around cycle 24 because input RNAs are all ~21–23 nt in length.

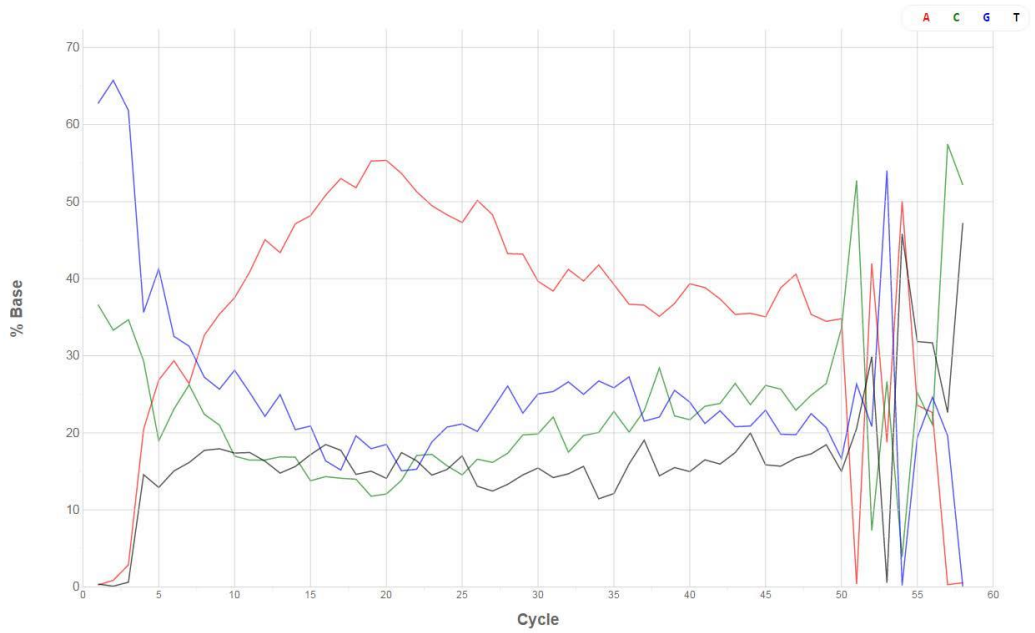

Figure 8. Percentage base calling from a typical MiSeq run following gel-free (AMPure beads) size selection. Size selection was performed as described in Section V.E. The proportions of A:T:G:C for each cycle are presented in graph form using software included on Illumina's BaseSpace platform. Cycles 1–50 correspond to Read1, while cycles 51–58 correspond to the library barcode. Note that overrepresentation of As begins around cycles 8–10 due to the inclusion of input RNAs <15 nt in length.

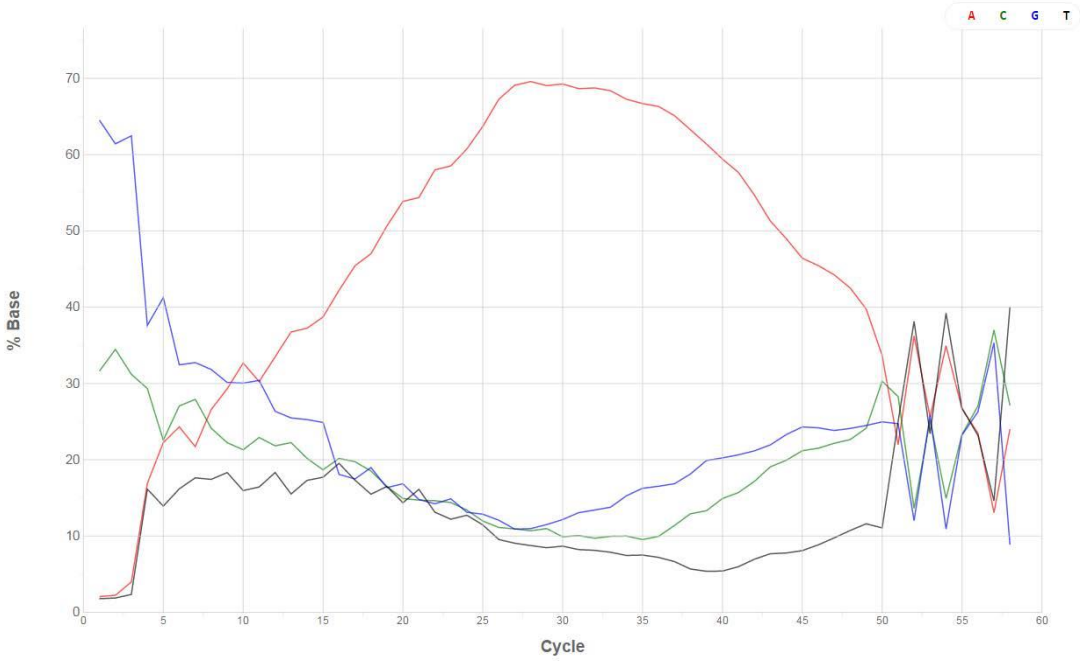

Figure 9. Percentage base calling from a typical MiSeq run following BluePippin size selection. Size selection was performed as described in Section V.F. The proportions of A:T:G:C for each cycle are presented in graph form using software included on Illumina's BaseSpace platform. Cycles 1–50 correspond to Read1, while cycles 51–58 correspond to the library barcode. Note that overrepresentation of As begins around cycles 8–10 due to the inclusion of input RNAs <15 nt in length.

C. Analysis of Sequencing Reads

1. Important information about read trimming

Proper trimming of the reads is important for efficient mapping. The first three nucleotides of the first sequencing read (Read 1) are derived from the template-switching oligo. **These three nucleotides must be trimmed prior to mapping.**

Please note that unambiguous determination of input RNA termini is not possible. See Figure 10 for a schematic of an example read. For the 3′ end, it is not possible to tell if RNA ends with an A due to the use of dT priming; the oligo(dT) primer cannot distinguish between naturally occurring and artificially added As at RNA termini. For the 5′ end, it is not possible to identify with 100% confidence the beginning of the 5′ sequence, because at low frequencies, template switching can add more than 3 nt to cDNA 5′ ends.

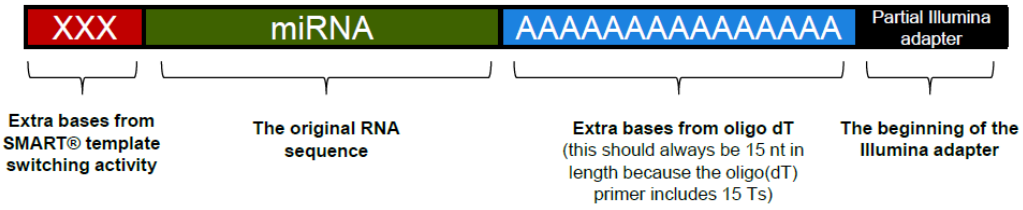

Figure 10. Example of a 50-bp read from a library generated with the SMARTer smRNA-seq kit.

Because reverse transcription involves dT-priming on artificial poly(A) tails, a good proportion of reads will include stretches of As (particularly when 20–25 bp-sized library inserts are selected with the BluePippin system). Figure 11 is a schematic representation of what the reads may look like, before and after trimming. It is common practice to discard reads shorter than 15 nt after trimming.

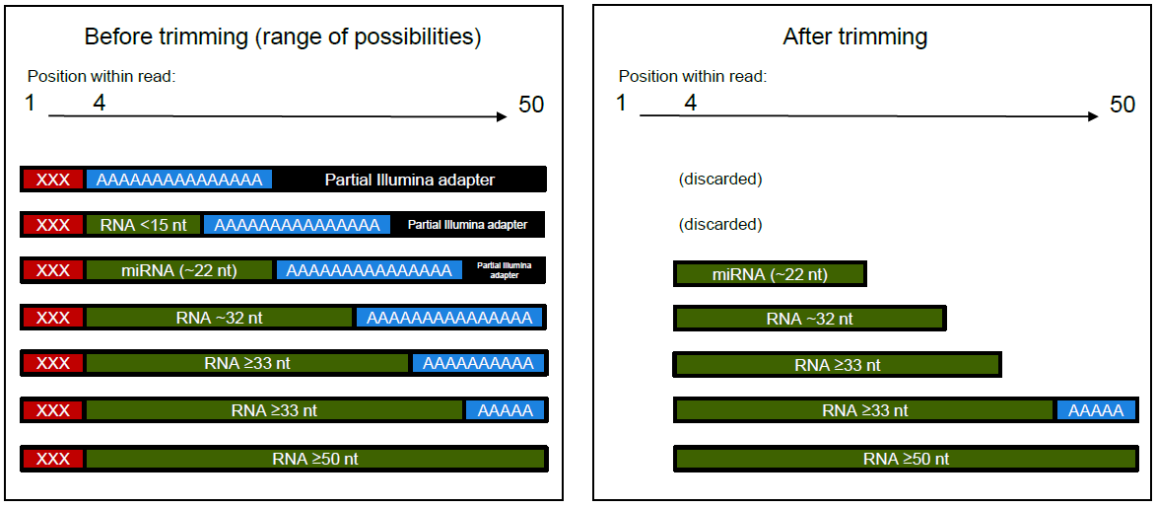

Figure 11. Example of possible reads obtained before and after trimming.

2. Recommended trimming tool

One of the trimming tools that can be used with a command-line interface is cutadapt (Martin 2011): https://cutadapt.readthedocs.org/en/stable/index.html.

The following command works well for the SMARTer smRNA-seq kit:

```
cutadapt -m 15 -u 3 -a AAAAAAAAAA input.fastq > output.fastq
```

- `-m 15`: after trimming, only reads 15 nt or longer will be retained, while shorter ones will be discarded. Use `-m 15` instead if a more stringent cutoff is desired.
- `-u 3`: trim the first 3 nt of all reads. This takes care of removing extra nt inserted due to the SMART template-switching mechanism
- `-a AAAAAAAAAA`: adapter sequence to be identified and removed. The adapter sequence and everything 3′ of the adapter (including any Illumina adapter sequence) will be removed. By default, cutadapt will accept an error rate of 10%.

V. References

Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* **17,** 10–12. (2011).

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