Takara Bio USA, Inc.

SMARTer® Stranded Total RNA-Seq Kit - Pico Input Mammalian User Manual

Cat. Nos. 635005, 635006, 635007
(112216)

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Table of Contents

Table of Figures

Table of Tables

I. Introduction

SMARTer cDNA Synthesis for Illumina® Sequencing Platforms

The **SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian** (Cat. Nos. 635005, 635006 & 635007) includes all components needed to generate indexed cDNA libraries suitable for next-generation sequencing (NGS) on any Illumina platform, with recommended input ranging from 250 pg to 10 ng of total mammalian RNA.

The entire library construction protocol, starting with total RNA, can be completed in about 5 hours (Figure 1). This kit incorporates SMART® (**S**witching **M**echanism **A**t 5' end of **R**NA **T**emplate) technology (Chenchik et al. 1998) and locked nucleic acid (LNA) technology, included as part of the template-switching oligo (TSO). The inclusion of LNA technology into the TSO (LNA-TSO) stabilizes the interaction between the TSO and non-templated nucleotides added by the reverse transcriptase (Picelli et al. 2013). PCR amplification generates Illumina-compatible libraries without the need for adapter ligation. The directionality of the template-switching reaction preserves the strand orientation of the original RNA, making it possible to obtain strand-specific sequencing data from the synthesized cDNA. See Figure 2 for an illustration of the cDNA library construction process.

The SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian is compatible with picogram-inputs of total RNA from high-quality or partially degraded samples (250 pg–10 ng) and FFPE samples (5–10 ng). In order to generate library inserts of an appropriate size for compatibility with Illumina sequencing, high-quality or partially degraded RNA samples are processed to the suitable fragment size prior to cDNA synthesis. For highly degraded, low-quality starting material, the RNA fragmentation step should be skipped. Please refer to the protocol Option 2 (Section V.A.) for guidance on how to proceed if you are skipping the fragmentation step.

Ribosomal RNA (rRNA) comprises a significant proportion (~90%) of total RNA samples. Depleting these abundant transcripts from total RNA samples prior to generating libraries provides benefits by lowering sequencing costs and improving mapping statistics. However, with very low input amounts, initial rRNA depletion from total RNA is not very effective and often leaves an insufficient amount of material for preparation of a good library. The workflow used in this kit takes advantage of a novel technology allowing removal of ribosomal cDNA (cDNA fragments originating from rRNA molecules) after cDNA synthesis using probes specific to mammalian rRNA. These R-Probes hybridize to ribosomal RNA and mitochondrial rRNA sequences; however, the mitochondrial sequences are derived from the human mitochondrial genome and are therefore strictly human specific. The rRNA depletion method used in this kit makes it especially well-suited for working with very small quantities of total RNA.

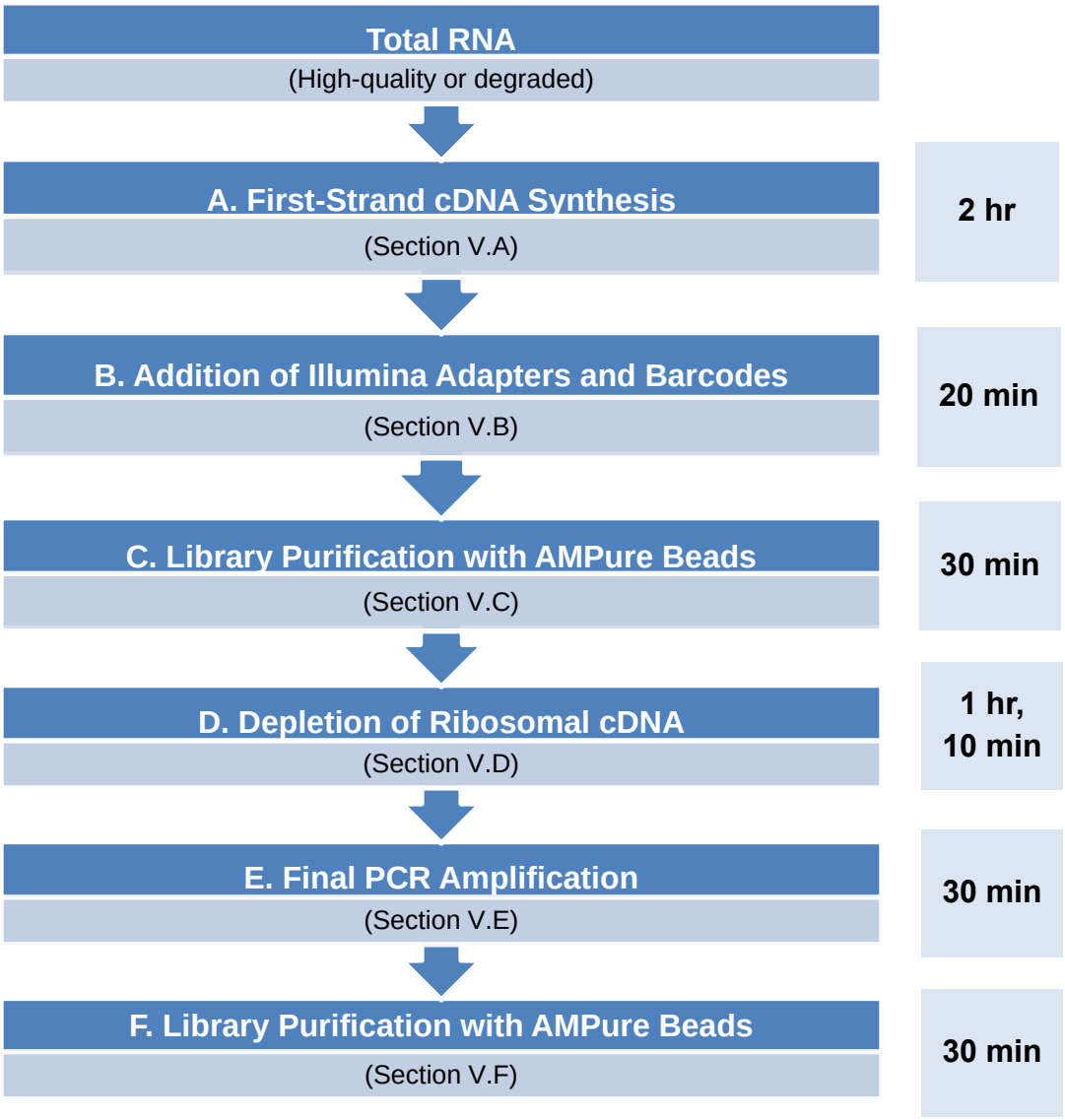

Figure 1. SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian protocol overview. This kit features an easy workflow that generates Illumina-compatible RNA-seq libraries in approximately 5 hr. First, total RNA is converted to cDNA (Step A), and then adapters for Illumina sequencing (with specific barcodes) are added through PCR using only a limited number of cycles (Step B). The PCR products are purified (Step C), and then ribosomal cDNA is depleted (Step D). The cDNA fragments from Step D are further amplified (Step E) with primers universal to all libraries (they work regardless of the barcode added in Step B). Lastly, the PCR products are purified once more to yield the final cDNA library (Step F).

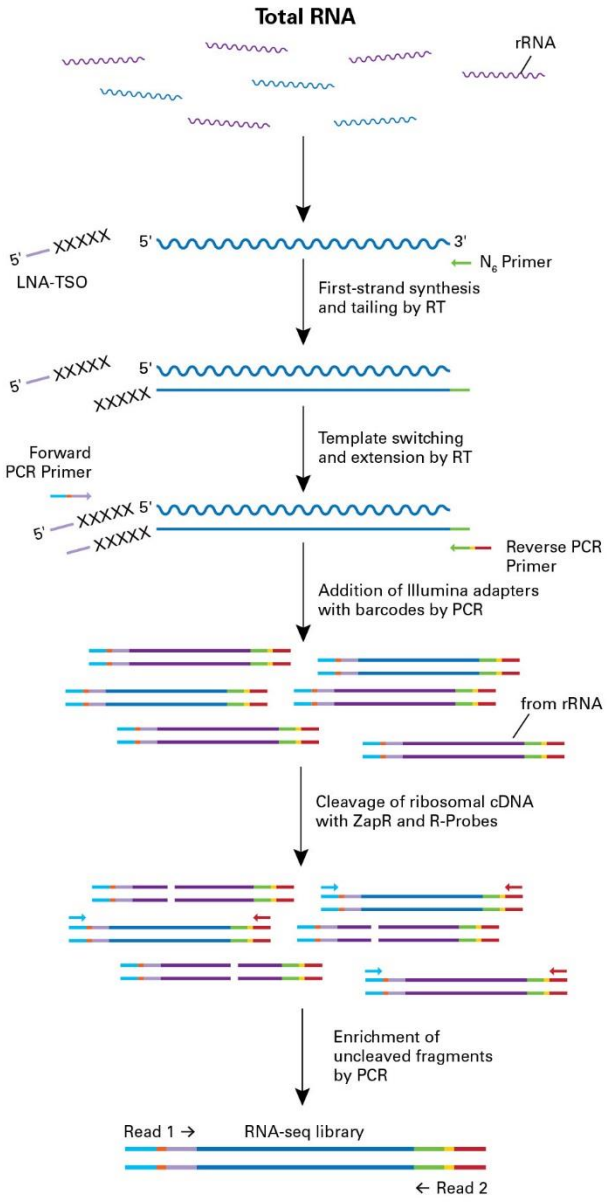

Figure 2. Schematic of the Technology in the SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian. SMART technology is used in this ligation-free protocol to preserve strand-of-origin information. Random priming (represented as the green N6 Primer) allows the generation of cDNA from all RNA fragments in the sample, including rRNA. When the SMARTScribe™ Reverse Transcriptase (RT) reaches the 5' end of the RNA fragment, the enzyme's terminal transferase activity adds a few non-templated nucleotides to the 3' end of the cDNA (shown as Xs). The carefully designed LNA-TSO (included in the Template Switching Oligo Mix) base-pairs with the non-templated nucleotide stretch, creating an extended template to enable the RT to continue replicating to the end of the oligonucleotide. The resulting cDNA contains sequences derived from the random primer and the LNA-TSO used in the reverse transcription reaction. In the next step, a first round of PCR amplification (PCR1) adds full-length Illumina adapters, including barcodes. The Forward PCR Primer binds to the LNA-TSO sequence (light purple), while the Reverse PCR Primer binds to sequence associated with the random primer (green). The ribosomal cDNA (originating from rRNA) is then cleaved by ZapR in the presence of the mammalian-specific R-Probes. This process leaves the library fragments originating from non-rRNA molecules untouched, with priming sites available on both 5' and 3' ends for further PCR amplification. These fragments are enriched via a second round of PCR amplification (PCR2) using primers universal to all libraries. The final library contains sequences allowing clustering on an Illumina flow cell (P5 shown in light blue, P7 shown in red), Illumina (TruSeq® HT) indexes (Index 1 [i7] sequence shown in yellow, and Index 2 [i5] sequence shown in orange) as well as the regions recognized by sequencing primers Read Primer 1 (Read 1, purple) and Read Primer 2 (Read 2, green).

II. List of Components

The SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian consists of the SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian 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. Please make sure to spin down tubes to collect all the liquid at the bottom before first use.

SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian		**635005** (12 rxns)	**635006** (48 rxns)	**635007** (96 rxns)
	SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian Components (Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)			
Cap Color	**Package 1 (Store at –70°C)**			
Red	TSO Mix[1, 2]	54 µl	216 µl	432 µl
Blue	R-Probes[1]	12 µl	50 µl	100 µl
Blue	ZapR	15 µl	60 µl	120 µl
	Control Total RNA[3] (1 µg/µl)	5 µl	5 µl	5 µl
	Package 2 (Store at –20°C)			
Pink	SMART Pico Oligos Mix[1]	12 µl	50 µl	100 µl
Pink	5X First-Strand Buffer	50 µl	200 µl	400 µl
Purple	SMARTScribe RT (100 U/µl)	25 µl	100 µl	200 µl
White	RNase Inhibitor (40 U/µl)	6 µl	25 µl	50 µl
Blue	10X ZapR Buffer	50 µl	200 µl	400 µl
	Stranded Elution Buffer	250 µl	1 ml	2 x 1 ml
Orange	PCR2 Primers[4]	25 µl	100 µl	200 µl
	Nuclease-Free Water	1.25 ml	2 x 1.25 ml	4 x 1.25 ml
	SeqAmp DNA Polymerase[5]	50 µl	200 µl	2 x 200 µl
	SeqAmp PCR Buffer (2X)	1.25 ml	3 x 1.25 ml	6 x 1.25 ml

[1] Takara Bio proprietary sequences

[2] TSO Mix contains the template-switching oligo (TSO) with locked nucleic acid (LNA) technology.

[3] Control Total RNA is from mouse brain.

[4] Do not freeze-thaw the PCR2 Primers more than 10 times.

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

WARNING: Do not freeze/thaw ZapR and R-Probes more than 3 times! We recommend aliquoting ZapR and R-Probes into multiple vials to avoid repeated freeze-thaw cycles.

WARNING: The Nuclease-Free Water is used in Steps V.A, V.B, and V.E. When using this kit for the first time, set aside a small amount of water to be used only for first-strand synthesis (Step V.A). This helps to avoid contamination during the kit's subsequent uses from previously introduced molecules with library adapters from Steps V.B and V.E.

Indexing Primer Sets:

Indexing Primer Set version		**HT for Illumina - 12**	**HT for Illumina - 48 A**	**HT for Illumina**
Cat. No.		635005	635006	635007
Size		12 rxns	48 rxns	96 rxns

(Not sold separately. Store at –20°C.)

Forward Primers 12 µM Full names of primers have been shortened[a]	F1[b]		2 x 15 µl	15 µl
	F2	2 x 15 µl	2 x 15 µl	15 µl
	F3		2 x 15 µl	15 µl
	F4		2 x 15 µl	15 µl
	F5			15 µl
	F6			15 µl
	F7			15 µl
	F8			15 µl
Reverse Primers 12 µM Full names of primers have been shortened[a]	R1	12 µl	12 µl	12 µl
	R2	12 µl	12 µl	12 µl
	R3	12 µl	12 µl	12 µl
	R4	12 µl	12 µl	12 µl
	R5	12 µl	12 µl	12 µl
	R6	12 µl	12 µl	12 µl
	R7	12 µl	12 µl	12 µl
	R8	12 µl	12 µl	12 µl
	R9	12 µl	12 µl	12 µl
	R10	12 µl	12 µl	12 µl
	R11	12 µl	12 µl	12 µl
	R12	12 µl	12 µl	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 correspond to Illumina TruSeq HT indexes D501–D508; R1–R12 correspond to Illumina TruSeq HT 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

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, two each (one for pre-PCR amplification steps and one dedicated for PCR amplification)
- Hot-lid PCR thermal cyclers: two (one dedicated for pre-PCR amplification steps and one dedicated for PCR amplification)

  NOTE: The final RNA-seq library amplification (Section V.E) is intended to be carried out with thermal cyclers that can accommodate 100 µl sample volumes. If your thermal cyclers only accommodate ≤50 µl sample volumes, we recommend splitting each reaction equally into two tubes so the PCR proceeds optimally.
- Multi-channel pipettes: 20 µl and 200 µl (eight- or twelve-channel pipettes are recommended when performing multiple reactions in a single experiment)
- Filter pipette tips: 10 µl, 20 µl, and 200 µl
- Quickspin Minicentrifuge for 0.2 ml tubes
- 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 High Sensitivity DNA Kit (Agilent, Part Number 5067-4626)
- Qubit dsDNA HS kit (Life Technologies, Cat. No. Q32851)
- Nuclease-free thin-wall PCR tubes (0.2 ml; USA Scientific, Item No. 1402-4700)
- Nuclease-free nonsticky 1.5 ml tubes (USA Scientific, Item No. 1415-2600)

For Purification Using AMPure Beads:

- Agencourt AMPure XP PCR Purification Kit (5 ml; Beckman Coulter, Item No. A63880 or 60 ml; Beckman Coulter, Item No. A63881). Kit size needed depends on the number of reactions performed.

  NOTE: The Agencourt AMPure beads need to come to room temperature before the container is opened. Therefore, we recommend aliquoting the beads into 1.5 ml tubes upon receipt, and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min). This aliquoting process is also essential to minimize the chances of bead contamination.

  NOTE: Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.
- Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011) or a magnetic separation device assembled from rare earth magnets (Applied Magnets, Model #NB026 or similar). See Appendix A for assembly instructions.

IMPORTANT: Very strong magnets produce the best-quality library with the highest yield. See Appendix A for instructions on how to make your own magnetic separation device. The recommended magnetic separation devices have been successfully tested with the SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian protocol. In order to prevent cross-contamination, we strongly recommend using separate magnets for library purification in Section V.C. and for post-PCR purification in Section V.F.

- 80% ethanol: freshly made for each experiment

IV. 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.A.)
    - **A second work station located in the general laboratory** where you will perform PCR1 (Section V.B.) and PCR2 (Section V.E.), and cleave ribosomal cDNA with ZapR and R-Probes (Section V.D.)
    - **A third work station located in the general laboratory** where you will purify the library (Sections V.C. and V.F.) and measure its concentration (Section V.G.)

    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 clean 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 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 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 SMARTer cDNA synthesis need to be stored in a DNA-free, closed cabinet. Ideally, reagents for SMARTer 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 mixture 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 amplification 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 Total RNA) control reactions.
- Because of the large volume and viscosity of the SeqAmp PCR buffer, AMPure bead purification requires a very strong magnet, particularly for the last purification (Step V.F). Do not only rely on the time indicated; leave samples on the magnet long enough for the samples to be completely clear. (See Appendix A for instructions on how to make your own magnetic stand.)

C. Sample Requirements

Input RNA Quality

- Degraded, partially degraded, or high-quality RNA can be used in this protocol. Please determine the quality of your RNA (RIN score) before starting this protocol using the **Agilent RNA 6000 Pico Kit** (Cat. No. 5067-1513). The first-strand cDNA synthesis protocol includes a fragmentation step for partially degraded or high-quality RNA, while the alternate protocol proceeds without fragmentation for highly degraded RNA, such as material extracted from FFPE samples. Please refer to Section V.A for guidance.

Input RNA Purity and Quantity

- **Purity of input RNA:** Input RNA should be free from genomic or carrier DNA and contaminants that would interfere with oligo annealing. Samples should have been treated with DNase I prior to use with this kit, as the random priming used in this protocol may lead to amplification of any DNA present in the starting material.

  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 RNA fragmentation and the efficiency of reverse transcription.

- **Volume and amount of input RNA:** This kit accommodates up to 8 µl of input RNA. This protocol has been optimized for cDNA synthesis with 250 pg–10 ng of total mammalian RNA.

  NOTE: We recommend using inputs in the range of 250 pg–10 ng of high-quality or degraded total RNA. For FFPE samples, which contain chemically damaged RNA that decreases the efficiency of reverse transcription, we recommend using inputs of 5–10 ng. Inputs higher than 10 ng have not been validated. It is strongly recommended that working conditions for your samples be established before trying inputs below the recommended range. For total RNA samples <250 pg, the yield may be low and is highly dependent on the RNA source (e.g., the mRNA content in

a particular sample/cell type/tissue) and the strength of the magnet used for bead purification steps. Libraries from <250 pg input RNA will also contain a significantly higher amount of PCR duplicates due to the low complexity of the starting material, and are more likely to contain undesirable environmental contaminants.

Diluting the Control Total RNA

1. Dilute the Control Total RNA (mouse brain) to 50 ng/µl by mixing 38 µl of Nuclease-Free Water with 2 µl of 1 µg/µl concentrated Control Total RNA in a sterile microcentrifuge tube.

   NOTE: Fresh dilutions should be made before use. If desired, make single-use aliquots of the 50 ng/µl dilution and store at –70˚C until needed, then further dilute.

2. Further dilute Control Total RNA to 5 ng/µl by mixing 45 µl of Nuclease-Free Water with 5 µl of 50 ng/µl Control Total RNA in a sterile microcentrifuge tube.
3. Further dilute Control Total RNA to 0.25 ng/µl by mixing 95 µl of Nuclease-Free Water with 5 µl of 5 ng/µl Control Total RNA in a sterile microcentrifuge tube.
4. Use 1 µl or more of 0.25 ng/µl Control Total RNA as a positive control RNA input for the kit and proceed alongside your samples.

   NOTE: Try to match the amount of Control Total RNA input to the amount of your own samples, and use the same number of PCR cycles. Due to the high quality of the Control Total RNA (RIN >8), a 4-min fragmentation is necessary, regardless of the amount of fragmentation needed for your experimental samples.

V. Protocols

A. Protocol: First-Strand cDNA Synthesis

Fragmentation of RNA into a size appropriate for sequencing on Illumina platforms is performed in the first step of the cDNA synthesis protocol. Fragmentation time is adjusted depending on the quality of the RNA input. Option 1 (with fragmentation) should be used when starting from samples with RIN ≥4, and Option 2 (without fragmentation) should be used when starting from samples with RIN ≤3. Please refer to Table 1 for guidelines.

Table 1. Recommended Fragmentation Protocol Options and Fragmentation Times.

RIN	Use protocol	Fragmentation time (min)
≥7	Option 1	4
5–6	Option 1	3
4	Option 1	2*
≤3 or FFPE	Option 2	–

* In most cases, samples with RIN ~4 will be optimally processed after 1–2 min of fragmentation. However, we recommend optimizing the fragmentation time, as overfragmentation may lead to inefficient ribosomal cDNA depletion while performing the protocol in Section V.D.

For this protocol, you will need the following components: SMART Pico Oligos Mix (pink cap), 5X First-Strand Buffer (RNase-Free) (pink cap), Nuclease-Free Water, Template Switching Oligo Mix (TSO Mix) (red cap), RNase Inhibitor (white cap), and SMARTScribe Reverse Transcriptase (purple cap).

Option 1 (With Fragmentation): Starting from High-Quality or Partially Degraded RNA (RIN ≥4)

1. Mix the following components on ice:

1–8 µl	RNA (0.25–10 ng)
1 µl	SMART Pico Oligos Mix
4 µl	5X First-Strand Buffer (RNase-Free)
0–7 µl	Nuclease-Free Water
13 µl	Total volume per reaction

2. Incubate the tubes at **94°C** in a preheated, hot-lid thermal cycler for the amount of time recommended in Table 1 or for an experimentally determined, optimal amount of time, then immediately place the samples on ice (or PCR chiller rack) for 2 min.

NOTE: The samples should be taken out of the thermal cycler immediately after the time indicated in order to avoid over-fragmentation. Make sure to wait by the thermal cycler when the incubation time is over and immediately chill the samples.

NOTE: The next reaction steps (Step 4–5) are critical for first-strand synthesis and should not be delayed after Step 2. Start Step 3, preparing the First Strand Master Mix, while your tubes are incubating (Step 2), or have it almost ready before starting Step 2.

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

4.5 µl	Template Switching Oligo Mix (TSO mix)
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe Reverse Transcriptase
7 µl	Total volume per reaction

4. Add 7 µl of the First Strand Master Mix to each reaction tube from Step 2. Mix the contents of the tube by gently vortexing or tapping the tubes with your finger, and spin the tubes briefly to collect the contents at the bottom.
5. Incubate the tubes in a preheated hot-lid thermal cycler with the following program:

42°C	90 min
70°C	10 min
4°C	forever

Leave the samples in the thermal cycler at 4°C until the next step.

NOTE: For convenience, samples can be left overnight in the thermal cycler at 4°C.

Option 2 (Without Fragmentation): Starting from Highly Degraded RNA (RIN ≤3)

1. Mix the following components on ice:

1–8 µl	RNA (0.25–10 ng)
1 µl	SMART Pico Oligos Mix
0–7 µl	Nuclease-Free Water
9 µl	Total volume per reaction

2. Incubate the tubes at **72°C** in a preheated, hot-lid thermal cycler for exactly 3 min, then immediately place the samples on ice (or PCR chiller rack) for 2 min.

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

4 µl	5X First-Strand Buffer (RNase-Free)
4.5 µl	Template Switching Oligo Mix (TSO mix)
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe Reverse Transcriptase
11 µl	Total volume per reaction

4. Add 11 µl of the First Strand Master Mix to each reaction tube. Mix the contents of the tubes by gently vortexing or tapping with your finger, and spin the tubes briefly to collect the contents at the bottom.
5. Incubate the tubes in a preheated hot-lid thermal cycler with the following program:

42°C	90 min
70°C	10 min
4°C	forever

6. Leave the samples in the thermal cycler at 4°C until the next step.

NOTE: For convenience, samples can be left overnight in the thermal cycler at 4°C.

B. Protocol: PCR1—Addition of Illumina Adapters and Indexes

The indexes (barcodes) that are used to distinguish pooled libraries from each other after sequencing are added at this step. Great care must be taken to select the right indexes.

For this protocol, you will need the following components: Nuclease-Free Water, 2X SeqAmp PCR Buffer, SeqAmp DNA Polymerase, and Forward and Reverse PCR Primer HT sets.

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:

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

NOTE: If the forward index is going to be the same for all libraries, the Forward Primer can also be added to the master mix (1 µl/reaction). Typically, a single forward index can be used if fewer than 12 libraries will be pooled for sequencing.

2. Add 28 µl (29 µl if the Forward Primer is included) of PCR Master Mix to each sample from Step A.6.
3. Add 1 µl of each Forward and Reverse PCR Primer HT to each sample. Mix by gentle vortexing or tapping of the tubes, then spin down briefly.
4. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the following program:

94°C		1 min
5 cycles:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
68°C		2 min
4°C		forever

C. Protocol: Purification of the RNA-Seq Library Using AMPure Beads

The amplified RNA-seq library is purified by immobilization onto AMPure beads. The beads are then washed with 80% ethanol and the cDNA is eluted in Nuclease-Free Water.

For this protocol, you will need the following components: AMPure beads (at room temperature), 80% ethanol (freshly prepared), Nuclease-Free Water, and a magnetic separation device.

1. Allow AMPure beads to come to room temperature before use (~30 min). Add 45 µl of AMPure beads to each sample.

   NOTE: Mix by vortexing for 5 sec (recommended) or by pipetting the entire volume up and down at least 10 times. The beads are viscous; pipette the entire volume up and then out slowly.

2. Incubate at room temperature for 8 min to allow the DNA to bind to the beads.
3. Briefly spin the sample tubes to collect the liquid at the bottom. 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 and discard.
5. Keeping 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 and discard the supernatant. cDNA will remain bound to the beads during the washing process.
6. Repeat Step 5 once.
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 carefully remove any remaining ethanol with a pipette, without disturbing the beads.
8. Let the open sample tubes rest at room temperature for ~3–5 min until the pellets appear dry.

   NOTE: You may see a tiny crack in each pellet when dry. Do not overdry.

9. Once the beads are dry, add 52 µl of Nuclease-Free Water to cover the beads. Remove the tubes from the magnetic stand and mix thoroughly by pipetting up and down until all the beads have been washed off the sides of the tubes.
10. Incubate at room temperature for 5 min to rehydrate.
11. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 1 min or longer, until the solution is completely clear.
12. Pipette 50 µl supernatant from each sample into respective wells of a new 8-well strip.
13. Perform a second bead clean-up by repeating Steps 1–8.

    NOTE: Drying of the beads (Step C.8) will be faster the second time. 1–2 min should be sufficient. Do not overdry.

14. Once the beads are dried, add 18 µl of Nuclease-Free Water to cover the beads. Remove the tubes from the magnetic stand and mix thoroughly by pipetting up and down or by gently tapping the tubes to resuspend the beads.
15. Incubate at room temperature for 5 min to rehydrate.
16. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 1 min or longer, until the solution is completely clear.
17. Label a new strip of 8-strip nuclease-free 0.2-ml thin wall PCR tubes.
18. Pipette out 16 µl of each supernatant, being careful not to disturb the beads, into respective tubes of the new PCR strip. Keep samples on ice (or PCR chiller rack) until the next step.

    NOTE: This step can be performed with a multi-channel pipette.

D. Protocol: Depletion of Ribosomal cDNA with ZapR and R-Probes

In this section, the library fragments originating from rRNA (18S and 28S) and mitochondrial rRNA (m12S and m16S) are cut by ZapR in the presence of R-Probes (mammalian-specific). These R-Probes hybridize to ribosomal RNA and mitochondrial rRNA sequences; however, the mitochondrial sequences are derived from the human mitochondrial genome and are therefore strictly human specific.

For this protocol, you will need the following components: R-Probes (blue cap), ZapR (blue cap), and 10X ZapR Buffer (blue).

1. Thaw R-Probes, ZapR, and ZapR buffer at room temperature. Place R-Probes and ZapR on ice as soon as they are thawed, but keep the buffer at room temperature.
2. Pipette into a PCR tube an R-Probes aliquot large enough for the number of reactions desired (1 µl per reaction), plus 5–10% to account for pipetting errors.
3. Incubate the PCR tube containing R-Probes at 72°C in a preheated hot-lid thermal cycler using the following program:

72°C	2 min
4°C	forever

4. Leave the R-Probes tube in the thermal cycler at 4°C for at least 2 min, but no more than 10 minutes, before using it in next step.
5. Prepare enough Master Mix for all reactions, plus 10%, by combining the following reagents at room temperature in the order shown. Store ZapR and R-Probes components at –70°C immediately after use. Mix the components well by vortexing briefly, and spin the tubes briefly in a microcentrifuge.

2 µl	10X ZapR Buffer
1 µl	R-Probes
1.25 µl	ZapR
4.25 µl	Total volume per reaction

6. Add 4.25 µl of the Master Mix to the purified RNA-seq libraries from Step C.18. The total volume is now 20.25 µl.
7. Mix the contents of the tubes by gently vortexing, and spin the tubes briefly to collect the contents at the bottom.
8. Incubate the tubes in a preheated hot-lid thermal cycler using the following program:

37°C	60 min
72°C	10 min
4°C	forever

E. Protocol: PCR2—Final RNA-Seq Library Amplification

In this section, the library fragments not cleaved by the ZapR reaction in Section V.D will be further enriched in a second round of PCR. Since barcodes have already been added to the libraries, a single pair of primers can be used for all libraries.

For this protocol, you will need the following components: Nuclease-Free Water, 2X SeqAmp PCR Buffer, PCR2 Primers (orange cap), and SeqAmp DNA Polymerase.

1. Prepare a PCR Master Mix for all reactions (plus 10%). Combine the following reagents in the order shown, then mix well and spin the tubes briefly in a microcentrifuge:

26 µl	Nuclease-Free Water
50 µl	2X SeqAmp PCR Buffer
2 µl	PCR2 Primer mix
2 µl	SeqAmp DNA Polymerase
80 µl	Total volume per reaction

NOTE: 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 (prior to Step 3).

2. Add 80 µl of PCR Master Mix to each tube from Step D.8. Mix by tapping gently, then spin down.
3. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the following program:

94°C		1 min
12–16 cycles:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
4°C		forever

NOTE: The actual number of cycles varies depending on the starting material. The guidelines below must be validated with your material. We do not recommend performing more than 16 cycles, as it will lead to background amplification. We recommend that you perform a pilot experiment with a small number of samples to determine the optimal number of cycles for your input material.

Table 2. Cycling Guidelines Based on Amount of Starting Material.

Amount of input RNA (ng)	Typical number of PCR cycles
10	12
1	14–15
0.25	16

F. Protocol: Purification of Final RNA-Seq Library Using AMPure Beads

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

For this protocol, you will need the following components: AMPure beads (at room temperature), 80% ethanol (freshly prepared), Stranded Elution Buffer, and a magnetic separation device.

1. Allow AMPure beads to come to room temperature before use (~30 min). Add 100 µl of AMPure beads to each sample.

   NOTE: Mix by vortexing for 5 sec or by pipetting the entire volume up and down at least 10 times. The beads are viscous; pipette the entire volume up, and then out slowly.

2. Incubate at room temperature for 8 min to let the cDNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 5–10 min or longer, until the solution is completely clear.

   NOTE: This step will take more time than in Protocol C (Section V.C) due to the high volume.

4. While the tubes are sitting on the magnetic stand, pipette out the supernatant and discard.
5. Keep the tubes on the magnetic stand. Without disturbing the beads, add 200 µl of **freshly made** 80% ethanol to each sample to wash away contaminants. Wait for 30 sec and carefully pipette out the supernatant. cDNA will remain bound to the beads during the washing process.
6. Repeat Step 5 once.
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 carefully remove all remaining ethanol with a pipette, without disturbing the beads.
8. Let the sample tubes rest open at room temperature for ~10 min until the pellets appear dry.

   NOTE: You may see a tiny crack in each pellet. Do not overdry.

9. Once the beads are dry, add 20 µl of Stranded Elution Buffer to cover the beads. Remove the tubes from the magnetic stand and mix thoroughly by pipetting up and down several times until all the beads have been washed off the sides of the tubes.

   NOTE: Consider eluting in 12 µl instead of 20 µl if anticipated yield is low.

10. Incubate at room temperature for 5 min to rehydrate.
11. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 2 min or longer, until the solution is completely clear.
12. Transfer the supernatants to nonsticky tubes. Proceed to validation immediately or store at –20°C.

G. Protocol: Validation Using the Agilent 2100 Bioanalyzer

1. Quantify libraries with Qubit dsDNA HS kit (Life Technologies). A yield >3 ng/µl will provide enough material for further library validation and sequencing. Consider adding one PCR cycle in subsequent experiments if yield is insufficient, or reducing cycles if yield is more than 10 ng/µl. Eluting the final libraries in a smaller volume (e.g., 12 µl instead of 20 µl) is also a simple way to achieve more concentrated libraries.
2. Evaluate library size distribution by running samples on the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent, Part Number 5067-4626) or an equivalent microfluidic device/kit. Dilute libraries to about 1.5 ng/µl prior to loading the chip (for a consistent library-to-library profile). See Figure 3 for an example of a successful library.
3. Compare the results for your samples and controls (if performed) to determine whether samples are suitable for further processing. Successful cDNA synthesis and amplification should produce a distinct curve spanning 200–1,000 bp, with a local maximum at ~300–400 bp, in the positive control RNA sample (see Figure 3A) and no product or very minimal background over the corresponding range in the negative control (see Figure 3B).

   NOTE: Library preparation adds 139 bp to the size of the original RNA molecules.

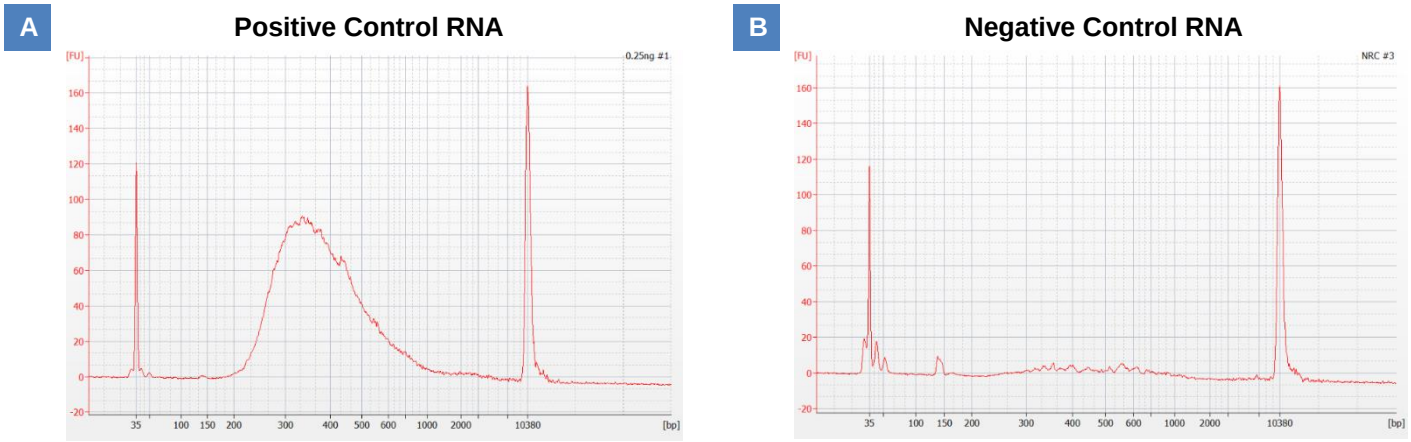

Figure 3. Electropherogram example results from the Agilent 2100 Bioanalyzer. Libraries were generated using 0.25 ng Control Total RNA (mouse brain; Panel A—1:3 library dilution) and a no-RNA control (Panel B—no library dilution). For both examples, PCR2 was performed using 16 cycles. Note that the no-RNA control exhibits a small amount of background (indicated by minimal product in the 200–1,000 bp range), which is acceptable as long as the libraries from the RNA samples contain a significantly larger amount of material.

VI. References

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

Picelli, S. *et al.* Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat. Methods* **10,** 1096–1098 (2013).

Appendix A: 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.

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

As seen in Figure 4, 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).

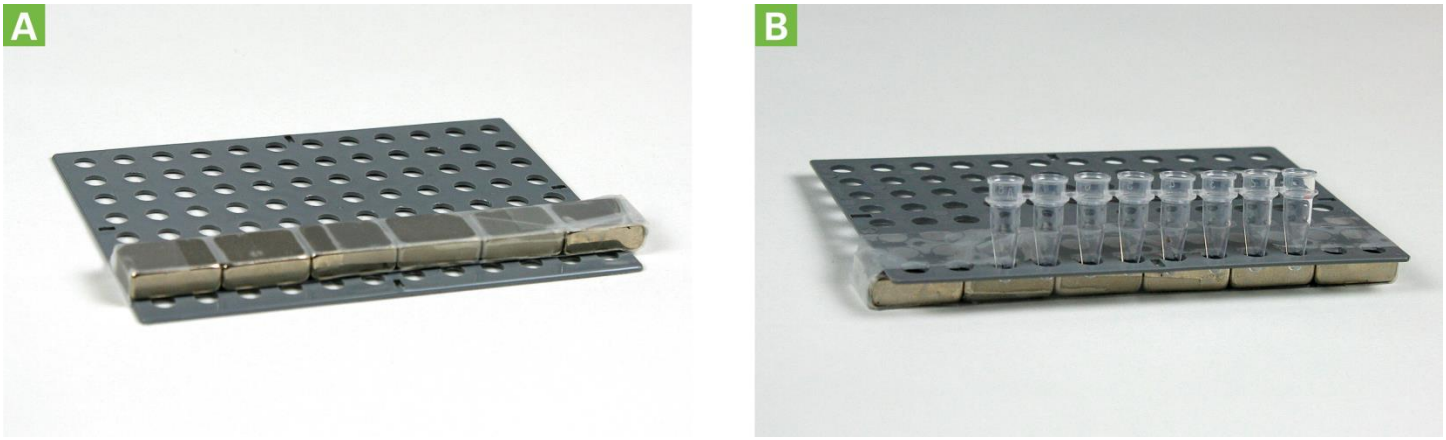

Figure 4. Constructing a magnetic separation device for 0.2-ml tubes from rare earth magnets. **Panel A.** Six 0.75" x 0.25" x 0.5" neodymium bar magnets (Applied Magnets, Model # NB026) taped together on the underside of the top section of a 20-µl tip rack. **Panel B.** The upright rack, with an 8-tube strip of 0.2-ml tubes inserted.

Appendix B: 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 average library size determined by the Bioanalyzer, the concentration in ng/µl can be converted to nM. The following web tool is convenient for the conversion: http://www.molbiol.edu.ru/eng/scripts/01_07.html. Alternatively, libraries can be quantified by qPCR using the Library Quantification Kit (Takara Bio USA, 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 error, 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 error.

3. Depending on the Illumina sequencing library preparation protocol, use a 5 µl aliquot (for the 4-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 2) 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 nt. 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 (Illumina, Cat. No. FC-110-3001) spike-in. Make sure to use a fresh and 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 2).

Table 3. PhiX Control Spike-in Guidelines for Various Illumina Sequencing Instruments.

Sequencing instrument	PhiX (%)
MiSeq	5–10 (optional[1])
HiSeq 1500/2000/2500	10
HiSeq 3000/4000	20
NextSeq/MiniSeq	20

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

Extra Precautions When Using NextSeq and MiniSeq Instruments

The libraries generated with the SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian can be used for sequencing on NextSeq and MiniSeq instruments. However, care must be taken to ensure that you get the most out of the sequencing run.

- Both systems use automatic adapter trimming by default. This can unexpectedly shorten your reads and 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. This can be problematic if the RNA input was very fragmented, considering that the SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian is good at retaining small inserts. 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 nt of Read 1), NextSeq and MiniSeq runs without PhiX spike-in may cause 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 and MiniSeq sequencing reagent kits.

Extra Precautions When Using HiSeq 3000/4000 Instruments

Similar to the NextSeq and MiniSeq instruments, the HiSeq 3000/4000 instruments are sensitive to low-diversity nucleotide stretches. Customers have reported to us that the inclusion of 20% of PhiX spike-in was sufficient to alleviate the problem.

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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