Takara Bio USA

SMARTer® Universal Low Input RNA Kit for Sequencing User Manual

Cat. Nos. 634938, 634940, 634945, 634946
(112219)

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I. Introduction: SMARTer cDNA Synthesis for Next-Generation Sequencing from Compromised Samples

A variety of techniques have been developed for transcriptome analysis by next-generation sequencing. Oligo dT-primed reverse transcriptase kits provide excellent results from well-preserved mRNA (RIN>8), but many common sample preparation techniques, including FFPE (formaldehyde fixed paraffin embedded tissue) and laser-capture dissection lead to degraded samples (RIN <7). The SMARTer Universal Low Input RNA Kit uses random (N6) primers to produce NGS-quality cDNA from low concentrations of degraded samples.

The SMARTer Universal Low Input RNA Kit allows high-quality cDNA synthesis starting from as little as 200 pg of input RNA. The kit has been validated to prepare cDNA samples for sequencing and RNA expression analysis with next-generation sequencing instruments. The entire library construction protocol can be completed in two days (Figure 1). SMART® technology offers unparalleled sensitivity and unbiased amplification of RNA transcripts, while random priming allows for amplification of damaged RNA and maintains the true representation of the original RNA sample. Both of these factors are critical for transcriptome sequencing and gene expression analysis.

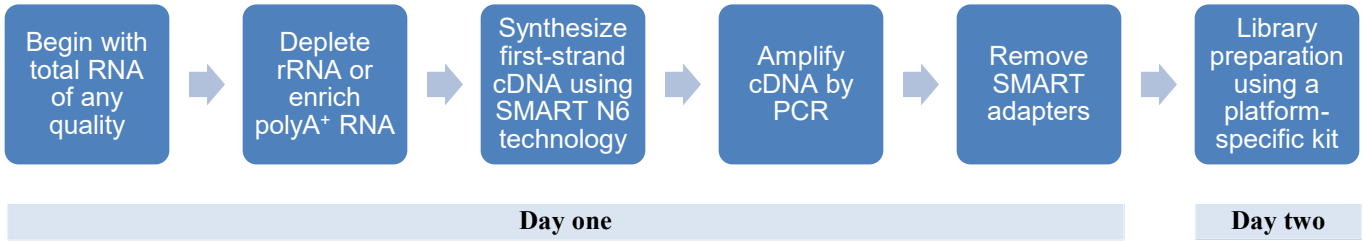
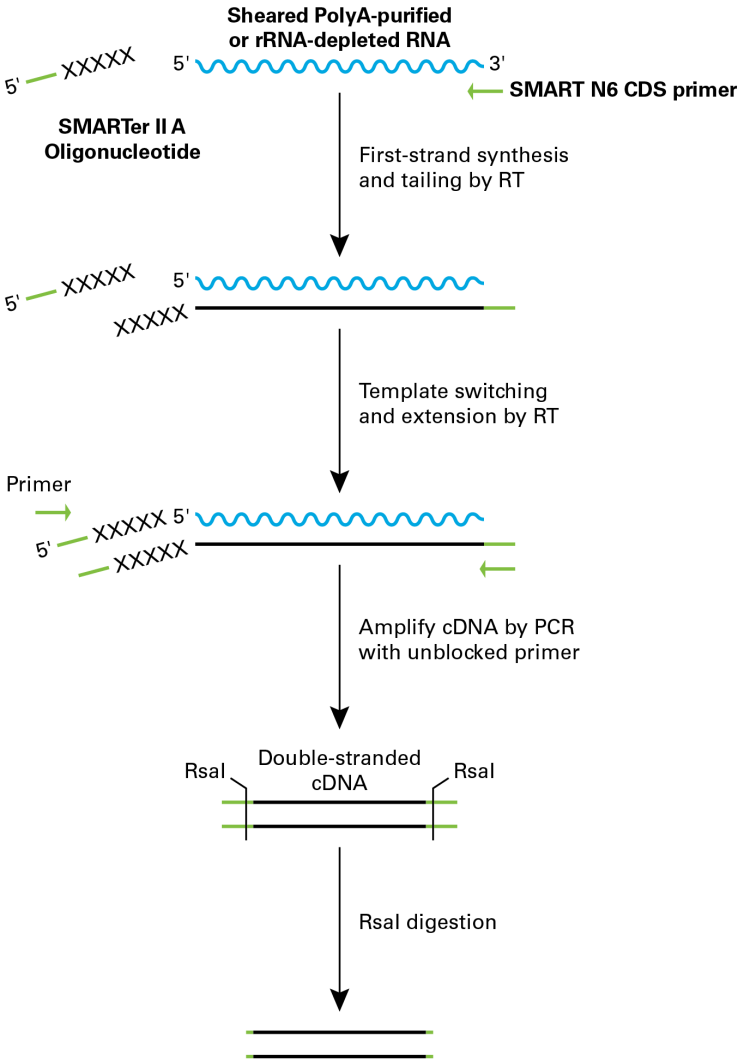


Figure 1. NGS workflow: SMARTer Universal Low Input RNA Kit for Sequencing.

The SMARTer Universal Low Input RNA Kit starts with picogram amounts of input RNA. A modified N6 primer (the SMART N6 CDS Primer) primes the first-strand synthesis reaction (Figure 2). When SMARTScribe™ Reverse Transcriptase reaches the 5' end of the RNA fragment, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3' end of the cDNA. The carefully-designed SMARTer Oligonucleotide base-pairs with the non-template nucleotide stretch, creating an extended template to enable SMARTScribe RT to continue replicating to the end of the oligonucleotide (Chenchik et al. 1998). The resulting single-stranded (ss) cDNA contains sequences that are complementary to the SMARTer Oligonucleotide. The SMARTer anchor sequence and the N6 sequence serve as universal priming sites for DNA amplification by PCR.



1. **First-strand synthesis and tailing by SMARTScribe Reverse Transcriptase.** The SMARTer Universal Low Input RNA Kit for Sequencing starts with sheared polyA-purified or rRNA-depleted RNA and a modified N6 primer called the SMART N6 CDS Primer. The SMARTScribe Reverse Transcriptase produces the complementary DNA strand. When SMARTScribe reaches the 5' end of the RNA, its terminal transferase activity adds a few additional nucleotides to the 3' end of the cDNA.
2. **Template switching and extension by SMARTScribe Reverse Transcriptase.** The SMARTer Oligonucleotide base-pairs with this non-template nucleotide stretch, creating an extended template to enable SMARTScribe to continue replicating to the end of the oligonucleotide (Chenchik et al. 1998).
3. **cDNA amplification.** The SMARTer anchor sequence and the modified N6 sequence serve as universal priming sites for cDNA amplification by PCR.
4. **Adapter removal.** Finally, amplified cDNA is digested with RsaI to remove the SMART adapter prior to sequencing.

Figure 2. SMARTer technology for cDNA synthesis from compromised samples. The SMARTer Universal Low Input RNA Kit for Sequencing starts with 200 pg–10 ng of input RNA and a modified N6 primer (where N = A, G, T, or C) called the SMART N6 CDS Primer, and produces cDNA libraries suitable for transcriptome profiling. The SMARTer II A Oligonucleotide, 3' SMART N6 CDS Primer II A, and PCR Primer I IA all contain a stretch of identical sequence.

II. Required Materials

The following components have been specifically designed to work together and are optimized for this protocol. Please do not make any substitutions. Substituting reagents in the kit and/or modifying the protocol may lead to unexpected results.

A. Kit Components

Unlike many cDNA synthesis kits for RNA-Seq, the SMARTer Universal Low Input RNA Kit for Sequencing is a complete solution which includes SMARTScribe Reverse Transcriptase (RT) and an Advantage® 2 PCR Kit. SMARTScribe RT amplifies rare transcripts, while preserving the relative transcript proportions of the original RNA sample. The Advantage 2 PCR Kit provides exceptionally high yields and sensitivity. Its fidelity is three times higher than that of regular *Taq*, making Advantage 2 perfect for SMARTer cDNA synthesis.

SMARTer Universal Low Input RNA Kit for Sequencing	**634938** (10 rxns)	**634940** (25 xns)
SMARTer Universal Low Input RNA Kit Components (Not sold separately. Storage conditions are listed below for Package 1 and Package 2.)		
Package 1 (Store at –70°C.)		
SMARTer II A Oligonucleotide (24 µM)	10 µl	25 µl
Control Sheared Total RNA (50 ng/µl)	10 µl	25 µl
Package 2 (Store at –20°C. Once thawed, store the Purification Buffer at Room Temperature. Continue to store all other reagents at –20°C.)		
3’ SMART N6 CDS Primer II A (24 µM)	10 µl	25 µl
PCR Primer IIA (12 µM)	20 µl	50 µl
5X First-Strand Buffer (RNase-Free)	40 µl	100 µl
SMARTer dNTP Mix (dATP, dCTP, dGTP, and dTTP, 20 mM each)	10 µl	25 µl
Dithiothreitol (DTT; 100 mM)	10 µl	25 µl
BSA (0.1%)	20 µl	50 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	20 µl	50 µl
Nuclease-Free Water	1 ml	2 x 1 ml
RNase Inhibitor (40 U/µl)	55 µl	140 µl
Purification Buffer (10 mM Tris-Cl, pH 8.5)	1 ml	2 x 1 ml
RsaI (10 U/µl)	10 µl	25 µl
RsaI Buffer (10X)	20 µl	50 µl

Advantage 2 PCR Kit (Store at –20°C.)

50X Advantage 2 Polymerase Mix	30 µl	2 x 30 µl
10X Advantage 2 PCR Buffer	200 µl	2 x 200 µl
10X Advantage 2 SA PCR Buffer	200 µl	2 x 200 µl
50X dNTP Mix (10 mM each)	50 µl	2 x 50 µl
Control DNA Template (100 ng/µl)	30 µl	2 x 30 µl
Control Primer Mix (10 µM each)	30 µl	2 x 30 µl
PCR-Grade Water	2 x 1.25 ml	4 x 1.25 ml

NOTE: The SMARTer Universal Low Input RNA Kit Components (Cat. Nos. 634939 & 634941) and Advantage 2 PCR Kit (Cat. No. 639207) both include dNTP mixes.

- Use the SMARTer dNTP Mix (20 mM each dNTP) for first-strand cDNA synthesis (Section IV.A.5).
- Use the Advantage 2 dNTP Mix (10 mM each dNTP) for double-stranded cDNA amplification (Section IV.C.1).

Storage Conditions:

- Store Sheared Control Total RNA and SMARTer IIA Oligonucleotide at –70°C.
- Store Purification Buffer at –20°C. (Once thawed, the buffer can be stored at room temperature.)
- Store all other reagents at –20°C.

B. Additional Materials Required

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

- Single channel pipette: 10 µl, 20 µl and 200 µl, one each
- Eight channel pipette: 20 µl and 200 µl, one each
- Filter pipette tips: 10 µl, 20 µl and 200 µl, one box each
- One QuickSpin minicentrifuge for 1.5 ml tubes
- One QuickSpin minicentrifuge for 0.2 ml tubes

For PCR Amplification & Validation:

- A dedicated PCR thermal cycler, used *only* for first-strand synthesis
- High Sensitivity DNA Kit (Agilent, Cat No. 5067-4626)
- IsoFreeze Flipper Rack (MIDSCI, Cat. No. TFL-20)
- IsoFreeze PCR Rack (MIDSCI, Cat. No. 5640-T4)
- Nuclease-free thin-wall PCR tubes (0.2 ml; USA Scientific, Cat. No.1402-4700)
- Nuclease-free nonsticky 1.5 ml tubes (USA Scientific, Cat. No. 1415-2600)

For SPRI Bead Purification:

- Agencourt AMPure PCR Purification Kit
  (5 ml kit: Beckman Coulter, Part No. A63880; 60 ml kit: Beckman Coulter, Part No. A63881)
- MagnaBot II Magnetic Separation Device (Promega Part No. V8351)
  Use this stand for the first purification (Protocol IV.B)
- Magnetic Stand-96 (Ambion Part No. AM10027)
  Use this stand for the second purification (Protocols IV.D and IV.F)
- 96-well V-bottom Plate (500 µl) (VWR Cat. No. 47743-996)
- MicroAmp Clear Adhesive Seal (Life Technologies, Part No. 4306311)
- 80% ethanol

For Ribosomal RNA Removal:

We strongly recommend removing ribosomal RNA prior to cDNA synthesis using the SMARTer Universal kit. For this purpose, we have developed the RiboGone™ - Mammalian rRNA removal kit (Cat. Nos. 634846 and 634847). This kit specifically and efficiently degrades *5S*, *5.8S*, *18S*, and *28S* nuclear rRNA and *12S* mtRNA from human, mouse, and rat total RNA samples, and is compatible with 10–100 ng of input RNA.

For Illumina® Library Preparation:

We recommend our ThruPLEX® DNA-Seq Kit (Cat. Nos. R400674, R400675, R400676, or R400677, depending on the number of reactions being processed). Indexes are also required.

III. General Considerations

A. Requirements for Preventing Contamination

Before you set up the experiment, **make sure you have two physically separated work stations**:

- **A PCR Clean Work Station** for all pre-PCR experiments that require clean room conditions, e.g. first-strand cDNA synthesis (Protocol IV.A) and first-strand cDNA purification (Protocol IV.B).

  NOTES:
  - 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.
  - Strictly obey clean room operation rules.

- **A second work station located in the general laboratory** where you will perform PCR (Protocol IV.D), purify digested cDNA (Protocol IV.F), and measure cDNA concentration (Protocol IV.G).

B. General Requirements

- **The success of your experiment depends on the quality of your starting RNA sample. Prior to cDNA synthesis, please make sure that your RNA is free of contaminants.**
- The assay is very sensitive to variations in pipette volume, etc. Please make sure that all your pipettes are calibrated for reliable delivery, and that nothing is attached to the external surface of the tips.
- All lab supplies related to SMARTer cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for SMARTer cDNA synthesis need to be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Add enzymes to reaction mixtures last. Thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not increase (or decrease) the amount of enzyme added or the concentration of DNA in the reactions. The amounts and concentrations have been carefully optimized for the SMARTer amplification reagents and protocol.
- If you are using this protocol for the first time, we strongly recommend that you perform negative and positive control reactions to verify that the kit components are working properly.

C. Sample Preparation

Ribosomal RNA (rRNA) depletion

We strongly recommend removing rRNA from the total RNA sample prior to using the SMARTer Universal Low Input RNA Kit for Sequencing. If your sample has not been adequately depleted of rRNA you may not get sufficient reads for analysis, and any results you do obtain may be compromised. We recommend the RiboGone - Mammalian kit (Cat. Nos. 634846 and 634847) for this purpose, as it has been specifically designed to remove rRNA from low-input samples (10–100 ng).

Input RNA length

The SMARTer Universal Low Input RNA Kit produces a cDNA output library that is ready for the addition of sequencing adapters. This kit has been validated using input RNA with RIN values between 2 and 3.

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 or reverse transcriptase reactions.
- Volume and amount of input RNA: This kit accommodates 10 µl of input RNA. It can make cDNA libraries from 200 pg to 10 ng of input RNA.

D. Sequencing Library Preparation and Analysis

Library Preparation for Sequencing

The cDNA output of the kit is between 2 and 10 ng. The cDNA library you create with this kit is ready for addition of sequencing adapters. For Illumina sequencing, we recommend the ThruPLEX DNA-Seq Kit (Cat. Nos. R400674, R400675, R400676, or R400677, depending on the number of reactions being processed). Indexes are also required.

Trimming Sequences during Analysis

Up to seven nucleotides from the SMART adapter will remain at each end of your sequences after RsaI digestion. We recommend trimming seven bases *in silico* from both ends of the reads prior to mapping.

IV. Protocols

A. Protocol: First-Strand cDNA Synthesis

Perform in a PCR Clean Work Station

Sheared RNA is converted to single-stranded (ss) cDNA that contains sequences complementary to the SMARTer Oligonucleotide.

1. Prepare a stock solution of Reaction Buffer by mixing Nuclease-Free Water with the RNase Inhibitor. Scale up as needed.

19 µl	Nuclease-free Water
1 µl	RNase Inhibitor
20 µl	Total volume

2. Serially dilute the control RNA in Reaction Buffer to the same concentration as your sample. Run the control RNA in parallel with your samples.
3. Place your samples, including the Diluted Control RNA, on a –20°C prechilled IsoFreeze PCR rack in a PCR Clean Work Station, and add 1 µl of 3' SMART N6 CDS Primer II A (24 µM). Mix the contents and spin the tubes briefly in a microcentrifuge.

10 µl	RNA
1 µl	3' SMART N6 CDS Primer II A (24 µM)
11 µl	Total volume

4. Incubate the tubes at 72°C in a pre-heated, hot-lid thermal cycler for 3 minutes, and then put the samples on the IsoFreeze PCR rack.

NOTE: Steps 6–8 are critical for first-strand synthesis and should not be delayed after Step 4. You can prepare your master mix, except for SMARTScribe Reverse Transcriptase, (for Step 5) while your tubes are incubating (Step 4) in order to jump start the cDNA synthesis.

5. Prepare a Master Mix for all reactions plus 10% by combining the following reagents in the order shown at room temperature.

4 µl	5X First-Strand Buffer (RNase-Free)
0.5 µl	DTT (100 mM)
1 µl	SMARTer dNTP Mix (20 mM)
1 µl	SMARTer II A Oligonucleotide (24 µM)
0.5 µl	RNase Inhibitor (40 U/µl)
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl)*
9 µl	Total volume per reaction

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

NOTE: The SMARTer Universal Low Input RNA Kit Components (Cat. Nos. 634939 & 634941) and Advantage 2 PCR Kit (Cat. No. 639207) both include dNTP mixes. *Use the SMARTer dNTP Mix (20 mM each dNTP) for first strand cDNA synthesis.*

6. Add 9 µl of Master Mix to each reaction tube from Step 4. Mix the contents of each tube by gently pipetting, and spin the tubes briefly to collect the contents at the bottom.
7. Incubate the tubes in a pre-heated thermal cycler at 42°C for 90 minutes.
8. Terminate the reaction by heating the tubes at 70°C for 10 minutes, then leave in thermal cycler at 4°C until proceeding to the next step.

NOTE: This is a break point. You can leave the tubes at 4°C overnight.

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

Perform in a PCR Clean Work Station

The first-strand cDNA selectively binds to SPRI beads, leaving contaminants in solution which is removed by a magnetic separation. The beads (bound to the purified first-strand cDNA) are then directly used for PCR amplification.

NOTES:

- Before use, beads should be brought to room temperature and mixed well to disperse.
- In order to ensure proper and steady positioning of the tubes containing first-strand cDNA (from Protocol A), you may place the tubes in the top part of an inverted P20 or P200 Rainin Tip Holder which is taped to the MagnaBlot II Magnetic Separator (Figure 3).

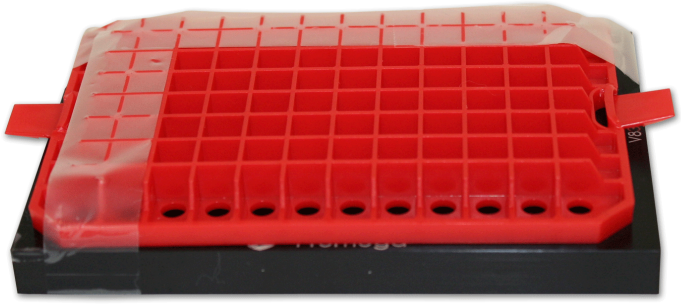


Figure 3. Setup for positioning tubes containing first-strand cDNA.

To purify the SMART cDNA from unincorporated nucleotides and small (< 50 bp) cDNA fragments, follow this procedure for each reaction tube:

1. Add 30 µl of SPRI AMPure beads to each sample using a 200 µl pipette.
    - Adjust the pipette to 50 µl, and pipette the entire volume up and down 10 times to mix thoroughly.
    - The beads are viscous; suck the entire volume up, and push it out slowly.
2. Incubate at room temperature for 8 minutes to let DNA bind to the beads.
3. Briefly spin the sample tubes to collect the liquid from the side of the wall. Place the sample tubes on the Promega MagnaBot II Magnetic Separation Device for 5 minutes or longer, until the solution is completely clear.
4. While the tubes are in the magnetic stand, pipette out the supernatant.
5. Add 150 µl of freshly made 80% ethanol to each sample without disturbing the beads, in order to wash away contaminants. Wait for 30 seconds and carefully pipette out the supernatant.
6. Repeat step 5.
7. Perform a brief spin of the tubes (~2,000g) for 30 seconds to collect remaining ethanol. Remove all of the remaining ethanol with a pipette.
8. Let the sample tubes rest open at room temperature for ~3–5 minutes until the pellet appears dry. You may see a tiny crack in the pellet.

NOTE: If you over-dry the beads, you will see many cracks in the pellet. If you under-dry the beads, the PCR amplification yield will be lower, due to the remaining ethanol.

C. Protocol: Double-Stranded cDNA Amplification by PCR

Perform Steps 1 and 2 in a PCR Clean Work Station

The purified first-strand cDNA is amplified into ds cDNA using Advantage 2 Polymerase and PCR Primer IIA.

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

Table 1. Cycling Guidelines Based on the Input Amount of rRNA-Depleted RNA

Input RNA (ng) from Section IV.A.3	Typical Number of PCR Cycles
0.2	15–16
1	12–13
10	9–10

We strongly recommend the **Advantage 2 PCR Kit** (included in this product; also available as Cat. Nos. 639206 & 639207) for PCR amplification. Advantage 2 Polymerase Mix has been specially formulated for efficient and accurate amplification of cDNA templates by PCR (Barnes 1994).

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

5 µl	10X Advantage 2 PCR Buffer
2 µl	PCR Primer IIA (12 µM)
2 µl	50X dNTP Mix (10 mM)
2 µl	50X Advantage 2 Polymerase Mix
39 µl	Nuclease-Free Water
50 µl	Total volume per reaction

NOTE: The SMARTer Universal Low Input RNA Kit Components (Cat. Nos. 634939 & 634941) and Advantage 2 PCR Kit (Cat. No. 639207) both include dNTP mixes. *Use the Advantage 2 dNTP Mix (10 mM each dNTP) for double-stranded cDNA amplification.*

2. Add 50 µl of PCR Master Mix to each tube containing DNA bound to the beads from Section IV.B.8. Mix well.

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

95°C		1 min
X[a] cycles:		
	95°C	15 sec
	65°C	30 sec
	68°C	3 min
72°C		10 min
4°C		forever

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

D. Protocol: Double-Stranded cDNA Library Purification using SPRI AMPure Beads

The PCR-amplified cDNA library is purified by immobilizing it onto SPRI beads. The beads are then washed with 80% ethanol and eluted in Purification Buffer.

1. Cover all the wells of a 96-well Axygen V-bottom plate with a MicroAmp Clear Adhesive Seal. Uncover only the wells that you want to use with a razor blade or scalpel. Vortex SPRI AMPure Beads until even, then add 90 µl of beads to each well that you are using on the 96-well plate.
2. Transfer the entire PCR product from Section IV.C.3 (including the SPRI beads) to the wells of the plate containing the SPRI beads (from Step 1, directly above). Pipette the entire volume up and down 10 times to mix thoroughly. Incubate at room temperature for 8 minutes to let the DNA bind to the beads.

   NOTE: The beads are viscous; suck the entire volume up, and push it out slowly.
3. Place the 96-well plate on the Ambion Magnetic Stand-96 for ~5 minutes or longer (until the liquid appears completely clear and there are no beads left in the supernatant).
4. Leave the plate on the magnetic stand and pipette out the supernatant.
5. While the plate is still 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 seconds and carefully pipette out the supernatant. The ds cDNA will remain bound to the beads during the wash process.
6. Repeat Step 5.
7. Seal the sample wells on the plate and briefly spin down for 10 seconds at 1,000 rpm to collect the liquid at the bottom of the well.
8. Place the plate on the magnetic stand for 30 seconds, and then remove all the remaining ethanol.
9. Let the plate rest at room temperature for ~3–5 minutes, until the pellet appears dry. You may see a tiny crack in the pellet.

   NOTE: If you over-dry the beads, you will see many cracks in the pellet. If you under-dry the beads, the DNA recovery rate will be lower, due to the remaining ethanol.
10. Once the beads are dried, add 12 µl of Purification Buffer to each sample well in order to cover the beads. Remove the plate from the magnetic stand and incubate at room temperature for 2 minutes to rehydrate.
11. Mix the pellet by pipetting up and down 10 times to elute the cDNA from the beads. Then put the plate back on the magnetic stand for 1 minute or longer until the solution is completely clear.
12. Transfer the clear supernatant containing your purified cDNA library from each well to a nuclease-free, nonsticky tube.

E. Protocol: RsaI Digestion to Remove Adaptors

Amplified and purified ds cDNA is digested with RsaI to remove the SMART adapter prior to sequencing.

1. Prepare a Digestion Master Mix for all reactions plus 10%. Combine the following reagents in the order shown, then mix well by vortexing and spin the tube briefly in a microcentrifuge:

2 µl	RsaI Buffer (10X)
2 µl	BSA (0.1%)
1 µl	RsaI (10 U/µl)
5 µl	Nuclease-Free Water
10 µl	Total volume per reaction

2. Add 10 µl of Digestion Master Mix to each tube containing double-stranded cDNA library from Section IV.D.12. Mix well and briefly spin down.
3. Incubate at 37°C for 30 minutes.
4. Incubate at 65°C for 20 minutes to deactivate.

F. Protocol: RsaI-Digested, Double-Stranded cDNA Library Purification Using SPRI AMPure Beads

The RsaI-digested cDNA is purified by immobilizing it onto SPRI beads. The beads are then washed with 80% ethanol and the cDNA library is eluted in Purification Buffer.

1. Cover all the wells of a 96-well Axygen V-bottom plate with a MicroAmp Clear Adhesive Seal. Uncover only the wells that you want to use with a razor blade or scalpel. Vortex SPRI beads until even; then add 36 µl of SPRI AMPure Beads to the wells of the 96-well plate.
2. Transfer the entire digestion reaction (from Section IV.E.4) to the wells of the plate containing the SPRI beads. Pipette the entire volume up and down 10 times to mix thoroughly. Incubate at room temperature for 8 minutes to let the DNA bind to the beads.

   NOTE: The beads are viscous; suck the entire volume up, and push it out slowly.
3. Place the 96-well plate on the Ambion Magnetic Stand-96 for ~5 minutes or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
4. Leave the plate on the magnetic stand and pipette out the supernatant.
5. Keep the plate 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 seconds and carefully pipette out the supernatant. The ds cDNA library will remain bound to the beads during the wash process.
6. Repeat Step 5.
7. Seal the sample wells on the plate and briefly spin down for 10 seconds at 1,000 rpm to collect the liquid at the bottom of the well.
8. Place the 96-well plate on the magnetic stand for 30 seconds, then remove all the remaining ethanol.
9. Let the plate rest at room temperature for ~3—5 minutes, until the pellet appears dry. You may see a tiny crack in the pellet.

   NOTE: If you over-dry the beads, you will see many cracks in the pellet. If you under-dry the beads, the DNA recovery rate will be lower due to the remaining ethanol.
10. Once the beads are dried, add 12 µl of Purification Buffer to cover the beads. Remove the plate from the magnetic stand and incubate at room temperature for 2 minutes to rehydrate.
11. Mix the pellet by pipetting up and down 10 times to elute the cDNA from the beads. Then put the plate back on the magnetic stand for 1 minute or longer until the solution is completely clear.

12. Transfer the clear supernatant containing your purified cDNA library from each well to a nuclease-free, nonsticky tube.

G. Protocol: Validation Using the Agilent 2100 Bioanalyzer

Assess the quality of your cDNA library before proceeding to sequencing and analysis.

1. Use 1 µl of the amplified cDNA library for validation with the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Chip from Agilent's **High Sensitivity DNA Kit** (Cat. No. 5067-4626). For specific instructions, please refer to the Agilent website.
2. Compare the results for your samples and controls (Figure 4) to verify whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield a distinct peak spanning 100–1,000 bp, peaked at ~200 bp for the positive control RNA sample (Figure 4, Panel A), yielding approximately 2–10 ng of cDNA (depending on the input and number of cycles)—and no product in the negative control (Figure 4, Panel B).

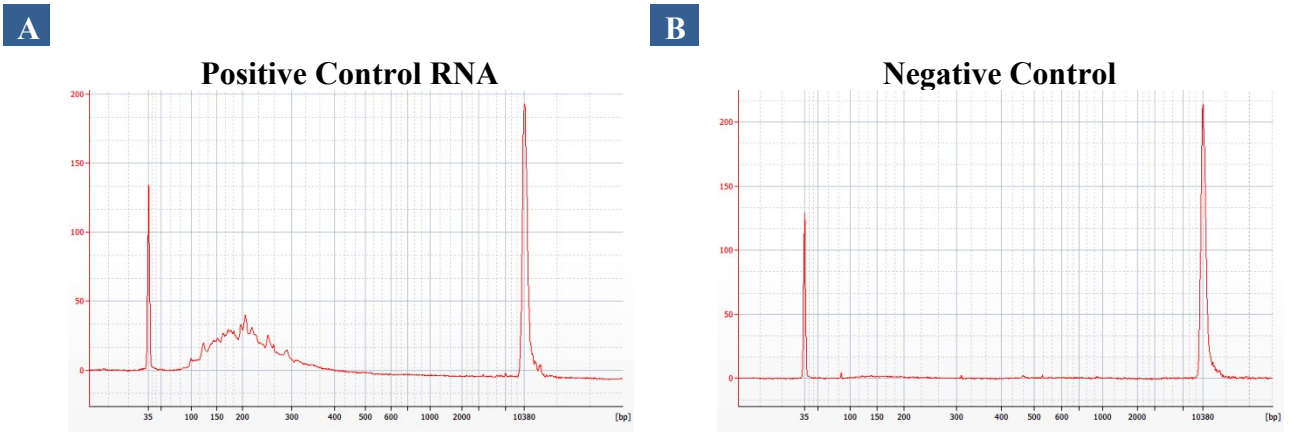


Figure 4. Electropherogram example results from the Agilent 2100 Bioanalyzer. All samples were subjected to SMARTer cDNA synthesis and amplification as described in protocol. FU= Fluorescence absorption units. **Panel A.** Positive Control RNA: SMARTer Amplification of 200 pg of sheared control RNA (15 PCR cycles). **Panel B.** SMARTer Negative Control Product (18 PCR cycles).

V. References

Barnes, W. M. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proc. Natl. Acad. Sci.* **91,** 2216–2220 (1994).

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

Appendix A: Sequencing Guidelines for the Illumina Sequencing Platform

lllumina libraries, generated from the SMARTer Universal cDNA, can have a lower than average filter pass rate due to the low complexity of the first two cycles followed by the low complexity of the next five cycles, before complexity of bases becomes random. lllumina software has problems interpreting low complexity libraries. To alleviate this issue, libraries should be combined with a PhiX Control v3 (lllumina, 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 2. 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.

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