Takara Bio USA, Inc.

SMARTer® Stranded RNA-Seq Kit User Manual

Cat. Nos. 634836, 634837, 634838, 634839, 634861, 634862
(102319)

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I. Introduction

SMARTer cDNA Synthesis for the Illumina® Sequencing Platform

The **SMARTer Stranded RNA-Seq Kit** (Cat. Nos. 634836, 634837, 634838, 634839 & 634861) and the **SMARTer Stranded RNA-Seq Kit HT** (Cat. No. 634862) include the components needed to generate indexed cDNA libraries suitable for next-generation sequencing (NGS) on any Illumina platform, starting from as little as 100 pg of polyA-purified or ribosomal RNA-depleted RNA. The kits consist of the SMARTer Stranded RNA-Seq Components, SeqAmp™ DNA Polymerase, and the llumina Indexing Primer Set or the Indexing Primer Seq HT for Illumina (PCR primers for the amplification of indexed, paired-end Illumina-compatible sequencing libraries, which enable multiplexing of NGS library analysis).

The entire library construction protocol can be completed in less than 4 hr (Figure 1). The SMARTer Stranded RNA-Seq Kits utilizes our proprietary SMART® (**S**witching **M**echanism **A**t 5' end of **R**NA **T**emplate) technology, coupled with PCR amplification, to generate Illumina-compatible libraries without the need for enzymatic clean-up or adapter ligations. The directionality of the template-switching reaction preserves the strand orientation of the RNA, making it possible to obtain strand-specific sequencing data from the synthesized cDNA.

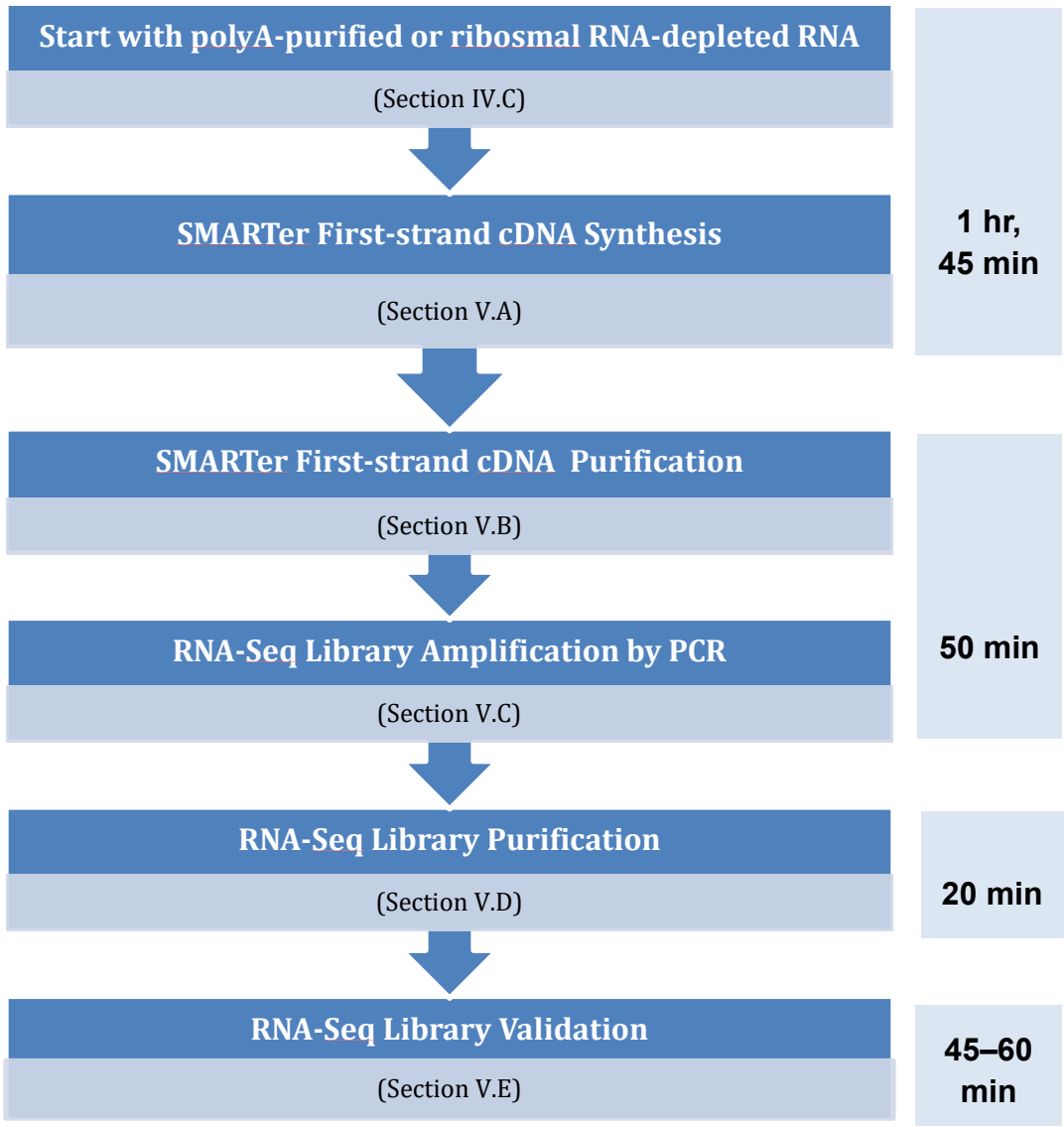

Figure 1. SMARTer Stranded RNA-Seq Kit protocol overview. You can complete this protocol in less than 4 hr.

The SMARTer Stranded RNA-Seq Kits start with sub-nanogram amounts of RNA. A modified N6 primer (the SMART Stranded N6 Primer) primes the first-strand synthesis reaction (Figure 2). For added simplicity, the RNA is chemically fragmented during denaturation.

NOTE: If your sample is degraded or of low quality (RNA Integrity Number; RIN <3), see Appendix A for a fragmentation-free protocol.

When the SMARTScribe™ Reverse Transcriptase reaches the 5’ end of the RNA fragment, the enzyme’s terminal transferase activity adds a few additional nucleotides to the 3’ end of the cDNA. The carefully-designed SMARTer Stranded Oligo base-pairs with the non-templated nucleotide stretch, creating an extended template to enable the SMARTScribe RT to continue replicating to the end of the oligonucleotide (Chenchik *et al.*, 1998). The resulting full-length, single-stranded (ss) cDNA contains the complete 5’ end of the mRNA, as well as sequences that are complementary to the SMARTer Stranded Oligo.

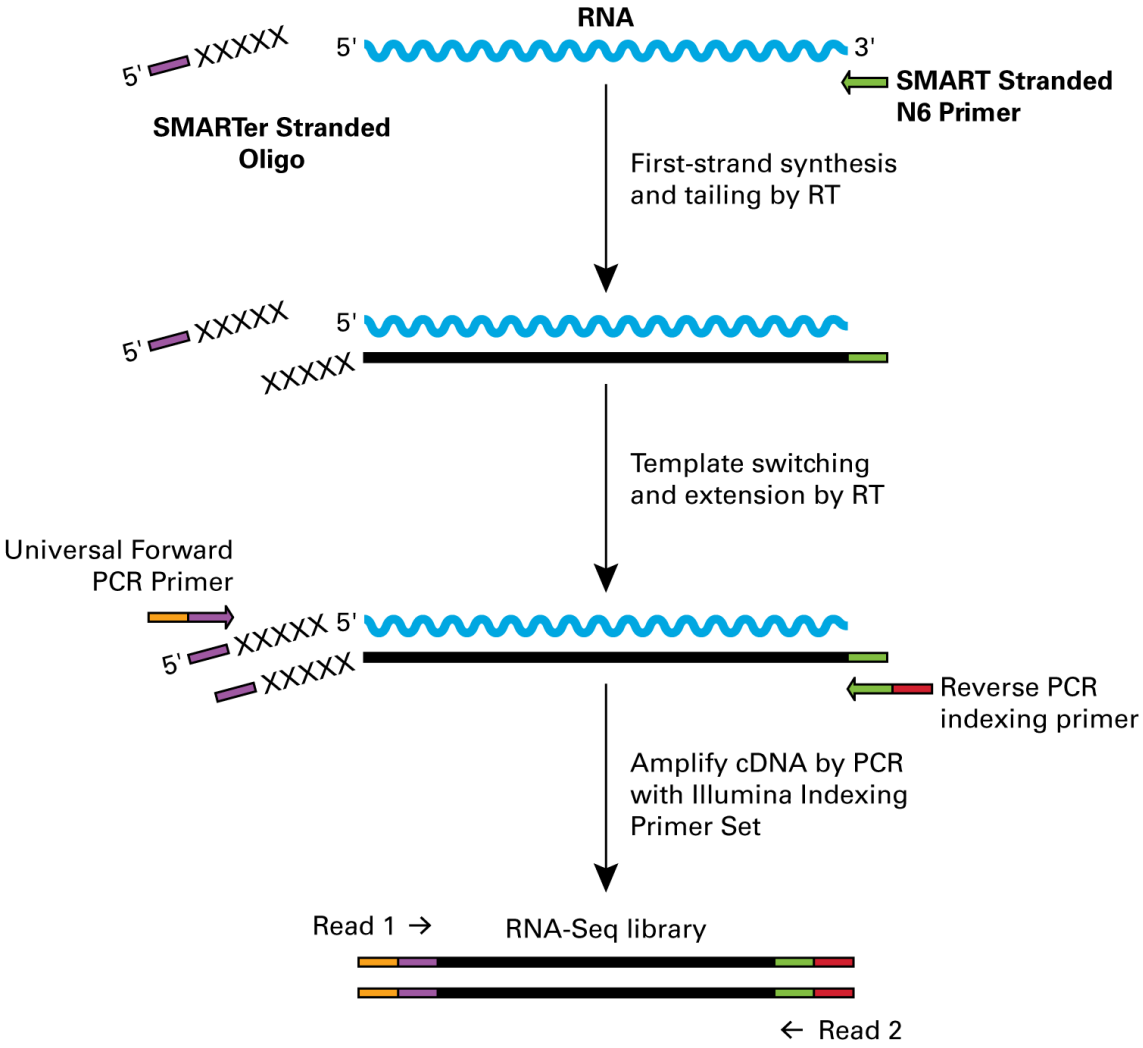

Figure 2. Flowchart of SMARTer Stranded RNA-Seq Kit library generation. The SMARTer Stranded RNA-Seq Kit HT follows the same method for library generation, but uses eight different Forward PCR indexing primers along with the twelve Reverse PCR indexing primers to generate 96 uniquely-indexed cDNA libraries.

II. List of Components

The SMARTer Stranded RNA-Seq Kit consists of the SMARTer Stranded RNA-Seq Components (not sold separately), the Illumina Indexing Primer Set (not sold separately), 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.

SMARTer Stranded RNA-Seq Kit	**634836** (12 rxns)	**634837** (24 rxns)	**634838** (48 rxns)	**634839** (96 rxns)
SeqAmp DNA Polymerase (Store at –20°C.)				
SeqAmp DNA Polymerase	50 µl	50 µl	2 x 50 µl	2 x 50 µl
SeqAmp PCR Buffer (2x)	1.25 ml	1.25 ml	2 x 1.25 ml	2 x 1.25 ml
SMARTer Stranded RNA-Seq Components (Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)				
Package 1 (Store at –70°C.)				
SMARTer Stranded Oligonucleotide (12 µM)*	24 µl	48 µl	96 µl	192 µl
Control Mouse Liver Total RNA (1 µg/µl)	5 µl	5 µl	5 µl	5 µl
Package 2 (Store at –20°C. Once thawed, store Stranded Elution Buffer at Room Temperature. Continue to store all other reagents at –20°C)				
SMARTer Stranded N6 Primer (12 µM)*	12 µl	24 µl	48 µl	96 µl
5X First-Strand Buffer (RNase-Free)	48 µl	96 µl	192 µl	384 µl
dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM)	24 µl	48 µl	96 µl	192 µl
Dithiothreitol (DTT; 100 mM)	12 µl	24 µl	48 µl	96 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	24 µl	48 µl	96 µl	192 µl
Nuclease-Free Water	1 ml	1 ml	2 x 1 ml	3 x 1 ml
RNase Inhibitor (40 U/µl)	55 µl	55 µl	55 µl	55 µl
Stranded Elution Buffer	240 µl	480 µl	960 µl	2 ml

* Takara Bio USA, Inc. proprietary sequences

Illumina Indexing Primer Set (Not sold separately. Store at –20°C.)				
Universal Forward PCR Primer (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 1 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 2 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 3 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 4 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 5 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 6 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 7 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 8 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 9 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 10 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 11 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl
Reverse PCR Primer Index 12 (12.5 µM)	12 µl	2 x 12 µl	48 µl	2 x 48 µl

Illumina Indexing Primer Set sequences:

Index	Barcode	Index	Barcode
1	ATCACG	**7**	CAGATC
2	CGATGT	**8**	ACTTGA
3	TTAGGC	**9**	GATCAG
4	TGACCA	**10**	TAGCTT
5	ACAGTG	**11**	GGCTAC
6	GCCAAT	**12**	CTTGTA

Note: The 6-nt indexes contained in the 12 reverse primers correspond to those in the Illumina TruSeq® DNA LT Sample Prep Kit (adapters AD001–AD012).

The SMARTer Stranded RNA-Seq Kit HT consists of the SMARTer Stranded RNA-Seq Components (not sold separately), the Indexing Primer Set HT for Illumina (not sold separately), 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.

SMARTer Stranded RNA-Seq Kit HT	**634862** (96 rxns)
SeqAmp DNA Polymerase (Store at –20°C.)	
SeqAmp DNA Polymerase	2 x 50 µl
SeqAmp PCR Buffer (2x)	2 x 1.25 ml
SMARTer Stranded RNA-Seq Components (Not sold separately.)	
Package 1 (Store at –70°C.)	
SMARTer Stranded Oligonucleotide (12 µM)*	192 µl
Control Mouse Liver Total RNA (1 µg/µl)	5 µl
Package 2 (Store at –20°C. Once thawed, store Stranded Elution Buffer at Room Temperature. Continue to store all other reagents at –20°C)	
SMARTer Stranded N6 Primer (12 µM)*	96 µl
5X First-Strand Buffer (RNase-Free)	384 µl
dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10 mM)	192 µl
Dithiothreitol (DTT; 100 mM)	96 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	192 µl
Nuclease-Free Water	3 x 1 ml
RNase Inhibitor (40 U/µl)	55 µl
Stranded Elution Buffer	2 ml

* Takara Bio USA, Inc. proprietary sequences

Indexing Primer Set HT for Illumina (Not sold separately. Store at –20°C.)	
Forward PCR Primer HT Index 1 (F1; 12.5 µM)	15 µl
Forward PCR Primer HT Index 2 (F2; 12.5 µM)	15 µl
Forward PCR Primer HT Index 3 (F3; 12.5 µM)	15 µl
Forward PCR Primer HT Index 4 (F4; 12.5 µM)	15 µl
Forward PCR Primer HT Index 5 (F5; 12.5 µM)	15 µl
Forward PCR Primer HT Index 6 (F6; 12.5 µM)	15 µl
Forward PCR Primer HT Index 7 (F7; 12.5 µM)	15 µl
Forward PCR Primer HT Index 8 (F8; 12.5 µM)	15 µl
Reverse PCR Primer HT Index 1 (R1; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 2 (R2; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 3 (R3; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 4 (R4; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 5 (R5; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 6 (R6; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 7 (R7; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 8 (R8; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 9 (R9; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 10 (R10; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 11 (R11; 12.5 µM)	12 µl
Reverse PCR Primer HT Index 12 (R12; 12.5 µM)	12 µl

Indexing Primer Set HT for Illumina sequences:

i5 Index (tube label)	**Barcode**	**i7 Index (tube label)**	**Barcode**
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

Notes:
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 correspond to Illumina TruSeq HT indexes D501–D508; R1–R12 correspond to Illumina TruSeq HT indexes D701–D712.

III. Additional Materials Required

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

- Single channel pipette: 10 µl, 20 µl and 200 µl, one each
- Eight channel pipette: 10 µl (for the SMARTer Stranded RNA-Seq Kit HT only)
- Twelve channel pipette: 10 µl (for the SMARTer Stranded RNA-Seq Kit HT only)
- Filter pipette tips: 10 µl, 20 µl and 200 µl, one box each (and two additional boxes of 10 µl tips for the SMARTer Stranded RNA-Seq Kit HT)
- One QuickSpin minicentrifuge for 0.2 ml tubes

For PCR Amplification & Validation:

- One dedicated PCR thermal cycler used only for first-strand synthesis
- High Sensitivity DNA Kit (Agilent, Cat No. 5067-4626)
- Nuclease-free thin-wall PCR tubes (0.2 ml; USA Scientific, Cat. No.1402-4700)
- Nuclease-free nonsticky 1.5 ml tubes (USA Scientific, Cat. No. 1415-2600)

For SPRI Bead Purification:

- Agencourt AMPure PCR Purification Kit (5 ml Beckman Coulter, Part No. A63880; 60 ml Beckman Coulter, Part No. A63881).
- Magnetic separation device for 0.2 ml tubes (see Appendix B)

  NOTE: We strongly recommend using separate magnets for purification of first-strand cDNA (Section V.B) and purification of the RNA-seq library (Section V.D) to prevent cross contamination.
- 80% ethanol

IV. General Considerations

A. Recommendations for Preventing Contamination

1. **Before you set up the experiment, it is advisable to have two 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.A) and purification of first-strand cDNA (Section V.B).
   - **A second work station located in the general laboratory** where you will perform PCR to amplify the RNA-seq library (Section V.C), purify the RNA-seq library (Section V.D), and measure its concentration (Section V.E).

   IMPORTANT: The PCR work station must be located in a clean room with positive air flow, as contamination may occur very easily. Once contamination occurs it can be difficult to remove.

2. **Guidelines for clean room operation:**

   - Only move materials/supplies from the clean room to the general lab, NOT the other way around. Don't share any equipment/reagents between the clean room and the general lab.
   - Use a separate PCR machine inside the PCR workstation for 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 quality of your starting RNA sample. Prior to cDNA synthesis, please make sure that your RNA is free of contaminants.**
- The assay is very sensitive to variations in pipette volume, etc. Please make sure that all your pipettes are calibrated for reliable delivery, and that nothing is attached to the outside of the tips.
- All lab supplies related to SMARTer cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for SMARTer cDNA synthesis need to 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 mixture up and down.
- Do not increase (or decrease) the amount of enzyme added or the concentration of DNA in the reactions. The amounts and concentrations have been carefully optimized for the SMARTer amplification reagents and protocol.
- If you are using this protocol for the first time we strongly recommend that you perform negative and positive control reactions to verify that kit components are working properly. The positive control should be at the same concentration as the sample RNA used in your experiment, and the negative control should have the same number of cycles as all of your samples. It can also be useful to perform an additional control reaction starting with your method of rRNA depletion or poly(A) enrichment, and using the Control Mouse Liver Total RNA processed along with your test samples.

C. Sample Preparation

The sequence complexity and the average length of SMARTer cDNA is noticeably dependent on the quality of starting RNA material.

- There are several commercially available products that enable purification of total RNA preparations from extremely small samples [e.g., we offer the **NucleoSpin RNA XS Kit** (Cat. No. 740902.10) for purification of RNA from 10^2 cells].
- When choosing a purification method (kit), ensure that it is appropriate for your sample amount.
- Input RNA should be free from genomic or carrier DNA, and free of contaminants that would interfere with the oligo-RNA template annealing or would inhibit the reverse transcriptase reaction.

D. Sample Requirements

Ribosomal RNA (rRNA) depletion

We strongly recommend removing rRNA from the sample prior to cDNA synthesis using the SMARTer Stranded RNA-Seq Kit. For 10–100 ng of input total RNA, we recommend the RiboGone™ - Mammalian (Cat. Nos. 634846 and 634847) rRNA depletion kit. This kit specifically and efficiently degrades *5S*, *5.8S*, *18S*, and *28S* nuclear rRNA and *12S* mtRNA from mouse, rat, and human total RNA samples.

Input RNA length

- The SMARTer Stranded RNA-Seq Kit is compatible with full-length or compromised RNA. Fragmentation times for RNA of intermediate lengths are provided in Section V.A. An alternative protocol for degraded samples is available in Appendix A.
- After RNA extraction, if your sample amount is not limiting, we recommend evaluating total RNA quality using the **Agilent RNA 6000 Pico Kit** (Cat. No. 5067-1513).

Input RNA purity and quantity

The input amounts indicated in this kit are for poly(A)-purified, rRNA-depleted, or otherwise-purified RNA samples.

- **Purity of input RNA:** Input RNA should be free from genomic or carrier DNA, and contaminants that would interfere with oligo annealing or reverse transcriptase reactions.

  IMPORTANT: Purified total RNA should be resuspended in nuclease-free water, **not in TE or other buffers containing EDTA.** Chelation of divalent cations by EDTA will interfere with RNA fragmentation.

- **Volume and amount of input RNA:** This kit accommodates up to 8 µl of input RNA. This protocol has been optimized for cDNA synthesis starting from 0.1 ng of RNA. However, if your RNA sample is not limiting, we recommend that you start with more than 1 ng of RNA.

E. Sequencing Analysis Guidelines

- Read 1 is derived from the sense strand of the input RNA. If you are performing paired-end sequencing, Read 2 corresponds to the antisense strand.

V. Protocols

A. PROTOCOL: First-Strand cDNA Synthesis

During this step, RNA is fragmented and converted to single-stranded (ss) cDNA that contains sequences complementary to the SMARTer Stranded Oligo.

IMPORTANT:

- This protocol was designed to fragment RNA for a final mean library insert size of ~180 nt. The first two steps will simultaneously fragment and prime the RNA for cDNA synthesis. See instructions below on RNA shearing conditions, depending on the RIN or its equivalent.
- For some RNA samples or sequencing applications, it may be appropriate to titrate the fragmentation time to achieve optimal yield and library size.
- **When working with degraded RNA samples with RIN <3,** use the First-Strand cDNA Synthesis Protocol for Degraded Samples in Appendix A instead, because additional fragmentation is unnecessary, and will result in lower library yields.

1. Mix the following components on ice:

1–8 µl	RNA (0.1–100 ng)
1 µl	SMART Stranded N6 Primer (12 µM)
4 µl	5X First-Strand Buffer (RNase-Free)
0–7 µl	Nuclease-Free Water
13 µl	Total volume

2. Incubate the tubes at 94°C in a preheated, hot-lid thermal cycler for an appropriate time depending on the RIN of the RNA sample, then place the samples on ice for 2 min.

RIN	Time
>7	5 min
4–7	4 min
3	3 min

NOTE: Steps 4–6 should not be delayed after completing Step 2, since they are critical for first-strand cDNA synthesis. You can prepare your master mix, except for SMARTScribe Reverse Transcriptase, (for Step 3), while your tubes are incubating (Step 2) in order to jump start the cDNA synthesis.

3. Prepare enough Master Mix for all reactions, plus 10%, by combining the following reagents in the order shown on ice.

0.5 µl	DTT (100 mM)
0.5 µl	RNase Inhibitor
2 µl	dNTP Mix (10 mM)
2 µl	SMARTer Stranded Oligo (12 µM)
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl)*
7 µl	Total volume per reaction

* Add the reverse transcriptase to the master mix immediately prior to use. Mix well by gently vortexing and spin the tubes briefly in a microcentrifuge.

4. Add 7 µl of the Master Mix to each reaction tube from Step 2. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
5. Incubate the tubes in a preheated thermal cycler at 42°C for 90 min.

6. Terminate the reaction by heating the tubes at 70°C for 10 min, then leave them in the thermal cycler at 4°C until the next step (Section V.B.1).

NOTE: If desired, you may stop here and store the reaction tubes at 4°C overnight before proceeding to Section V.B.

B. PROTOCOL: Purification of First-Strand cDNA using SPRI AMPure Beads

The first-strand cDNA selectively binds to SPRI beads leaving contaminants in solution which is removed by a magnetic separation. The beads are then directly used for RNA-seq library amplification.

NOTES:

- Aliquot SPRI beads and allow them to come to room temperature for 30 min prior to use.
- Before use, beads should be brought to room temperature and mixed well to disperse.
- You will need a Magnetic Separation Device for 0.2 ml tubes. If you do not have such a device, we recommend constructing one using the instructions in Appendix B.
- Clean-up of SMARTer reactions must be performed using Ampure XP beads. **Spin columns do not adequately remove adapter-dimers from the reactions and will result in experimental failure!**

To purify the SMART cDNA from unincorporated nucleotides and small (<100 bp) cDNA fragments, follow this procedure for each reaction tube:

1. Add 20 µl of SPRI AMPure beads to each sample using a 20 µl pipetter.
    - Mix by vortexing for 5 sec or by pipetting the entire volume up and down at least 10 times.
    - The beads are viscous; suck the entire volume up, and push it out slowly.
2. Incubate at room temperature for 8 min to let DNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid from the walls of the tube. Place the sample tubes on the Magnetic Separation Device for 5 min or longer, until the solution is completely clear.
4. While the tubes are sitting on the magnetic stand, pipette out the supernatant.
5. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads, in order to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant.
6. Repeat Step 5.
7. Perform a brief spin of the tubes (~2,000 g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes rest open at room temperature for ~3–5 min until the pellet appears dry. You may see a tiny crack in the pellet. If using more than 10 ng of input RNA, proceed immediately to Section V.C.

NOTE: Under- or over-drying the beads will reduce PCR efficiency, resulting in lower yields.

9. If using less than 10 ng of input RNA or if performing more than 12 PCR cycles, elute the cDNA in 20 µl of Nuclease-Free Water as described below (Steps 9a–d), this ensures complete removal of adapter primers. Then **repeat Steps 1–8 above** (this ensures complete removal of adapter primers) before proceeding to Section V.C.

    a. Add 20 µl Nuclease-Free Water to the pellet from Step 8.
    b. Thoroughly resuspend the beads and allow to rehydrate for 2 min.
    c. Briefly spin the sample tubes to collect the liquid from the walls of the tube. Place the sample tubes on the Magnetic Separation Device for 1 min or longer, until the solution is completely clear.
    d. Transfer the supernatant to a fresh 0.2-ml tube. Repeat Steps 1–8 above before proceeding to section V.C.

C. PROTOCOL: RNA-Seq Library Amplification by PCR

The purified first-strand cDNA is amplified into RNA-seq libraries using SeqAmp DNA Polymerase, the Universal Forward PCR Primer, and the Reverse PCR Primers from the Illumina Indexing Primer Set.

IMPORTANT: Optimal parameters may vary with different templates and thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles.

Table I. Cycling Guidelines Based on the Input Amount of rRNA-depleted or poly(A)-enriched RNA.

Amount of Input RNA (ng)	Typical Number of PCR Cycles
0.1	18
1	16
10	12
100	9

NOTE: If you are using the SMARTer Stranded RNA-Seq Kit HT (Cat. No. 634862) use the alternative protocol below.

1. Prepare a PCR Master Mix for all reactions. Separate master mixes should be prepared for different library indexes. Combine the following reagents in the order shown, then mix well and spin the tube briefly in a microcentrifuge:

25 µl	2X SeqAmp PCR Buffer
1 µl	Universal Forward PCR Primer (12.5 µM)
1 ul	Reverse PCR Primer* (12.5 µM)
1 µl	SeqAmp DNA Polymerase
22 µl	Nuclease-Free Water
50 µl	Total volume per reaction

*Your selection of the Reverse PCR Primer will determine which of the 12 indexing sequences in the Illumina Indexing Primer Set will be associated with your library.

2. Add 50 µl of PCR Master Mix to each tube containing DNA bound to the beads from Section V.B., Step 8. Mix well, making sure that the beads are uniformly resuspended.

3. Place the tube in a preheated thermal cycler with a heated lid. Start thermal cycling using the following program:

94°C		1 min
X[a] cycles:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
4°C		forever

[a] The number of cycles depends on the amount of input RNA. See Table 1 (above) for guidelines.

For the SMARTer Stranded RNA-Seq Kit HT: The purified first-strand cDNA is amplified into 96 uniquely-indexed RNA-seq libraries using SeqAmp DNA Polymerase, and the Forward and Reverse PCR Primers from the Indexing Primer Set HT for Illumina.

1. Prepare a PCR Master Mix for all reactions. Combine the following reagents in the order shown, then mix well and spin the tube briefly in a microcentrifuge:

25 µl	2X SeqAmp PCR Buffer
1 µl	SeqAmp DNA Polymerase
22 µl	Nuclease-Free Water
48 µl	Total volume per reaction

2. Add 48 µl of PCR Master Mix to each well containing DNA bound to the beads from Section V.B., Step 8. Mix well, making sure that the beads are uniformly resuspended.
3. Using an 8-channel pipette, add 1 µl of each Forward PCR Primer HT to the wells of the 96-well plate containing DNA bound to beads and the Master Mix being careful not to cross-contaminate each well. Each well in a row will have the same Forward PCR Primer HT Index.
4. Using a 12-channel pipette, add 1 µl of each Reverse PCR Primer HT to the wells of the 96-well plate being careful not to cross-contaminate each well. Each well in a column will have the same Reverse PCR Primer HT Index.
5. Place the tube in a preheated thermal cycler with a heated lid. Start thermal cycling using the following program:

94°C		1 min
X[a] cycles:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
4°C		forever

[a] The number of cycles depends on the amount of input RNA. See Table 1 (above) for guidelines.

D. PROTOCOL: Purification of the RNA-Seq Library using SPRI AMPure Beads

The amplified RNA-seq library is purified by immobilizing it onto SPRI beads. The beads are then washed with 80% ethanol and eluted in Stranded Elution Buffer.

1. Add 50 µl of SPRI AMPure beads to each sample.
    - Mix by vortexing 5 sec or by pipetting the entire volume up and down at least 10 times.
    - The beads are viscous; suck the entire volume up, and push it out slowly.
2. Incubate at room temperature for 8 min to let the DNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid from the side of the wall. Place the sample tubes on the Magnetic Separation Device for 5 min or longer, until the solution is completely clear.
4. While the tubes are sitting on the magnetic stand, pipette out the supernatant.
5. Keep the tubes on the magnetic stand. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. DNA will remain bound to the beads during the washing process.
6. Repeat Step 5 one more time.
7. Perform a brief spin of the tubes (~2,000 g) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic stand for 30 sec, then remove all the remaining ethanol with a pipette.
8. Let the sample tubes rest open at room temperature for ~3–5 min until the pellet appears dry. You may see a tiny crack in the pellet.

    NOTE: Be sure to dry the pellet enough.
    - If you under-dry the pellet, ethanol will remain in the sample tubes. The ethanol will reduce your RNA-seq library recovery rate and ultimately your yield. Allow the tubes to sit at room temperature until the pellet is dry.
    - If you over-dry the pellet, it will take longer than 2 min to rehydrate (Step V.D.9).
9. Once the beads are dried, add 20 µl of Stranded Elution Buffer to cover the beads. Remove the tubes from the magnetic stand and mix thoroughly to resuspend the beads.

    NOTE: Be sure the beads are completely resuspended. The beads can sometimes stick to the sides of the tube. We recommend vortexing or directly pipetting the beads up and down to ensure complete dispersion.
10. Incubate at room temperature for 2 min to rehydrate.
11. Briefly spin the sample tubes to collect the liquid from the side of the wall. Place the sample tubes on the Magnetic Separation Device for 1 min or longer, until the solution is completely clear.
12. Transfer the clear supernatant containing the purified RNA-seq library from each tube to a nuclease-free nonsticky tube.

E. PROTOCOL: Validation Using the Agilent 2100 Bioanalyzer

1. Dilute 1 µl of the amplified RNA-seq library with 3 µl Stranded Elution Buffer.
2. Use 1 µl of the diluted sample for validation using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Chip from Agilent's **High Sensitivity DNA Kit** (Cat. No. 5067-4626). See the user manual for the Agilent High Sensitivity DNA Kit for instructions.
3. Compare the results for your samples and controls (if performed) to determine whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield no product in the negative control (Figure 3, Panel B), and a distinct peak spanning 150–1,000 bp, peaked at ~300 bp for the positive control RNA sample (Figure 3, Panel A), yielding >7.5 nM RNA-seq library (depending on the input and number of cycles).

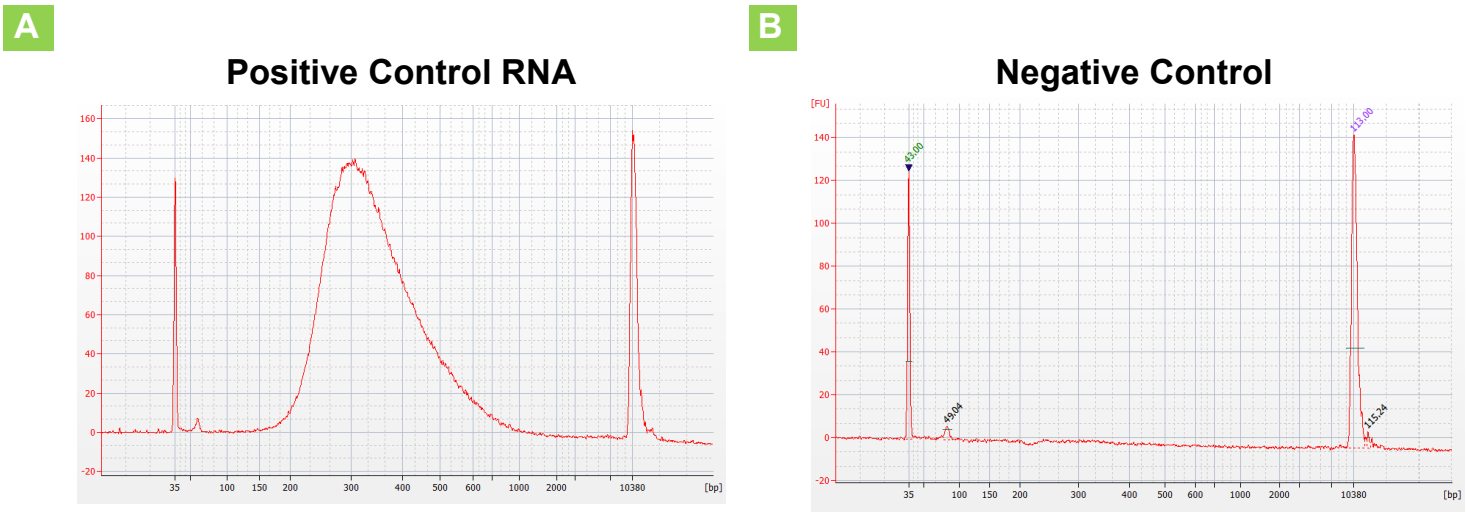

Figure 3. Electropherogram example results from Agilent 2100 Bioanalyzer.

VI. References

Chenchik, A. et al. (1998). In *RT-PCR Methods for Gene Cloning and Analysis*. (BioTechniques Books, MA), pp. 305-319.

Appendix A: First-Strand cDNA Synthesis Protocol for Degraded Samples

Our typical protocol for first-strand cDNA synthesis (Section V.A) includes simultaneous RNA fragmentation. If your RNA has a RIN <3 (or equivalent), use this alternative protocol for first-strand cDNA synthesis.

1. Mix the following components on ice:

1–8 µl	RNA (0.1–100 ng)
1 µl	SMART Stranded N6 Primer (12 µM)
0–7 µl	Nuclease-Free Water
9 µl	Total volume

2. Incubate the tubes at 72°C in a preheated, hot-lid thermal cycler for 3 min, then put the samples on ice for 2 min.

NOTE: Steps 4–6 should not be delayed after completing Step 2, since they are critical for first-strand cDNA synthesis. You can prepare your master mix, except for SMARTScribe Reverse Transcriptase, (for Step 3), while your tubes are incubating (Step 2) in order to jump start the cDNA synthesis.

3. Prepare enough Master Mix for all reactions, **plus 10%, by combining the following reagents in the order shown on ice.**

4 µl	5X First Strand Buffer
0.5 µl	DTT (100 mM)
0.5 µl	RNase Inhibitor
2 µl	dNTP Mix (10 mM)
2 µl	SMARTer Stranded Oligo (12 µM)
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl)*
11 µl	Total volume per reaction

* Add the reverse transcriptase to the master mix immediately prior to use. Mix well by gently vortexing and spin the tubes briefly in a microcentrifuge.

4. Add 11 µl of the Master Mix to each reaction tube from Step 2. Mix the contents of the tubes by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
5. Incubate the tubes in a preheated thermal cycler at 42°C for 90 minutes.
6. Terminate the reaction by heating the tubes at 70°C for 10 min, then leave them in the thermal cycler at 4°C until the next step (Section V.B.1).

NOTE: If desired, you may stop here and store the reaction tubes at 4°C overnight before proceeding to Section V.B.

Appendix B: Constructing a Magnetic Separation Device for 0.2 ml PCR Tubes

It can be difficult to find magnetic separation devices designed specifically to handle 0.2 ml PCR strip tubes. Often, one can place strip tubes in a column/row of a magnetic separation device designed for use with 96-well plates. Alternatively, one can construct a suitable low-cost separation device from common laboratory materials.

Example 1: Using a 96-well separation device with strip tubes

As seen in Figure 4, you may place the tubes in the top part of an inverted P20 or P200 Rainin Tip Holder which is taped to a MagnaBlot II Magnetic Separator (Promega Part No. V8351)

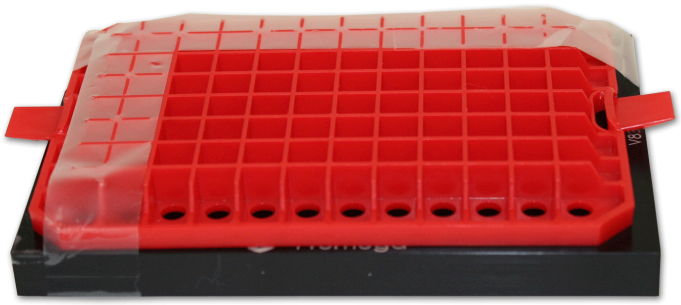

Figure 4. Setup for positioning 0.2 ml tubes containing first-strand cDNA on a MagnaBlot II Magnetic Separator.

Example 2: Building a 0.2ml tube magnetic separation device from rare earth bar magnets and a tip rack

As seen in Figure 5, neodymium bar magnets are taped together on the underside of the top section of a 20 µl tip rack (Panel A), and the rack is inverted so the tubes can be inserted (Panel B).

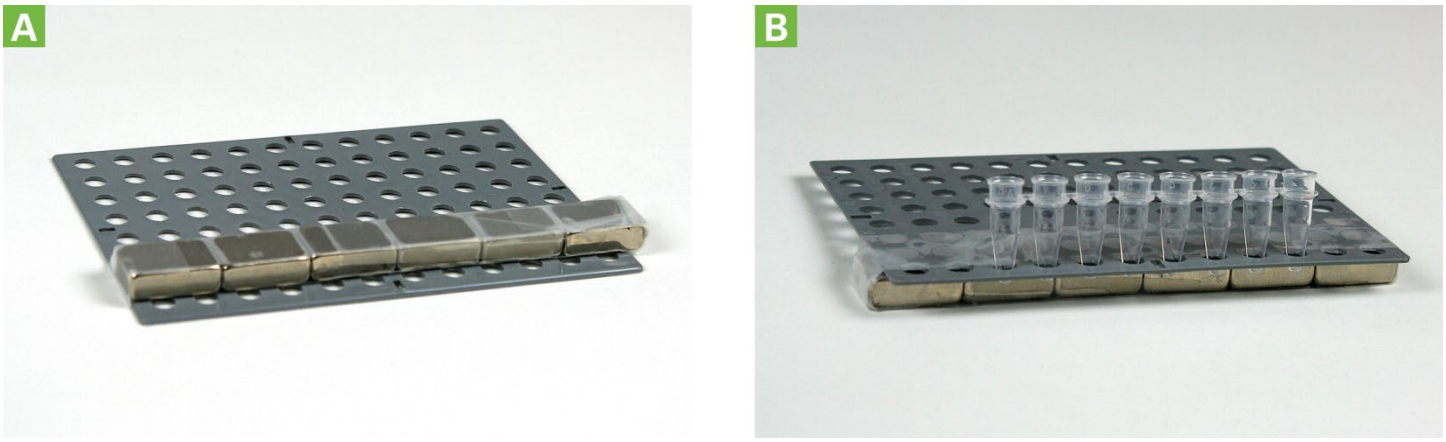

Figure 5. Constructing a magnetic separation device for 0.2 ml tubes from rare earth magnets. **Panel A** shows six 0.75" x 0.25" x 0.5" neodymium bar magnets (Applied Magnets Model # NB026) taped together on the underside of the top section of a 20 µl tip rack. **Panel B** shows the upright rack, into which an 8-tube strip of 0.2 ml tubes has been inserted.

Appendix C: Sequencing Guidelines

Pooling Recommendations

Following library validation by Qubit and Bioanalyzer, prepare the desired library pools for the sequencing run. Prior to pooling, libraries must be carefully quantified. By combining the quantification obtained with the Qubit with the library size as determined by the Bioanalyzer, the concentration in ng/µl can be converted to nM. The following web tool is convenient for this conversion: http://www.molbiol.edu.ru/eng/scripts/01_07.html. Alternatively, libraries can be quantified by qPCR using the Library Quantification Kit (Cat. No. 638324).

Most Illumina sequencing library preparation protocols require 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.

Prepare a pool of 2 nM (or 4 nM) as follows:

1. Dilute each library to 2 nM (or 4 nM) in nuclease-free water. To avoid pipetting errors, use at least 2 µl of each original library for dilution.
2. Pool the diluted libraries by combining an equal amount of each library in a low-bind, 1.5-ml tube. Mix by vortexing at low speed or by pipetting up and down. Use at least 2 µl of each diluted library to avoid pipetting errors.
3. Depending on the Illumina sequencing library preparation protocol, use a 5-µl aliquot (for the 10 nM concentration) or a 10-µl aliquot (for the 2 nM concentration) of the pooled libraries. Follow the library denaturation protocol according to the latest edition of your Illumina sequencing instrument's user guide.

If you are planning to include a PhiX control spike-in, make sure to combine the aliquot with an appropriate amount of the PhiX control.

- See our recommendations below (Table II) regarding the amount of PhiX control to include with SMARTer stranded libraries
- Follow Illumina guidelines on how to denature, dilute, and combine a PhiX control library with your own pool of libraries

PhiX Control Spike-In Recommendations

Illumina cluster detection algorithms are optimized around a balanced representation of A, T, G, and C nucleotides. SMARTer stranded libraries can have a lower than average pass filter rate, due to the low complexity observed in the first three cycles. To alleviate this issue, libraries should be combined with a PhiX Control v3 spike-in (Illumina, Cat. No. FC-110-3001). Make sure to use a fresh, reliable stock of the PhiX control library. Spike in a PhiX control at about 10% or more of the total library pool, depending on the instrument (see Table II).

Table II. PhiX Control Spike-In Guidelines for Various Illumina Sequencing Instruments.

Sequencing instrument	PhiX (%)
MiSeq	5–10 (optional*)
HiSeq 1500/2000/2500	10
HiSeq 3000/4000	20
NextSeq/MiniSeq	20

*A typical MiSeq® run generates a high passing filter rate. While the inclusion of PhiX spike-in can be beneficial, it does not significantly improve overall performance.

Extra Precautions When Using NextSeq® and MiniSeq™ Instruments

- Both systems use automatic adapter trimming by default. This can unexpectedly shorten your reads and cause the 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. This can be problematic if the RNA input was very fragmented. Therefore, we strongly recommend that you turn off automatic adapter trimming by creating a custom library prep kit program without adapter trimming. More information can be found on the Illumina website.
- Due to the algorithm's sensitivity to low complexity (found in the first three nucleotides of Read 1), NextSeq and MiniSeq runs without PhiX spike-ins may result in low-quality sequencing reads and incorrect base calling. Therefore, we strongly recommend adding 20% of PhiX spike-in when using current NextSeq 500/550 v2 or MiniSeq sequencing reagent kits.

Sequencing Analysis Guidelines

IMPORTANT: 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.

- Read 1 is derived from the sense strand of the input RNA
- If you are performing paired-end sequencing, Read 2 will correspond to the antisense strand

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