Takara Bio USA, Inc.

SMART-Seq® Stranded Kit User Manual

Cat. Nos. 634442, 634443, 634444
(022819)

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I. Introduction

Single-Cell cDNA Synthesis for Illumina® Sequencing Platforms

The **SMART-Seq Stranded Kit** (Cat. Nos. 634442, 634443, 634444) includes all components needed to generate indexed cDNA libraries suitable for next-generation sequencing (NGS) on Illumina platforms, starting directly from 1–1000 intact cells, or 10 pg–10 ng of mammalian total RNA. The entire library construction protocol, starting with cells or total RNA, can be completed in about 7 hours (Figure 1).

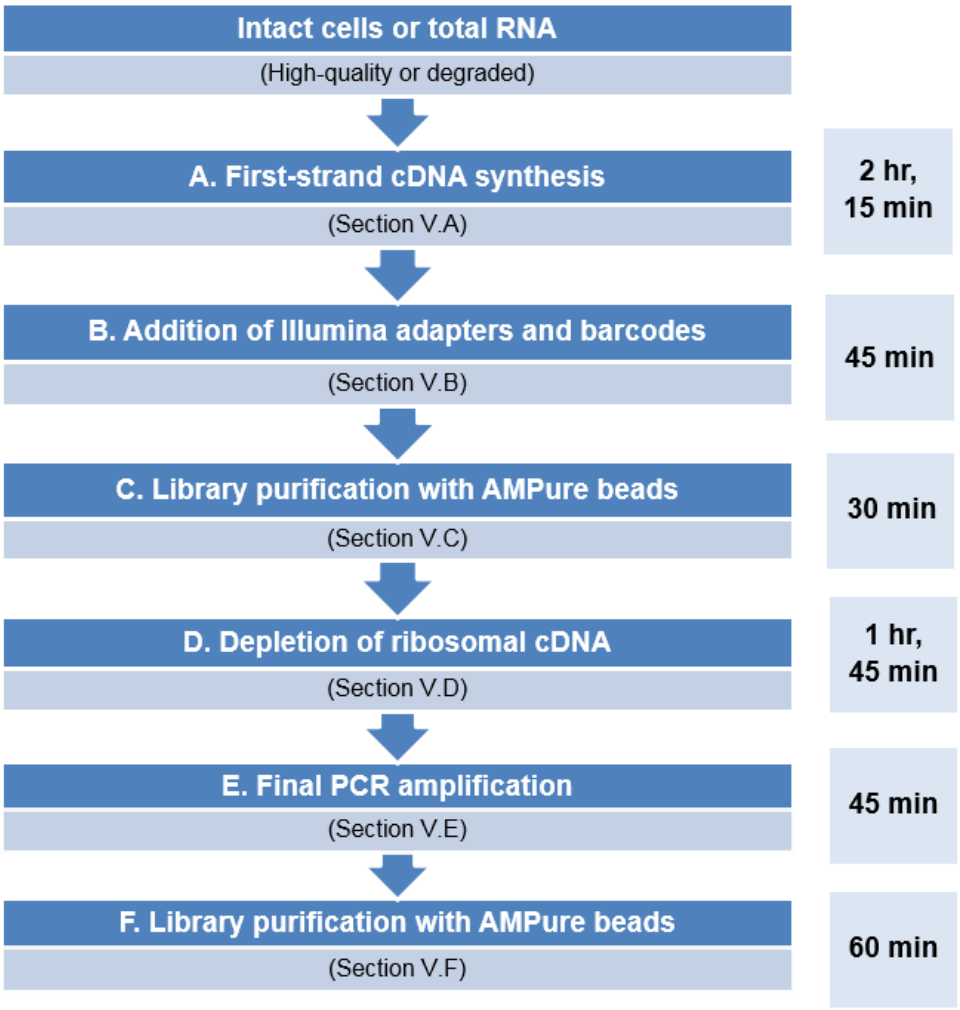

Figure 1. SMART-Seq Stranded Kit protocol overview. This kit features an easy workflow that generates Illumina-compatible RNA-seq libraries from single cells in approximately 7 hr. First, RNA is converted to cDNA (**A**), and then adapters for Illumina sequencing (with specific barcodes) are added through PCR using a limited number of cycles (**B**). The PCR products are purified (**C**), and then ribosomal cDNA is selectively depleted (**D**). The remaining cDNA fragments are further amplified (**E**) with primers universal to all libraries. Lastly, the PCR products are purified once more to yield the final cDNA library (**F**). Actual processing time may vary depending on the number of samples and cycling conditions, but a set of 24–48 samples can be easily processed from **A** to **F** within an 8-hr workday. As outlined in Section V, the kit workflow includes three safe stopping points following the completion of **A**, **B**, and **E**.

This kit incorporates SMART® (**S**witching **M**echanism **A**t 5' end of **R**NA **T**emplate) cDNA synthesis technology (Chenchik et al. 1998) and generates Illumina-compatible libraries via PCR amplification, avoiding 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. Illustrated below are the cDNA library construction process and technologies employed by the kit (Figure 2), and the structural details of final libraries (Figure 3). For sequencing libraries produced with this kit, Read 1 generates sequences antisense to the original RNA.

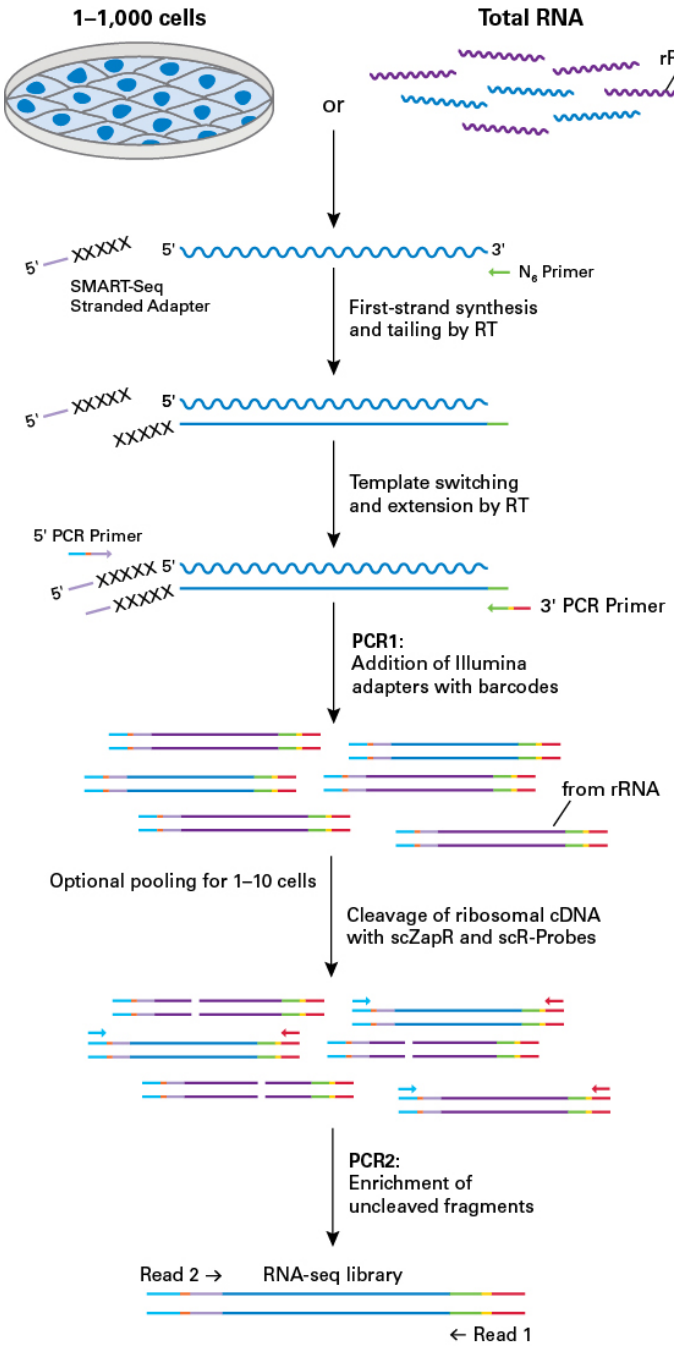

Figure 2. Schematic of technology in the SMART-Seq Stranded Kit. SMART technology is used in a ligation-free protocol to preserve strand-of-origin information. Random priming (through the SMART scN6 Primer) allows the generation of cDNA from all RNA fragments in the sample, including rRNA. When the SMARTScribe™ Reverse Transcriptase (RT) reaches the 5' end of the RNA fragment, the enzyme's terminal transferase activity adds a few nontemplated nucleotides to the 3' end of the cDNA (shown as Xs). The carefully designed SMART-Seq Stranded Adapter (included in the SMART scTSO Mix) base-pairs with the nontemplated 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 SMART scN6 Primer and the SMART-Seq Stranded Adapter. In the next step, a first round of PCR amplification (PCR1) adds full-length Illumina adapters, including barcodes. The 5' PCR Primer binds to the SMART-Seq Stranded Adapter sequence (light purple), while the 3' PCR Primer binds to sequence associated with the SMART scN6 sequence (green). The ribosomal cDNA (originating from rRNA) is then cleaved by scZapR in the presence of the mammalian-specific scR-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 any Illumina flow cell (see details in Figure 3). An optional pooling of up to 12 samples after PCR1 allows for greater ease of use by minimizing the number of samples to be processed downstream.

Figure 3. Structural features of final libraries generated with the SMART-Seq Stranded Kit. The adapters added during PCR1 contain sequences allowing clustering on Illumina flow cells (P7 shown in light blue, P5 shown in red), indexes for pooling multiple samples in a single sequencing lane (Index 1 [i7] sequence shown in orange and associated with the P7 sequence, and Index 2 [i5] sequence shown in orange and associated with P5 sequence), as well as the regions recognized by sequencing primers Read Primer 2 (Read 2, purple) and Read Primer 1 (Read 1, green). Read 1 generates sequences antisense to the original RNA, while Read 2 yields sequences sense to the original RNA (orientation of original RNA denoted by 5' and 3' in dark blue). The first three nucleotides of the second sequencing read (Read 2) are derived from the SMART-Seq Stranded Adapter (shown as Xs). It is best to trim these three nucleotides prior to mapping if performing paired-end sequencing.

Ribosomal RNA (rRNA) comprises a considerable proportion (~90% or more) of all RNA molecules in cells and is readily captured by random priming. Depleting these abundant transcripts from cell lysates or total RNA samples prior to generating libraries relying on random priming provides benefits by lowering sequencing costs and improving mapping statistics. However, with very low input amounts, initial rRNA depletion from total RNA often leaves an insufficient amount of material for preparation of high-quality libraries. In addition, initial rRNA depletion from the lysate of single cells is impossible. The workflow used in this kit takes advantage of an innovative technology allowing removal of ribosomal cDNA (cDNA fragments originating from rRNA molecules) after cDNA synthesis using probes specific to mammalian rRNA. These scR-Probes target nuclear rRNA and mitochondrial rRNA sequences; however, the mitochondrial rRNA scR-Probes 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 or cells, including intact single cells.

The new SMART-Seq Stranded Kit evolved from the SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian and is designed for handling cells directly without the need for RNA extraction. It generates RNA-seq data that are at par with the industry-leading SMART-Seq v4 Ultra® Low Input RNA Kit for Sequencing without the additional requirement for Nextera® library preparation. It provides additional benefits, including strand-of-origin information and a fuller representation of the transcriptome, thus allowing expanded applications such as analysis of lncRNA. Indeed, initiation of reverse transcription using random priming instead of the oligo(dT) priming used in the SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing leads to the capture of the full transcriptome instead of only the polyadenylated fraction.

II. List of Components

The SMART-Seq Stranded Kit consists of the SMART-Seq Stranded Kit Components (not sold separately) and the Indexing Primer Set HT for Illumina v2 (not sold separately). **These components have been specifically designed to work together and are optimized for this particular kit. 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 each use.

SMART-Seq Stranded Kit			634442 (12 rxns)	634443 (48 rxns)	634444 (96 rxns)
Box 1					
Cap color	**Cap label**				
Brown	scTSO Mix	SMART scTSO Mix[1, 2]	55 µl	225 µl	450 µl
Light Blue	scR-Probes	scR-Probes[1]	20 µl	80 µl	160 µl
		Control Total RNA[3] (1 µg/µl)	5 µl	5 µl	5 µl
Box 2					
	scZapR	scZapR	20 µl	80 µl	160 µl
Pink	scN6	SMART scN6[1]	15 µl	55 µl	110 µl
Red	scRT Buffer	scRT Buffer	50 µl	200 µl	400 µl
Purple	SMART Scribe	SMARTScribe RT (100 U/µl)	25 µl	100 µl	200 µl
White	RRI	RNase Inhibitor (40 U/µl)	10 µl	40 µl	80 µl
Blue	ZapR Buffer	ZapR Buffer (10X)	50 µl	200 µl	400 µl
Orange		Tris Buffer (5 mM)	1.25 ml	1.25 ml	2 x 1.25 ml
Khaki	PCR2	PCR2 Primers[4]	50 µl	200 µl	400 µl
		Nuclease-Free Water	1.25 ml	4 x 1.25 ml	10 ml
Green	SeqAmp	SeqAmp™ DNA Polymerase[5]	50 µl	200 µl	2 x 200 µl
	CB Buffer	SeqAmp CB PCR Buffer (2X)[6]	1.25 ml	4 x 1.25 ml	10 ml
		10X Lysis Buffer	0.5 ml	1 ml	1 ml

[1] Takara Bio proprietary sequences

[2] SMART scTSO Mix contains the SMART-Seq Stranded Adapter (a.k.a., template-switching oligo).

[3] Control Total RNA is from human brain.

[4] Do not freeze-thaw the PCR2 Primers v2 more than 10 times.

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

[6] Do not substitute regular SeqAmp PCR Buffer for SeqAmp CB PCR Buffer.

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

WARNING: The Nuclease-Free Water is used in Sections 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 (Section V.A). This helps to avoid contamination during the kit's subsequent uses from previously introduced molecules with library adapters from Sections V.B and V.E.

Indexing primer sets:

Kit Cat. No.		634442	634443	634444
Indexing primer set version		**HT for Illumina v2 - 12**	**HT for Illumina v2 - 48**	**HT for Illumina v2 - 96**
Size		**12 rxns**	**48 rxns**	**96 rxns**
(Not sold separately. Store at –20°C.)				
3' PCR primers 12.5 µM Full names of primers have been shortened[1]	3' 1[2]		20 µl	20 µl
	3' 2	20 µl	20 µl	20 µl
	3' 3		20 µl	20 µl
	3' 4		20 µl	20 µl
	3' 5			20 µl
	3' 6			20 µl
	3' 7			20 µl
	3' 8			20 µl
5' PCR primers 12.5 µM Full names of primers have been shortened[1]	5' 1	15 µl	15 µl	15 µl
	5' 2	15 µl	15 µl	15 µl
	5' 3	15 µl	15 µl	15 µl
	5' 4	15 µl	15 µl	15 µl
	5' 5	15 µl	15 µl	15 µl
	5' 6	15 µl	15 µl	15 µl
	5' 7	15 µl	15 µl	15 µl
	5' 8	15 µl	15 µl	15 µl
	5' 9	15 µl	15 µl	15 µl
	5' 10	15 µl	15 µl	15 µl
	5' 11	15 µl	15 µl	15 µl
	5' 12	15 µl	15 µl	15 µl

[1] Full names of primers have been shortened: for example, 3' PCR Primer HT Index 1 has been shortened to 3' 1 and 5' PCR Primer HT Index 1 has been shortened to 5' 1.

[2] 3' 1 to 3' 8 correspond to Illumina TruSeq® CD (formerly known as TruSeq HT) indexes D501–D508; 5' 1 to 5' 12 correspond to Illumina TruSeq CD indexes D701–D712 (see table below). They are combinatorial dual indexes and can be retrieved in BaseSpace or the Illumina Experiment Manager by selecting the Library Prep Workflow TruSeq Stranded Total RNA or TruSeq Stranded mRNA.

Indexing Primer Set HT for Illumina v2 adapter sequences:

i5 Index (Tube Label)	i5 Illumina Index Name	i5 Bases for Sample Sheet MiSeq®, NovaSeq™, HiSeq® 2000/2500	i5 Bases for Sample Sheet MiniSeq™, NextSeq®, HiSeq 3000/4000	i7 Index (Tube Label)	i7 Illumina Index Name	i7 Bases for Sample Sheet
3' 1	D501	TATAGCCT	AGGCTATA	5' 1	D701	ATTACTCG
3' 2	D502	ATAGAGGC	GCCTCTAT	5' 2	D702	TCCGGAGA
3' 3	D503	CCTATCCT	AGGATAGG	5' 3	D703	CGCTCATT
3' 4	D504	GGCTCTGA	TCAGAGCC	5' 4	D704	GAGATTCC
3' 5	D505	AGGCGAAG	CTTCGCCT	5' 5	D705	ATTCAGAA
3' 6	D506	TAATCTTA	TAAGATTA	5' 6	D706	GAATTCGT
3' 7	D507	CAGGACGT	ACGTCCTG	5' 7	D707	CTGAAGCT
3' 8	D508	GTACTGAC	GTCAGTAC	5' 8	D708	TAATGCGC
				5' 9	D709	CGGCTATG
				5' 10	D710	TCCGCGAA
				5' 11	D711	TCTCGCGC
				5' 12	D712	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, as you may not obtain the expected results.

- Nuclease-free thin-wall PCR tubes (0.2 ml; USA Scientific, Cat. No. 1402-4700)
- Nuclease-free non-sticky 1.5-ml tubes (Eppendorf DNA LoBind Microcentrifuge Tubes, Cat. No. 0224311021)
- Single-channel pipette: 10 µl, 20 µl, and 200 µl, two each (one for pre-PCR amplification steps and one dedicated for PCR amplification)
- Two hot-lid PCR thermal cyclers: one dedicated to pre-PCR amplification steps and one dedicated to 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 Cell Sorting

- 96-well polycarbonate PCR plates (USA Scientific Plastics, Cat No. 2796-3330) or 8-tube strips (Thermo Fisher, Cat No. AB-0264) inserted into a PCR rack
- BD FACS Pre-Sort Buffer (BD, Cat No. 563503)
- Dulbecco's phosphate-buffered saline (DPBS, without Ca^{2+} and Mg^{2+}; Sigma, Cat. No. D8537)
- Adhesive PCR Plate Foils (Thermo Fisher Scientific, Cat No. AB-0626) or 8-cap strips (Thermo Fisher Scientific, Cat No. AB-0784)
- Dry ice in a suitable container for flash freezing cells

For PCR Amplification & Validation:

- Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)
- Qubit dsDNA HS Kit (Thermo Fisher Scientific, Cat. No. Q32851)
- Nuclease-free thin-wall PCR tubes (0.2 ml; USA Scientific, Cat. No. 1402-4700)
- Nuclease-free non-sticky 1.5-ml tubes (Eppendorf DNA LoBind Microcentrifuge Tubes, Model No. 0224311021)

For Purification Using AMPure Beads:

- Agencourt AMPure XP PCR purification system (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: Agencourt AMPure XP beads need to come to room temperature before the container is opened. Therefore, we strongly 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 for minimizing 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.

- 80% ethanol: freshly made for each experiment
- Magnetic separation device for small volumes:
  - For 12–24 samples: SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio USA, Inc., Cat. No. 635011); accommodates two 8-tube or 12-tube strips
  - For 1.5-ml tubes (if pooling sequencing libraries prior to purification after PCR1): Takara Bio USA, Inc. Magnetic Stand (Cat. No. 631964) or NEB Magnetic Separation Rack (NEB, Cat. No. S1506S or S1509S).

IMPORTANT: Very strong magnets produce the best-quality libraries with the highest yields. The recommended magnetic separation device has been successfully tested with the SMART-Seq Stranded Kit protocol. In order to prevent cross-contamination, we strongly recommend using separate magnetic separation devices for the initial rounds (Sections V.C. and V.D) and final round of library purification (Section V.F).

For Library Validation

- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851)
- Agilent 2100 Bioanalyzer with High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)

IV. General Considerations

A. Recommendations for Preventing Contamination

When using the SMART-Seq Stranded Kit, capture of environmental contaminants is very likely, particularly with the ultra-low workflow. Contaminants such as bacterial, fungal, or viral DNA and RNA can be captured due to the random priming used to initiate the reverse transcription. For this reason, extreme care must be taken to minimize environmental contaminants (e.g., from air, bench, pipettes, tube racks, lab coat sleeves, etc.) and introduction of contaminants into reagents from the kit. The following guidelines should be followed to minimize 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 scZapR and scR-Probes (Section V.D.)
   - **A third work station located in the general laboratory** where you will purify the library (Sections V.C, V.D, 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 high-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 assay is very sensitive to variations in pipette volume. Please make sure that all pipettes are calibrated for reliable reagent delivery and that nothing adheres to the outsides 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.
- Do not change the amount or concentration of any of the components in the reactions; they have been carefully optimized for the SMART-Seq Stranded amplification reagents and protocol.
- **IMPORTANT:** If you are using this protocol for the first time, we strongly recommend that you perform negative (without RNA or cell) and positive (with provided Control Total RNA) control reactions. For the positive control, a good starting point is to use an input amount that matches the sample inputs. If working with single cells, a single-cell equivalent of 10 pg is desirable. However, first time users may experience difficulties getting library amounts above background; an input amount of 100 pg is recommended in this situation.
- **IMPORTANT:** Because this kit relies on random priming instead of priming specifically to the polyA tail, it is extraordinarily sensitive to environmental contaminants. The random primers can bind to any DNA or RNA molecule that finds its way into the sample. Therefore, extraordinary precautions need to be taken to ensure that both samples and kit reagents remain as clean as possible.
- Because of the large volume or viscosity of mixtures subject to the AMPure bead purification protocol, each round of purification requires a very strong magnet, particularly the final purification step (Section V.F). Never assume that bead separation will be completed within a given timeframe; when in doubt, leave samples on the magnet beyond the recommended time period until samples are completely clear.

C. Sample Recommendations and Requirements

This kit accommodates up to 7 µl of cells or purified RNA. The cDNA synthesis has been optimized for 1–1,000 intact mammalian cells, or 10 pg–10 ng of DNA-free mammalian total RNA.

Whether working with cells or purified RNA, we recommend processing at least eight samples at a time**.**

NOTE: If using RNA extracted from cells that have undergone fixation (e.g., RNA extracted from FFPE material), we recommend using no less than 1 ng. Lower inputs may generate acceptable results but need to be evaluated by the user on a case-by-case basis.

If starting directly from cells, this kit cannot be used with cells that have undergone fixation.

Input RNA Purity and Integrity

- **Volume and amount of input RNA:** Input RNA should be in a maximum volume of 7 µl. RNA quantity can vary from 10 pg to 10 ng.

- **Integrity of input RNA:** Degraded, partially degraded, or high-integrity RNA can be analyzed with this kit. Before starting the workflow, please determine the integrity of your RNA (RIN score or DV200) using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513). The section of this manual describing first-strand cDNA synthesis (Section V.A) includes alternate protocols for processing RNA inputs of varying integrity; **Option 1** includes a fragmentation step and is intended for cells as well as partially degraded or high-integrity RNA, while **Option 2** proceeds without fragmentation and is intended for highly degraded RNA. Please refer to Section V.A for further guidance.
- **Purity of input RNA:** Input RNA should be free from genomic or carrier DNA and contaminants such as organic compounds 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.

  - Environmental background can be captured during the RNA purification procedure and may become a significant proportion of the RNA sample if working with very low inputs. When purifying RNA from a small amount of starting material (e.g., plasma), a mock RNA purification should be performed and used for library preparation, even if it does not yield any measurable RNA. The nucleic acids obtained from a mock RNA purification will potentially generate a small amount of library—this can be sequenced and used to bioinformatically filter out the background.

Diluting the Control Total RNA

1. Prepare RNase Inhibitor Water (RRI Water) by combining 398 µl of Nuclease-Free Water with 2 µl of RNase Inhibitor. Mix by vortexing and keep on ice until the next step.
2. Dilute Control Total RNA (human brain) to 50 ng/µl by mixing 38 µl of RRI Water with 2 µl of Control Total RNA (1 µg/µl) in a sterile microcentrifuge tube.

NOTE: Fresh dilutions should be made before each use. If desired, make single-use aliquots of the 50 ng/µl dilution and store at –70˚C until needed, then further dilute (Steps 3–6). Make sure to change pipet tips for each dilution step described below. Non-sticky tubes should be used for all dilutions.

3. Further dilute Control Total RNA to 5 ng/µl by mixing 45 µl of RRI Water with 5 µl of 50 ng/µl Control Total RNA in a sterile microcentrifuge tube.
4. Further dilute Control Total RNA to 0.25 ng/µl by mixing 95 µl of RRI Water with 5 µl of 5 ng/µl Control Total RNA in a sterile microcentrifuge tube.
5. Further dilute Control Total RNA to 0.01 ng/µl by mixing 120 µl of RRI Water with 5 µl of 0.25 ng/µl Control Total RNA in a sterile microcentrifuge tube
6. Use 1 µl or more of 0.01 ng/µl Control Total RNA as a positive control RNA input for the kit and proceed alongside your samples.

NOTE: Try to match the input amount of Control Total RNA to the input amount of your own samples, and use the same number of PCR cycles. For first time users, it is highly recommended to start with no less than 100 pg.

Cell Input

- This protocol has been validated to generate libraries starting from intact cells, specifically cells that have been previously sorted and frozen. The cDNA synthesis protocol has been tested with cultured cells. **It cannot be used with cells that have undergone fixation.**
- If using fluorescence activated cell sorting (FACS), it is recommended to sort the cells in 7 µl of 1X PBS aliquoted in 96-well plates or 8-tube strips. Immediately after sorting, seal the samples with a foil or 8-cap strips, spin the plates/PCR strips for a few seconds to ensure the cells are within the liquid, and flash freeze in dry ice. Sorted cells should be stored at –70°C until use.
- When working with cultured cells, it is important to select a cell culture medium that does not inhibit first-strand cDNA synthesis. The protocol in this user manual was validated with cultured cells washed in cell-culture-grade PBS and labelled with a fluorescent antibody which recognizes a surface marker, then further washed in PBS and suspended in BD FACS Pre-Sort Buffer prior to sorting.

IMPORTANT: Cells should be washed and then resuspended in PBS that is free of Mg^{2+} and Ca^{2+} ions before sorting. The presence of media can interfere with the first-strand synthesis. If necessary, test the effect of your media on cDNA synthesis by performing a reaction with control RNA and the estimated amount of media that you expect to accompany your cell(s).

To maintain cells in a single-cell suspension prior to FACS, we recommend that the cells are resuspended in BD FACS Pre-Sort Buffer.

D. Sequencing Analysis Considerations

Trimming

When performing paired-end sequencing, the first three nucleotides of the second sequencing read (Read 2) are derived from the SMART-Seq Stranded Adapter (see Figure 3). For optimal mapping, these three nucleotides should be trimmed prior to mapping.

Strand-of-Origin Information

Read 1 matches the antisense sequence of the input RNA. If you are performing paired-end sequencing, Read 2 will correspond to the sense strand (see Figure 3).

E. Before You Start: Navigating Through Protocol Options

Shearing or No Shearing

In order to generate library inserts of an appropriate size for compatibility with Illumina sequencing, RNA molecules obtained from cell lysates as well as high-quality or partially degraded total RNA samples must be fragmented prior to cDNA synthesis. For highly degraded, low-quality starting material, the RNA fragmentation step should be skipped. Please refer to Option 2 for Protocol A (Section V.A) for guidance on how to proceed if you are skipping the fragmentation step.

Choosing Between the Ultra-Low- and Low-Input Workflow

The SMART-Seq Stranded Kit offers workflow choices which depend on input level, divided between ultra-low (1–50 cells or 10–500 pg total RNA) and low (50–1000 cells or 0.5–10 ng total RNA) inputs, with variances in pooling, PCR cycling, and cleanup steps (Figure 4 and Table I). As a general rule, ultra-low inputs require 10 cycles in the initial PCR (PCR1), and two AMPure bead cleanups after the final PCR (PCR2), while low inputs require only 5 cycles in PCR1 and a single AMPure bead cleanup after PCR2. Furthermore, the ultra-low workflow offers the possibility to pool samples after PCR1 if inputs are less than 10 cells or 100 pg total RNA. See Appendix A for extra protocol associated with pooling. Please note that while pooling is convenient when working with single cells, it is not required to achieve high-quality libraries, particularly for experienced users.

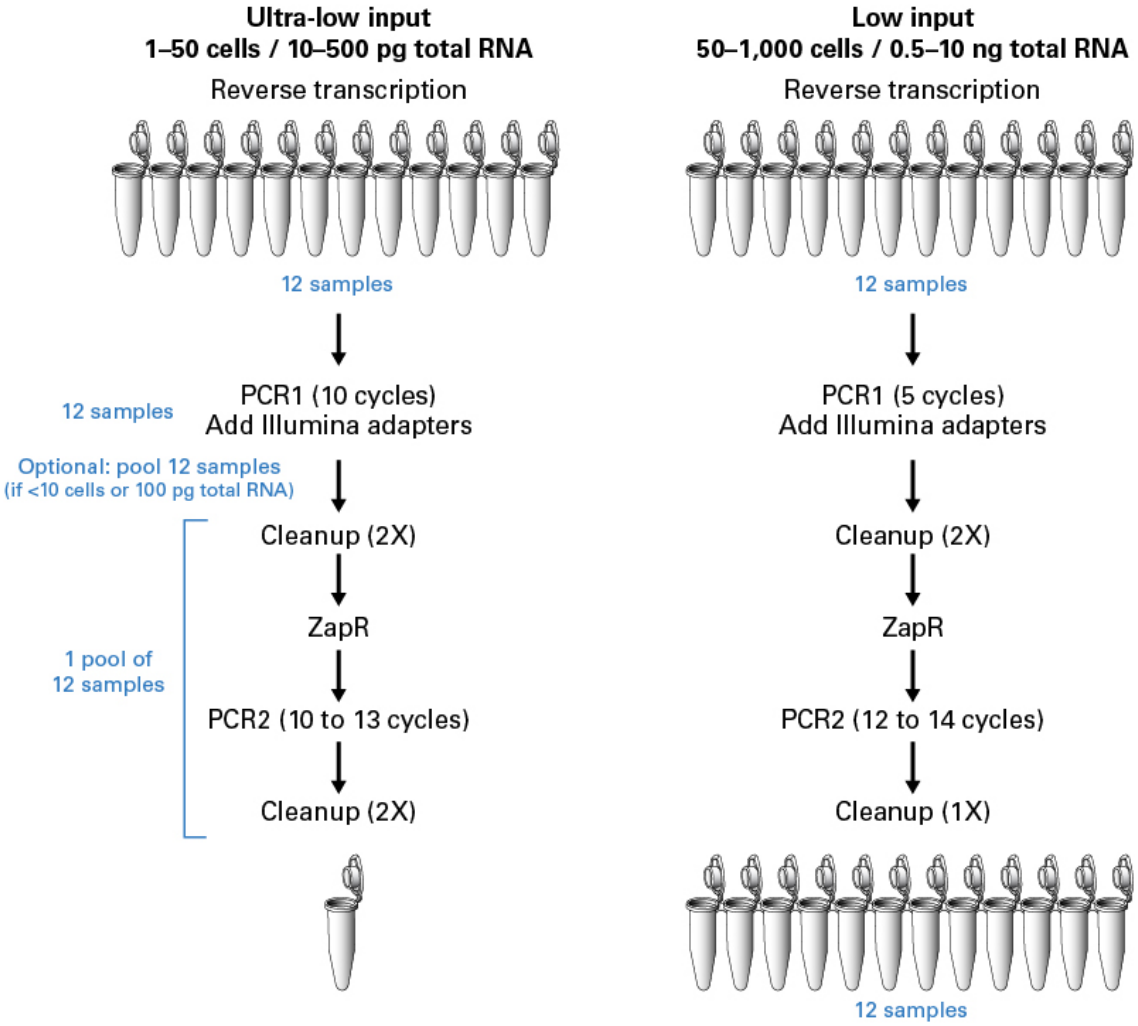

Figure 4. Summary of SMART-Seq Stranded Kit workflows based on input amounts. The SMART-Seq Stranded Kit can accommodate ultra-low input amounts (1–50 cells or 10–500 pg total RNA) as well as low input amounts (50—1,000 cells or 0.5–10 ng total RNA). The number of PCR cycles in PCR1 and PCR2 vary between the two options. The ultra-low input requires two rounds of cleanup with AMPure beads after PCR2, instead of a single cleanup in the low-input workflow. When following the ultra-low-input workflow with 1–10 cells or <100 pg total RNA, an optional pooling of 8–12 samples after PCR1 significantly reduces hands-on time for downstream steps.

Check Table I below for protocol details that correspond to your cell or RNA input.

Table I. Summary of SMART-Seq Stranded Kit protocols.

Input category	Cell input	RNA input	Post-PCR1 Pooling	PCR1 # of cycles	PCR2 # of cycles	# of final cleanups	Final elution volume (µl)
Ultra low	1	10 pg	Yes	10	12–13	2	12
			No	10	13		
	10	100 pg	Yes	10	10		
			No	10	11–12		
	10–50	100–500 pg	No	10	10		
Low	50–100	0.5–1 ng	No	5	14	1	22
	500	5 ng	No	5	13–14		
	1,000	10 ng	No	5	12–13		

NOTE: The guidelines in Table I must be validated with your material. When optimizing cycling conditions, only PCR2 cycles should be modified. We do not recommend performing more than 13 cycles for PCR2 in the ultra-low-input category, and no more than 16 cycles for PCR2 in the low-input category, as it will lead to substantial background amplification. We recommend that you perform a pilot experiment with a small number of samples to determine the optimal number of PCR2 cycles for your input material. In particular, an input of **500–1,000 cells** may require 1 extra PCR2 cycle because inhibitors associated with high cell input may make the library preparation less efficient than for lower cell inputs.

NOTE: Cycling recommendations in Table I are for cells or purified total RNA from normal tissues. If using RNA extracted from tissues that have been fixed or chemically modified in any way, the number of PCR2 cycles may need to be increased by at least 1–2 cycles.

F. Before You Start: Preprogramming the Thermal Cyclers

For convenience, set up the following programs (Table II) into your thermal cyclers before using this kit. Whenever indicated in the protocol, pre-heat the thermal cycler before or during reaction setup. Once the thermal cycler has reached the desired temperature for the first step, set it on hold. This ensures that the cycler is at the correct temperature when the samples are ready. Let the program resume after you place your samples in the thermal cycler. Run the program to completion.

Table II. Thermal cycler programs.

Program name	Program details		Used in
85-hold	85°C	forever	Section V.A (Option 1)
72-hold	72°C	forever	Section V.A (Option 2)
RT	42°C 70°C 4°C	90 min 10 min forever	Section V.A (Options 1 and 2)
PCR1	94°C 5 or 10 cycles: 98°C 55°C 68°C 68°C 4°C	1 min 15 sec 15 sec 30 sec 2 min forever	Section V.B
PreZap	72°C 4°C	2 min forever	Section V.D
Zap	37°C 72°C 4°C	60 min 10 min forever	Section V.D
PCR2	94°C 10–14 cycles: 98°C 55°C 68°C 4°C	1 min 15 sec 15 sec 30 sec forever	Section V.E

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. If starting from cells, the fragmentation step also contributes to cell lysis and release of the RNA content. Fragmentation time is adjusted depending on the quality of the RNA input. **Option 1 (with fragmentation)** should be used when starting from intact cells as well as purified RNA samples with RIN ≥4 or DV200 ≥60%, and **Option 2 (without fragmentation)** should be used when starting from samples that are already severely degraded. For samples with RIN ~4 and below, the RIN value is often unreliable, and DV200 is a better metric for evaluating the quality of the RNA input. Please refer to Table III (below) for guidelines. Option 2 should not be used for intact cells.

Table III. Recommended fragmentation protocol options and fragmentation time guidelines.

RNA quality	Protocol	Fragmentation time (min)
RIN ≥7 or cells	Option 1	6[1]
RIN 5–7	Option 1	4
RIN 4/DV200 ≥60%	Option 1	2[2]
DV200 <60%	Option 2	–

[1] The control RNA from this kit has a RIN of ~7.5 and should be fragmented for 6 min.
[2] We recommend optimizing the fragmentation time, as over-fragmentation may lead to reduced performance due to inefficient ribosomal cDNA depletion in Section V.D. When in doubt, choose a shorter fragmentation time or Option 2.

For this protocol, you will need the following components: 10X Lysis Buffer, SMART scN6, scRT Buffer, Nuclease-Free Water, SMART scTSO Mix, RNase Inhibitor, and SMARTScribe RT.

For all options:

1. Prepare RNase Inhibitor Water (RRI Water) by combining 199 µl of Nuclease-free water with 1 µl of RNase Inhibitor (scale up as needed). Mix by vortexing and keep on ice until needed.
2. Prepare a stock solution of 10X Lysis Mix by mixing 10X Lysis Buffer with RNase Inhibitor as indicated below (scale up as needed). Mix by pipetting up and down and keep on ice until needed.

19 µl	10X Lysis Buffer
1 µl	RNase Inhibitor
20 µl	Total volume

Option 1 (With Fragmentation): Starting from Intact Cells or High-Integrity / Partially Degraded Purified RNA

1. Make sure your RNA or cells are in a total volume of 7 µl, in 0.2-ml PCR tubes or strip tubes. If input is less than 7 µl, complete with RRI Water. Keep samples on ice.

   NOTE: Make sure to include a negative control with 7 µl of RRI water, in addition to any other mock or no-cell control.

2. Preheat the thermal cycler with the program "85-hold".
3. Prepare enough Shearing Master Mix for all reactions, plus 10%, by combining the following reagents on ice, in the order shown:

1 µl	10X Lysis Mix
1 µl	SMART scN6
4 µl	scRT Buffer
6 µl	Total volume per reaction

4. Add 6 µl of the Shearing Master Mix to each reaction tube and mix by tapping gently, then spin down.

   NOTE: When working with frozen cells, have the Shearing Master Mix ready before taking the cells out of the freezer. While keeping the cells on ice, add the shearing master mix to the cells as they are melting or no more than a few minutes after they have all melted. Mix by tapping gently, making sure there is no remaining ice, then spin down.

5. Incubate the tubes at **85°C** in a preheated, hot-lid thermal cycler for **6 min if starting from cells**; if starting from purified total RNA, follow the recommendations in Table II above (or use an experimentally determined, optimal amount of time), then immediately place the samples on an ice-cold PCR chiller rack for 2 min.

   NOTE: The samples should be taken out of the thermal cycler immediately after the time indicated to avoid over-fragmentation. Make sure to wait by the thermal cycler when the incubation time is over and immediately chill the samples. Then start the program "RT" and leave it on hold until Step 8.

   NOTE: The next reaction steps (Steps 6–7) are critical for first-strand synthesis and should not be delayed after Step 5. Start Step 6, preparing the First-Strand Master Mix, while your tubes are incubating (Step 5), or have it almost ready before starting Step 5.

6. 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	SMART scTSO Mix
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe RT
7 µl	Total volume per reaction

   NOTE: The SMART scTSO Mix is very viscous—it may be left at room temperature after thawing to facilitate accurate pipetting. Make sure to homogenize the First-Strand Master Mix very well by **vortexing for ~5 seconds** followed by a brief spin-down.

7. Add 7 µl of the First-Strand Master Mix to each reaction tube from Step 5. Mix the contents of the tubes by vortexing for ~5 sec, then spin the tubes briefly to collect the contents at the bottom.

   NOTE: The samples will be viscous—make sure to homogenize the content of the tubes very well.

8. Incubate the tubes in a preheated hot-lid thermal cycler with the program "RT":

42°C	90 min
70°C	10 min
4°C	forever

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

   SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not processed the next day, freeze the cDNA at –20°C for up to 2 weeks.

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

1. Make sure your RNA is in a total volume of 7 µl, in 0.2-ml PCR tubes. If input is less than 7 µl, complete with RRI Water. Keep samples on ice.

   NOTE: Make sure to include a negative control with 7 µl of RRI water, in addition to any other mock or no-cell control.

2. Preheat the thermal cycler with the program "72-hold".
3. Prepare enough Annealing Master Mix for all reactions, plus 10%, by combining the following reagents on ice, in the order shown:

1 µl	10X Lysis Mix
1 µl	SMART scN6
2 µl	Total volume per reaction

4. Add 2 µl of the Annealing Master Mix to each reaction tube and mix by tapping gently, then spin down.
5. Incubate the tubes at **72°C** in a preheated, hot-lid thermal cycler for exactly 3 min, then immediately place the samples on an ice-cold PCR chiller rack for 2 min. Start the program "RT" and leave it on hold until Step 7.

   NOTE: The samples should be taken out of the thermal cycler immediately after the time indicated to avoid fragmentation. Make sure to wait by the thermal cycler when the incubation time is over and immediately chill the samples. Then start the program "RT" and leave it on hold until Step 8.

   NOTE: The next reaction steps (Steps 6–7) are critical for first-strand synthesis and should not be delayed after Step 5. Start Step 6, preparing the First-Strand Master Mix, while your tubes are incubating (Step 5), or have it almost ready before starting Step 5.
6. Prepare enough First-Strand Master Mix for all reactions, plus 10%, by combining the following reagents on ice, in the order shown.

4 µl	scRT Buffer
4.5 µl	SMART scTSO Mix
0.5 µl	RNase Inhibitor
2 µl	SMARTScribe RT
11 µl	Total volume per reaction

   NOTE: The SMART scTSO Mix is very viscous—it may be left at room temperature after thawing to facilitate accurate pipetting. Make sure to homogenize the First-Strand Master Mix very well by **vortexing for 5 seconds** followed by a brief spin-down.
7. Add 11 µl of the First-Strand Master Mix to each reaction tube from Step 6. Mix the contents of the tubes by vortexing for ~2–3 sec, then spin the tubes briefly to collect the contents at the bottom.

   NOTE: The samples will be viscous—make sure to homogenize the content of the tubes very well.
8. Incubate the tubes in a preheated hot-lid thermal cycler with the program "RT":

42°C	90 min
70°C	10 min
4°C	forever

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

   SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not processed the next day, freeze the cDNA at –20°C for up to 2 weeks.

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, SeqAmp CB PCR Buffer (2X), SeqAmp DNA Polymerase, and 5' and 3' PCR Primer HT sets.

NOTE: If library purification (Section V.C) will be performed immediately following PCR1, remove aliquots of AMPure beads from the refrigerator to allow them to reach room temperature.

1. Preheat the thermal cycler with the program "PCR1".
2. Prepare a PCR1 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	SeqAmp CB PCR Buffer (2X)
1 µl	SeqAmp DNA Polymerase
28 µl	Total volume per reaction

NOTE: If the 3' index (i5) is going to be the same for all libraries, the 3' PCR Primer can also be added to the PCR1 Master Mix (1 µl/reaction). Typically, a single i5 index can be used if fewer than 12 libraries will be pooled for sequencing.

3. Add 28 µl (29 µl if the 3' PCR Primer is included) of PCR Master Mix to each sample from Step A.6.
4. Add 1 µl of each 5' and 3' PCR Primer HT to each sample. Mix by gentle vortexing or tapping of the tubes, then spin down briefly.
5. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the program "PCR1," making sure to choose the appropriate number of cycles for your input level (either 5 or 10 cycles, depending.)

94°C		1 min
5 or 10 cycles*:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
68°C		2 min
4°C		forever

***IMPORTANT: Use 10 cycles if following the ultra-low input workflow, and 5 cycles if following the low-input workflow (see Table I.)**

SAFE STOPPING POINT: Samples can be left for up to 1 hr in the thermal cycler at 4°C. If not processed within the next hour, freeze the PCR products at –20°C for up to 2 weeks.

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. The purification is then performed a second time, starting in Section V.C and finishing in Section V.D.

If inputs are less than 10 cells or 100 pg, it is possible to pool the samples (grouping 8–12 samples) prior to library preparation. Skip this section and follow the recommendations in Appendix A instead, then resume the current protocol with Section V.D.

IMPORTANT: Do not start Section V.C if you do not have enough time to perform all steps up to Section V.E.

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.

IMPORTANT: Remove ZapR Buffer from –20°C storage and thaw it at room temperature in preparation for Section V.D.

1. Allow AMPure beads to come to room temperature before use (~30 min). Add 35 µl of AMPure beads to each sample.

   NOTE: Mix by vortexing for 5 sec. The beads are viscous; pipette the entire volume up and then out slowly. Accurate pipetting of AMPure beads is critical.
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 separation device, pipette out the supernatant and discard.
5. Keeping 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 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. Briefly spin the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic separation device 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 5 min until the pellets appear dry.

   NOTE: You may see a tiny crack in each pellet when dry. Do not over-dry.
9. Once the beads are dry, add 52 µl of Nuclease-Free Water to cover the beads. Close the tubes, remove from the magnetic separation device and mix thoroughly by vortexing 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 5 min or longer, until the solution is completely clear.

    NOTE: The solution may appear clear after only 1 min, but still incubate for 5 min.
12. Pipette 50 µl of supernatant from each sample into respective wells of a new 8-well strip.
13. Add 40 µl of AMPure beads to each sample and mix well by vortexing for 5 sec.

    NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.
14. Incubate at room temperature for 8 min to allow the DNA to bind to the beads. During the incubation time, proceed immediately to Section V.D.

D. Protocol: Depletion of Ribosomal cDNA with scZapR and scR-Probes

In this section, the library fragments originating from rRNA (18S and 28S) and mitochondrial rRNA (m12S and m16S) are cut by scZapR in the presence of scR-Probes (mammalian-specific). These scR-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: scR-Probes, scZapR, ZapR Buffer, Nuclease-Free Water, and a magnetic separation device.

1. Thaw scR-Probes and ZapR Buffer at room temperature. Place scR-Probes on ice as soon as it is thawed, but keep ZapR Buffer at room temperature. scZapR should be kept on ice at all times and returned to the freezer immediately after use.
2. Preheat the thermal cycler with the program "PreZap".
3. Upon completion of the 8-min incubation in Step V.C.14, 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.

   NOTE: It is acceptable—and in some cases necessary—to leave the tubes on the magnetic separation device for more than 5 min.

4. During the 5-min incubation time in Step V.D.3, pipette into a pre-chilled PCR tube a sufficient volume of scR-Probes for the number of reactions to be performed—1.5 µl per reaction (see Step V.D.12)—plus 10% to account for pipetting errors. Keep the PCR tube containing scR-Probes on ice and immediately return the remaining unused scR-Probes to a –70°C freezer.
5. Incubate the PCR tube containing scR-Probes at 72°C in a preheated hot-lid thermal cycler using the program "PreZap":

72°C	2 min
4°C	forever

6. Leave the scR-Probes tube in the thermal cycler at 4°C for at least 2 min, but for no more than 10–15 min, before using it in the next step (Step V.D.12).
7. Once the 5-min incubation on the magnetic separation device is complete (Step V.D.3) and the samples are clear, pipette out the supernatant and discard, while keeping the tubes sitting on the magnetic separation device.
8. Keeping 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 sec and carefully pipette out and discard the supernatant. cDNA will remain bound to the beads during the washing process.
9. Repeat Step 8 once.
10. Briefly spin the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic separation device for 30 sec, then carefully remove any remaining ethanol with a pipette, without disturbing the beads.
11. Let the open sample tubes rest at room temperature until the pellets appear dry.

    NOTE: The beads will dry more quickly than in Step V.C.8. 1–2 min may be sufficient, but the beads can be left to dry for up to 5 min during preparation of the scZapR Master Mix in Step V.D.12.

12. While the beads are drying, prepare the scZapR Master Mix. Prepare enough Master Mix for all reactions, plus 10%, by combining the following reagents at room temperature in the order shown. Make sure to add the preheated and chilled scR-Probes from Step V.D.6 last. Return scZapR to a –20°C freezer immediately after use. Mix the components well by vortexing briefly, and spin the tubes briefly in a microcentrifuge.

16.8 µl	Nuclease-Free Water
2.2 µl	10X ZapR Buffer
1.5 µl	scZapR
1.5 µl	scR-Probes
22 µl	Total volume per reaction

13. To each tube of dried AMPure beads from Step V.D.11, add 22 µl of the scZapR Master Mix. Close the tubes, remove the tubes from the magnetic separation device and mix thoroughly by vortexing to resuspend the beads.
14. Incubate at room temperature for 5 min to rehydrate.
15. Briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 1 min or longer, until the solution is completely clear.
16. Pipet out 20 µl of supernatant, being careful not to disturb the beads, into a new PCR strip.
17. Incubate the tubes in a preheated hot-lid thermal cycler using the program "Zap":

37°C	60 min
72°C	10 min
4°C	forever

NOTE: Samples can be left in the thermal cycler at 4°C for up to 1 hr. However, we recommend proceeding immediately to Section V.E.

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

In this section, the library fragments not cleaved by the scZapR 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, SeqAmp CB PCR Buffer (2X), PCR2 Primers and SeqAmp DNA Polymerase.

1. Preheat the thermal cycler with the program "PCR2".
2. Prepare a PCR2 Master Mix for all reactions, plus 10%, by combine the following reagents in the order shown below. Then mix well and spin the tubes briefly in a microcentrifuge.

26 µl	Nuclease-Free Water
50 µl	SeqAmp CB PCR Buffer
2 µl	PCR2 Primers
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 4).

3. Add 80 µl of PCR2 Master Mix to each tube from Step V.D.17. Mix by tapping gently, then spin down.
4. Place the tubes in a preheated hot-lid thermal cycler. Perform PCR using the program "PCR2" and the recommended number of cycles according to Table I:

94°C		1 min
10–14 cycles:		
	98°C	15 sec
	55°C	15 sec
	68°C	30 sec
4°C		forever

SAFE STOPPING POINT: Samples can be left overnight in the thermal cycler at 4°C. If not processed within the next day, freeze the PCR products at –20°C for up to 2 weeks.

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

In this section, the amplified RNA-seq library is purified by immobilization onto AMPure beads. The beads are then washed with 80% ethanol and eluted in Tris Buffer. If following the low-input workflow, proceed until Step F.12. If following the ultra-low-input workflow, perform the entire protocol, which includes a second bead cleanup. This is necessary to completely remove all adapter dimers that may have accumulated in the ultra-low-input workflow.

For this protocol, you will need the following components: AMPure beads (at room temperature), 80% ethanol (freshly prepared), Tris 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 and mix well by vortexing for 5 sec.

   NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.
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 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 volumes.
4. While the tubes are sitting on the magnetic separation device, pipette out the supernatant and discard.
5. Keep the tubes on the magnetic separation device. 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 separation device 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.

   Once the beads are dry, add 22 µl of Tris Buffer to cover the beads. Close the tubes, remove from the magnetic separation device and mix thoroughly by vortexing until all the beads have been washed off the sides of the tubes.
9. Incubate at room temperature for 5 min to rehydrate.
10. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 5 min or longer, until the solution is completely clear.

11. Transfer the supernatants (20 µl) to new PCR tubes.

    NOTE: If following the low-input workflow, stop here and instead transfer the supernatants to non-sticky low-bind tubes. The supernatants obtained in Step F.12 are the final libraries and are ready for validation (Section G).

12. Perform a second beads clean-up by adding **20 µl** of AMPure beads to each sample. Mix by vortexing for 5 sec.

    NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.

13. Incubate at room temperature for 8 min to let the DNA bind to the beads.
14. 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.
15. While the tubes are sitting on the magnetic stand, pipette out the supernatant.
16. 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.
17. Repeat Step 16 one more time.
18. 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.
19. Let the sample tubes rest open at room temperature for 5 min until the pellet appears dry. You may see a tiny crack in the pellet.
20. Once the beads are dry, add 12 µl of Tris Buffer to cover the beads. Close the tubes, remove the tubes from the magnetic separation device and mix thoroughly by vortexing until all the beads have been washed off the sides of the tubes.
21. Incubate at room temperature for 5 min to rehydrate.
22. 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.
23. Transfer the supernatants (10–11 µl) to non-sticky low-bind tubes.
24. Proceed immediately to validation (Section V.G.) or store at –20°C.

G. Protocol: Validation Using the Qubit and Agilent 2100 Bioanalyzer

1. Quantify libraries with Qubit dsDNA HS kit (Thermo Fisher Scientific). A yield >3 ng/µl will provide enough material for further library validation and sequencing. Consider adding one PCR2 cycle in subsequent experiments if the yield is insufficient, or reducing cycles if the yield is more than 10 ng/µl. However, never perform more than 23 cycles total (PCR1 and PCR2 combined).

   NOTE: Background may be visible in your negative controls, particularly when using 22–23 cycles total. If performing 23 cycles total, a negative control yielding 0.5–1 ng/µl is not unusual, thus obtaining a detectable library in positive samples does not mean it is a successful library. Background is acceptable as long as the positive samples are 3x the value of the negative control.

2. Evaluate library size distribution by running samples on the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 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 5 for examples of a successful library.
3. Compare the results for your samples and controls to determine whether samples are suitable for further processing.
4. Validation guidance:

   - Successful cDNA synthesis and amplification should produce a distinct curve spanning 200–2,000 bp, with a local maximum at ~300–450 bp, in the positive control RNA sample (see Figure 5, Panels A and B) and no product or very minimal background over the corresponding range in the negative control (see Figure 5C).

   - No-template controls typically display a higher amount of PCR products between 1,000 and 2,000 bp than between 200 and 1,000 bp. No-template controls included in a pool cannot be evaluated based on profile—this is one drawback of the pooling option. However, as seen in Figure 5D, our internal validation has shown that the typical profile from a pool of 12 no-template controls is similar to the typical profile from an individual no-template control sample (Figure 5C).

   - A small amount of product ~150–200 bp in size, such as those found in the example in Figure 5D, will not interfere with sequencing. However, consider repeating the AMPure bead cleanup (with a bead:sample ratio of 1:1 as in Section V.F) if an excessive amount of product <200 bp in size is present.

   - Some first-time users may find that their library yield, particularly for the total RNA positive control (using human brain total RNA, provided in the kit), is lower than specified in our Certificate of Analysis (see also the Validation section). This could be due to extra losses in the bead cleanup after PCR1, or losses when mixing the samples at various steps (most steps call for mixing by vortexing to minimize losses, since pipetting up and down leads to loss of liquids in the pipette tips). If the total RNA control provides lower than anticipated yield, the yield of a no-template control would be similarly low. **Thus, it is important to include negative controls every time the kit is used, not only when it is tested the first time.**

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

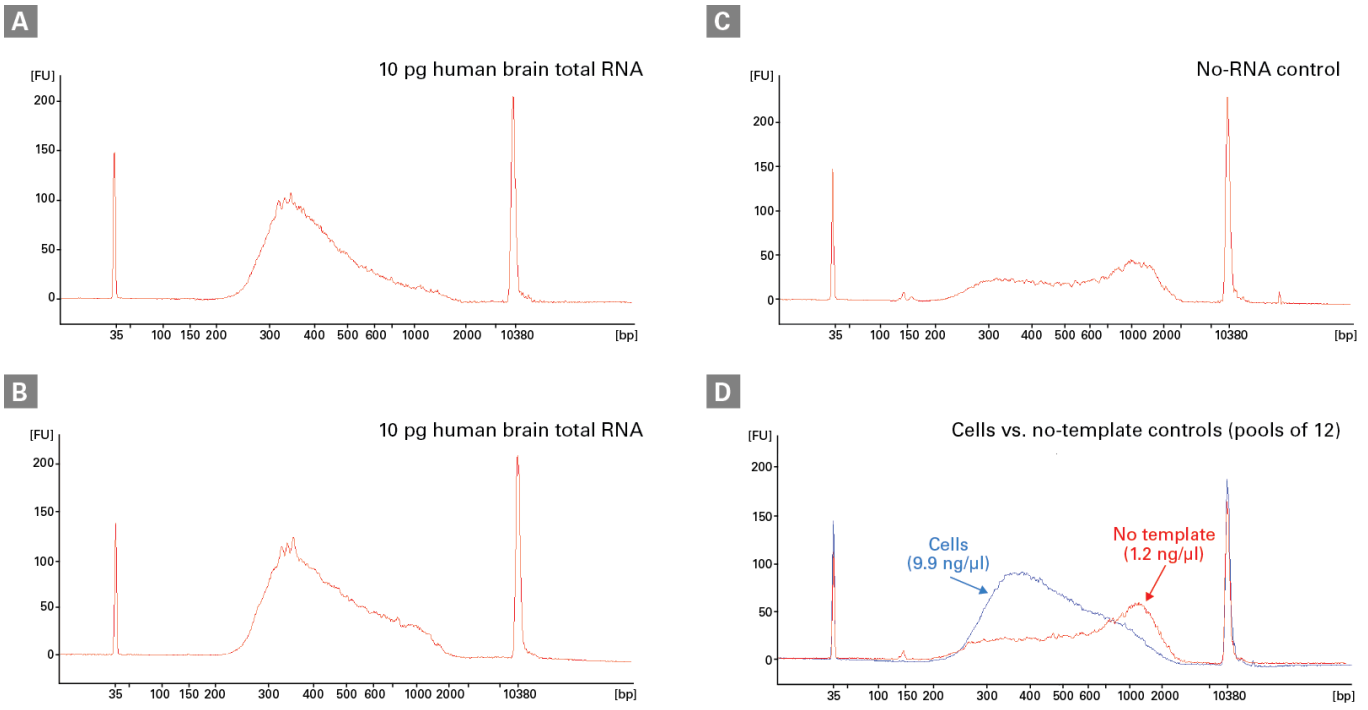

Figure 5. Example electropherogram results from the Agilent 2100 Bioanalyzer. Libraries were generated using 10 pg of Control Total RNA (human brain) (**Panels A and B**) or a no-RNA control (**Panel C**) using the ultra-low workflow, without any pooling and 13 cycles in PCR2. **Panel D** shows an example of libraries generated directly from single cells using the ultra-low workflow, with pooling of 12 samples and 12 cycles in PCR2. The no-template control also shown in **Panel D** was prepared similarly, with pooling of 12 samples, but generated a yield significantly lower than the yield obtained from the pool of 12 cells. All libraries were diluted to 1.5 ng/µl (based on Qubit quantification) prior to analysis with a High Sensitivity DNA Kit (Agilent), except for the negative controls which were analyzed without dilution. Note that the no-RNA control exhibits a small amount of background <200 bp, and typically shows some amount of PCR product between 200 and 1,000 bp, in addition to a larger amount of product >1,000 bp. This background is typically unavoidable but acceptable as long as the libraries from the RNA samples contain a significantly larger amount of material (at least three times more, as measured by Qubit).

VI. References

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Appendix A: Pooling Strategy for Single-Cell Applications

When processing single cells, it can be convenient to pool samples after PCR1 in order to minimize the number of samples to process downstream. Some users may find that the pooling helps them get better, more consistent data from single cells, because sample loss should be minimal. One caveat is that if re-sequencing of particular samples is going to be required, the entire pool will need to be re-sequenced. Failed wells—those that did not receive a cell or in which the cell was dead—are likely to have a yield similar to the no-template control. In general, we find that failed samples are only a small fraction of the reads, thus there should be no concern over wasting reads by sequencing failed samples using the pooling method. If the viability of the sorted cells is a concern, a small number of cells can be processed for a small-scale evaluation prior to processing a large number of samples.

We recommend pooling no less than 8 samples and no more than 12 samples at a time. Pooling more than 12 samples has not been validated and is not recommended. For example, 48 samples can be processed as 4 groups of 12 samples (or 6 groups of 8 samples) resulting in only 4 (or 6) samples to handle in subsequent steps. The final samples will then represent pools of 12 (or 8) individuals and will not be distinguishable from each other except through sequencing. In this example, the 4 or 6 samples should be carefully quantified and further mixed in equimolar amounts (see recommendations in Appendix B) prior to sequencing.

Pooling after PCR1 has been fully validated only for inputs ranging from 1–10 cells, or 10–100 pg. Do not use the pooling strategy for higher inputs. It is also critical to ensure that each sample receive a distinct set of i5 and i7 indexes during PCR1.

The pooling strategy still requires the use of the entire PCR1 reaction volume (50 µl) for each sample. Do not attempt to use less than the entire reaction volume. Any loss of material at this stage will affect the quality of the final library. The input amounts are so low that every effort should be taken to recover every last µl of each sample while pooling. The protocol below replaces protocol V.C. if you are pooling samples. When you are finished with this protocol, return to the main protocol and continue with Section V.D.

IMPORTANT: Do not start this protocol if you do not have enough time to perform all steps up to Section V.E.

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 for 1.5 ml tubes.

IMPORTANT: Remove ZapR Buffer from –20°C storage and thaw it at room temperature in preparation for Section V.D.

1. Allow AMPure beads to come to room temperature before use (~30 min).
2. After completion of PCR1 in Step B.5, transfer the entire volume (50 µl) into a 1.5-ml LoBind tube; transfer no less than 8 and no more than 12 samples. The volume should be between 400 µl and 600 µl of pooled PCR product.

NOTE: Use a single pipet tip for all the samples that will be included in any given pool, and after the last sample has been added to the pool, rinse the tip into the pooled samples by performing several up and down, then slowly expulse the content of the tip. Make sure to change pipet tip between pools.

3. Add AMPure beads as shown in Table IV below. The beads are viscous and even when using such a large volume, it is critical to pipette the entire volume up and then out very slowly.

Table IV. AMPure bead volume for various numbers of pooled samples.

Number of samples in pool	Total sample volume (µl)*	AMPure bead volume (µl)
8	400	260
9	450	293
10	500	325
11	550	358
12	600	390

*Because of the viscosity of the samples, actual volumes will be slightly lower than those listed in Table IV. Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.

4. Mix by vortexing for 5 sec.
5. Incubate at room temperature for 8 min to allow the DNA to bind to the beads.
6. Briefly spin the sample tubes to collect the liquid at the bottom. Place the sample tubes on the magnetic separation device for 10 min or longer, until the solution is completely clear.

NOTE: Because of the large volume involved, a very strong magnet is required. Make sure the solution is completely clear before moving on to the next step. Failure to recover all of the beads will lead to low-yield and low-quality libraries.

7. While the tubes are sitting on the magnetic separation device, pipette out the supernatant and discard.
8. Keeping the tubes on the magnetic separation device, add 1 ml of **freshly made** 80% ethanol to each sample—without disturbing the beads—to wash away contaminants. Wait for 1 min and carefully pipette out and discard the supernatant. cDNA will remain bound to the beads during the washing process.
9. Repeat Step 8 once.
10. Briefly spin the tubes (~2,000*g*) to collect the remaining ethanol at the bottom of each tube. Place the tubes on the magnetic separation device for 30 sec or more, then carefully remove any remaining ethanol with a pipette, without disturbing the beads.
11. Let the open sample tubes rest at room temperature for 10 min until the pellets appear dry.
12. Once the beads are dry, add 52 µl of Nuclease-Free Water to cover the beads. Close the tubes, remove the tubes from the magnetic separation device and mix thoroughly by vortexing until all the beads have been washed off the sides of the tubes.
13. Incubate at room temperature for 5 min to rehydrate.
14. Briefly spin the sample tubes. Place the sample tubes on the magnetic separation device for 5 min or longer, until the solution is completely clear. The solution may appear clear after only 1 min, but still incubate for 5 min.
15. Pipette 50 µl of supernatant from each sample into respective wells of a new 8-well strip.
16. Add 40 µl of AMPure beads to each sample and mix well by vortexing for 5 sec.

NOTE: Accurate pipetting of AMPure beads with a well-calibrated pipet is critical.

17. Incubate at room temperature for 8 min to allow the DNA to bind to the beads. During the incubation time, proceed immediately to Section V.D.

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, Inc., Cat. No. 638324).

Most Illumina sequencing library preparation protocols require libraries with a final concentration of 4 nM or higher, depending on the sequencing platform. Lower concentrations can also be accommodated, depending on the instrument. Below is an example when pooling libraries normalized to 4 nM.

Prepare a pool of 4 nM as follows:

1. Dilute each library to 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 errors.
3. Depending on the Illumina sequencing library preparation protocol, use a 5-µl aliquot or a 10-µl aliquot 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. Illumina recommends the systematic inclusion of ~1% PhiX to help assess run performance and troubleshooting. Libraries generated with the SMART-Seq Stranded Kit do not require the inclusion of extra PhiX beyond the typical 1%. However, we cannot guarantee that your particular sample type and RNA input amount will display the well-balanced nucleotide diversity required for base calling. If in doubt, include 5–10% PhiX.

- Follow Illumina guidelines on how to denature, dilute, and combine a PhiX control library with your own pool of libraries. Make sure to use a fresh and reliable stock of the PhiX control library.

Additional Considerations if Pooling Samples after PCR1

This kit offers the option of pooling samples after PCR1 if samples contain 10 cells or less (see Appendix A). In that case, each final library is already a group of 8–12 samples. If multiple groups have been generated (e.g., if analyzing a total of 48 cells as 4 groups of 12 samples, or 6 groups of 8 samples), all groups should be normalized to 4 nM and then combined in equimolar amounts as described in Appendix A.

Loading Guidelines for Various Illumina Instruments

Libraries generated with the SMART-Seq Stranded Kit cluster very efficiently and care must be taken to avoid over-clustering. The guidelines in Table V (below) are a good starting point and have been fully validated. The SMART-Seq Stranded Kit has not been validated on the HiSeq 3000/4000, but the related SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian has been validated and the sequencing performance should be similar.

Table V. Library loading guidelines for various Illumina sequencing instruments.

Sequencing instrument	Loading concentration (pM)
MiSeq – v2 chemistry	8
MiSeq – v3 chemistry	10
MiniSeq	1.2
NextSeq 500/550	1.1–1.2

Extra Precautions When Using NextSeq and MiniSeq Instruments

Libraries generated with the SMART-Seq Stranded Kit perform extremely well when sequenced 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 SMART-Seq Stranded Kit is good at retaining small inserts. Therefore, we strongly recommend that you turn off automatic adapter trimming by creating a custom library prep kit 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 2), NextSeq and MiniSeq runs may display incorrect base calling in the first three bases. Because those bases are trimmed, it does not affect the quality of the data obtained. However, we still strongly recommend avoiding overloading of the instrument.

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