Clontech Laboratories, Inc.

SMARTer® Ultra™ Low Input RNA Kit for Sequencing - v3 User Manual

Cat. Nos. 634848, 634849, 634850, 634851, 634852, 634853
(120514)

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I. Introduction

A. SMARTer cDNA Synthesis for Sequencing

The **SMARTer Ultra Low Input RNA Kit for Sequencing - v3** (Cat. Nos. 634848, 634849, 634850, 634851, 634852 & 634853) is designed to generate high-quality cDNA directly from 1–1,000 cells or 10 pg–10 ng of total RNA, in a convenient input volume of 1–9 µl. This kit improves on previous generations of SMARTer Ultra Low kits (SMARTer Ultra Low RNA Kit for Illumina® Sequencing and SMARTer Ultra Low Input RNA for Illumina Sequencing - HV) by simplifying the workflow, identifying more genes, and increasing the representation of GC-rich genes. This new kit also provides the benefit of generating cDNA that is compatible with both Ion Torrent and Illumina platform-specific library preparation kits. The protocol described in this user manual has been optimized for cDNA synthesis using the SMARTer Ultra Low Input RNA Kit for Sequencing - v3 only. If you are using a previous version of the SMARTer Ultra Low kit, please refer to its user manual.

The kit has been designed and validated to prepare cDNA samples for library preparation and sequencing with the following next-generation sequencing platforms: Ion Torrent Personal Genome Machine (PGM), Ion Proton, Illumina HiSeq®, and MiSeq®. The cDNA synthesis protocol can be completed in five hours, and the entire library construction protocol can be completed within two working days (Figure 1). SMART® technology offers unparalleled sensitivity and unbiased amplification of cDNA transcripts, and enables a direct start from your sample. Most importantly, SMART technology enriches for full-length transcripts and maintains the true representation of the original mRNA transcripts; these factors are critical for transcriptome sequencing and gene expression analysis.

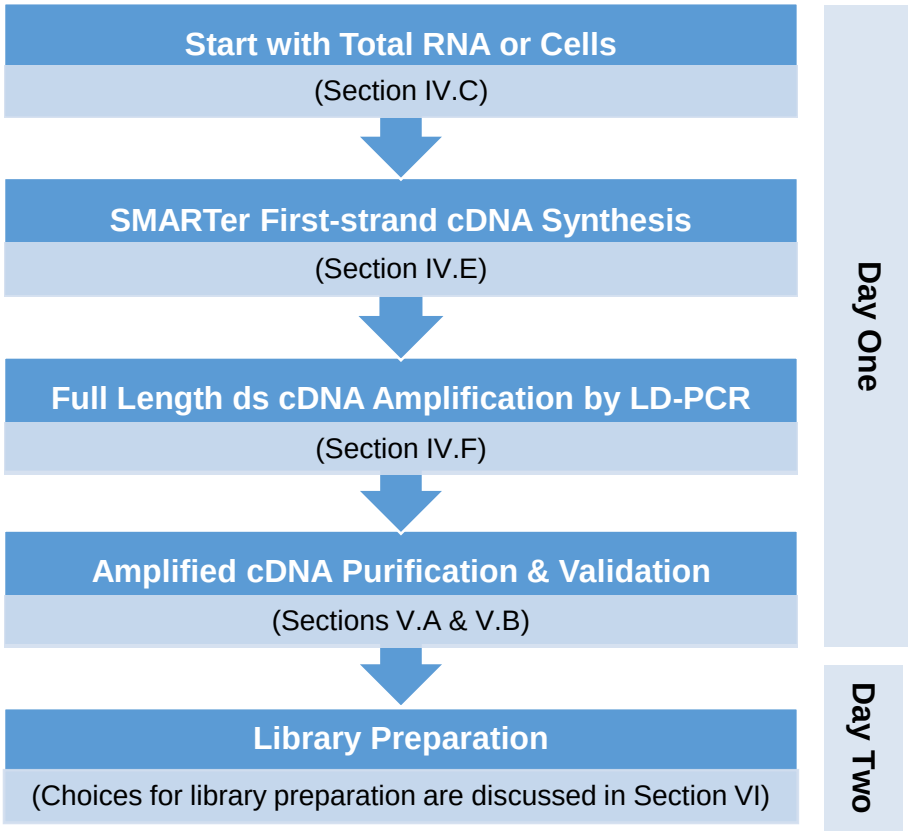

Figure 1. Protocol overview.

The SMARTer Ultra Low Input RNA Kit for Sequencing - v3 incorporates Clontech's patented SMART (**S**witching **M**echanism at 5' End of **R**NA **T**emplate) technology. This technology uses the template switching activity of reverse transcriptase to enrich for full-length cDNAs containing the 5' end of the mRNA and directly add defined PCR adapters to both ends of the first-strand cDNA (Chenchik *et al.*, 1998). SMARTer advances to the core SMART technology have significantly improved cDNA synthesis efficiency when starting with picogram amounts of total RNA. An mRNA-Seq protocol known as SMART-Seq (Ramsköld *et al.*, 2012) was first developed for single-cell transcriptome sequencing. Since then there has been a push to continue to improve sensitivity and robustness of single-cell mRNA-seq methods. The current SMARTer Ultra Low Input RNA kit for Sequencing - v3 is the latest generation of methods that clearly outperforms previously-published protocols, including SMART-seq. For more information on SMART technology, please visit **www.clontech.com.** A schematic outline of the technology and workflow is shown in Figure 2.

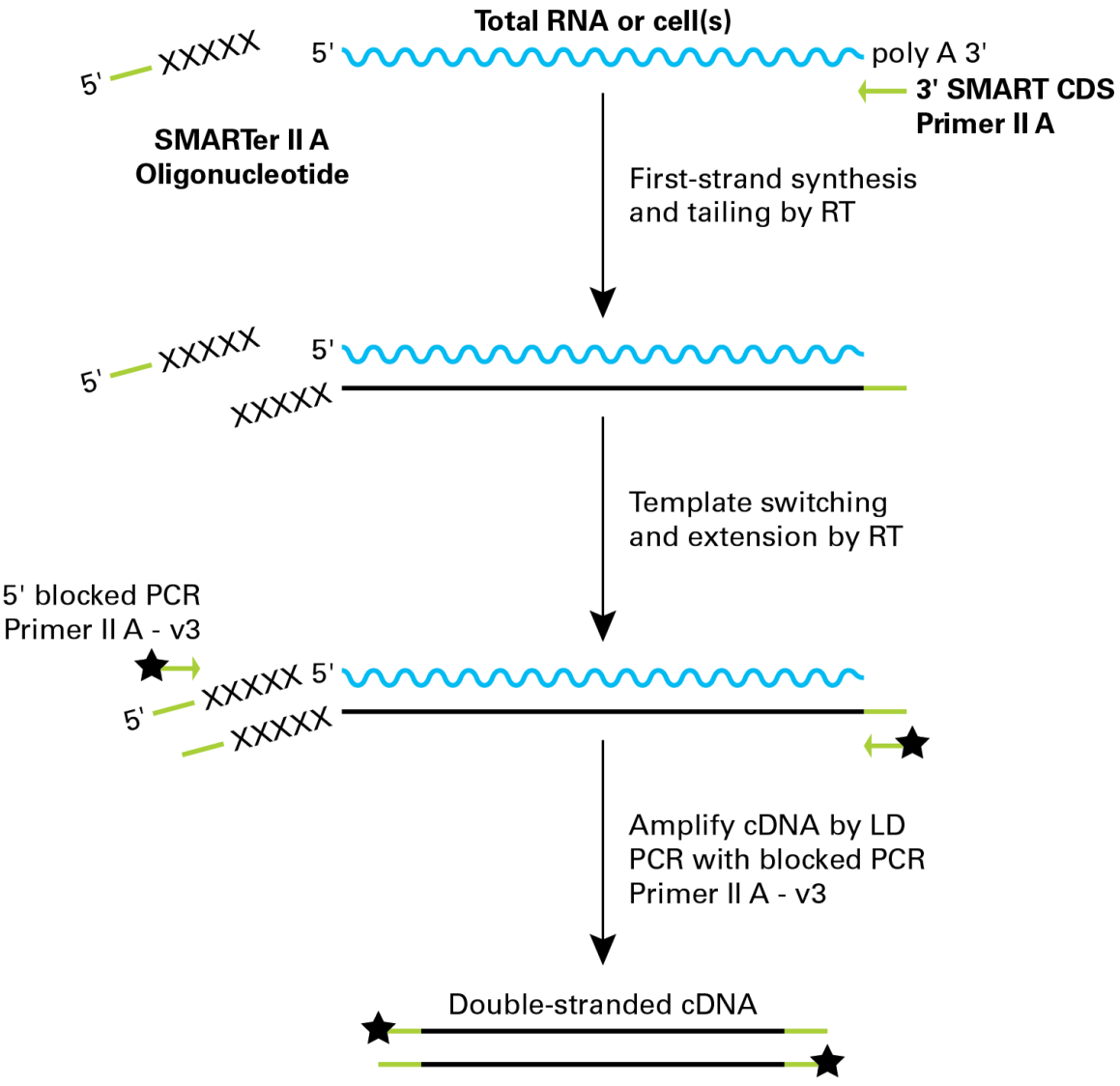

Figure 2. Flowchart of SMARTer cDNA synthesis. The SMARTer II A Oligonucleotide, 3' SMART CDS Primer II A, and PCR Primer II A - v3 all contain a stretch of identical sequence.

B. SMART Adapter in Illumina Primer 2 Read

The use of blocked PCR primers is especially useful when preparing cDNA for library construction on next-generation sequencing platforms. The primer used for amplification of the double-stranded cDNA is blocked (See Figure 2), which prevents ligation of the sequencing adapter at the 5' ends of the double-stranded cDNA fragments containing the SMART sequence.

In many library preparation methods for Illumina, the double-stranded adapters are added to the cDNA fragments through ligation. Unfortunately, in these reactions, ligation may also take place between the bottom strand of the cDNA fragment and the Illumina adapter containing Read Primer 2, at a low and somewhat variable rate. If ligation is also successful on the other, unblocked side of the same cDNA fragment, this bottom strand can be amplified by the subsequent PCR and can ultimately form clusters for sequencing on Illumina machines.

Upon sequencing these clusters, the SMART adapter will be present in the first 30 cycles in Read 2 of an Illumina sequencing run. In addition, the dT30 sequence from the 3' SMART CDS Primer II A will also be present after the adapter in a subset of these clusters. The presence of the SMART adapter in Read 2 commonly occurs at a high enough rate to be observed in the base distribution by cycle graph generated by the run analysis (Figure 3, cycles 77–106), as does the dT30 sequence (Figure 3, cycles 107–136).

If you wish to avoid sequencing the SMART adapter, there are three options:

1. Use the Low Input Library Prep Kit (Cat. No. 634947). This unique adapter addition method does not allow for erroneous ligation.
2. Use the Nextera® XT DNA Sample Preparation Kit from Illumina to prepare your library. We recommend using an input amount of 100–150 pg amplified DNA.
3. Sequence only from Read Primer 1.

If you have already sequenced with Read Primer 2, the SMART adapter sequence can be trimmed from reads prior to mapping to your transcriptome.

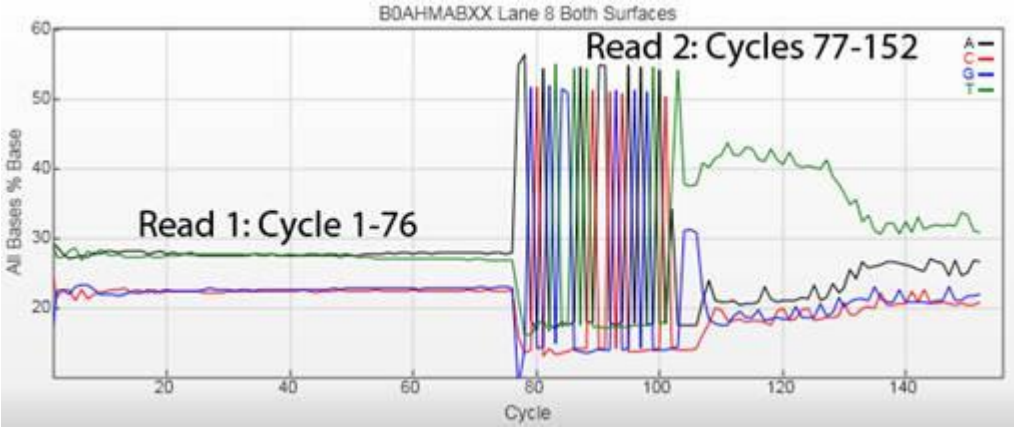

Figure 3. SMART adapter in Primer 2 Read. The presence of the SMART adapter in Read 2 commonly occurs at a high enough rate to be observed in the base distribution by cycle graph generated by the run analysis (cycles 77–106), as does the dT30 sequence (cycles 107–136).

II. List of Components

The SMARTer Ultra Low Input RNA Kit for Sequencing - v3 consists of the SMARTer Ultra Low Input RNA Kit for Sequencing - v3 Components (not sold separately) and SeqAmp DNA Polymerase. **These components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions.** The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results.

SMARTer Ultra Low Input RNA Kit for Sequencing - v3	**634848** (12 rxns)	**634849** (24 rxns)	**634850** (48 rxns)	**634851** (96 rxns)	**634852** (192 rxns)	**634853** (480 rxns)
SeqAmp DNA Polymerase (Store at –20°C.)						
SeqAmp DNA Polymerase	50 µl	50 µl	2 x 50 µl	200 µl	2 x 200 µl	3 x 200 µl
SeqAmp PCR Buffer	1. 25 ml	1. 25 ml	2 x 1. 25 ml	4 x 1.25 ml	8 x 1.25 ml	12 x 1.25 ml
SMARTer Ultra Low Input RNA Kit for Sequencing - v3 Components (Not sold separately. Storage conditions are listed below for Box 1 and Box 2.)						
Box 1 (Store at –70°C.)						
SMARTer II A Oligonucleotide (12 µM)	12 µl	24 µl	48 µl	96 µl	2 x 96 µl	5 x 96 µl
Control Total RNA (1 µg/µl)	5 µl	5 µl	5 µl	5 µl	2 x 5 µl	5 x 5 µl
Box 2 (Store at –20°C. Once thawed, store Lysis Buffer at 4°C and store Elution Buffer at –20°C. Continue to store all other reagents at –20°C)						
3’ SMART CDS Primer II A (12 µM)	12 µl	24 µl	48 µl	96 µl	2 x 96 µl	5 x 96 µl
PCR Primer II A - v3 (12 µM)	12 µl	24 µl	48 µl	96 µl	2 x 96 µl	5 x 96 µl
5X First-Strand Buffer	48 µl	96 µl	192 µl	384 µl	2 x 384 µl	5 x 384 µl
SMARTer dNTP Mix (20 mM each)	12 µl	24 µl	48 µl	96 µl	2 x 96 µl	5 x 96 µl
Dithiothreitol (DTT; 100 mM)	6 µl	12 µl	24 µl	48 µl	2 x 48 µl	5 x 48 µl
SMARTScribe™ Reverse Transcriptase (100 U/µl)	24 µl	48 µl	96 µl	192 µl	2 x 192 µl	5 x 192 µl
Nuclease-Free Water	2 x 1 ml	2 x 1 ml	2 ml	4 ml	2 x 4 ml	5 x 4 ml
RNase Inhibitor (40 U/µl)	30 µl	60 µl	120 µl	240 µl	2 x 240 µl	5 x 240 µl
10X Lysis Buffer - v3	230 µl	460 µl	920 µl	1.85 ml	2 x 1.85 ml	5 x 1.85 ml
Elution Buffer (10 mM Tris-Cl, pH 8.5)	1.7 ml	2 x 1.7 ml	6.8 ml	2 x 6.8 ml	4 x 6.8 ml	10 x 6.8 ml
10X AfaI Buffer	24 µl	48 µl	96 µl	192 µl	2 x 192 µl	5 x 192 µl
0.1% BSA	24 µl	48 µl	96 µl	192 µl	2 x 192 µl	5 x 192 µl
AfaI (10 U/µl)	12 µl	24 µl	48 µl	96 µl	2 x 96 µl	5 x 96 µl

Storage Conditions

- Store Control Total RNA and SMARTer IIA Oligonucleotide at –70°C.
- Store 10X Lysis Buffer - v3 at –20°C. Once thawed, the buffer can be stored at 4°C.
- Store Elution Buffer at –20°C. Once thawed, the buffer can be stored at 4°C.
- Store all other reagents at –20°C.

III. Additional Materials Required

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

- Single channel pipette: 10 µl, 20 µl, and 200 µl
- Eight channel pipette (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, and 200 µl
- Minicentrifuge for 1.5 ml tubes
- Minicentrifuge for 0.2 ml tubes or strips

For PCR Amplification & Validation:

- One dedicated thermal cycler used only for first-strand synthesis
- One dedicated thermal cycler used only for double-stranded cDNA amplification by PCR
- High Sensitivity DNA Kit (Agilent, Cat No. 5067-4626)
- Nuclease-free thin-wall PCR tubes or strips (0.2 ml PCR 8-tube strip; USA Scientific, Cat. No.1402-4700)
- Nuclease-free low adhesion 1.5-ml tubes (USA Scientific, Cat. No. 1415-2600) or LoBind tubes (Eppendorf, Cat. No. 022431021)

For SPRI (Solid Phase Reversible Immobilization) Bead Purifications:

- Agencourt AMPure XP PCR purification kit (5 ml Beckman Coulter Part No. A63880; 60 ml Beckman Coulter Part No. A63881)
  - Use this kit for the amplified cDNA purifications (Section V.A).
- Molecular biology grade 100% ethanol
- Magnetic separation device for small volumes (See Appendix A)
  - Use this magnetic stand for the amplified cDNA purifications (Section V.A).
- Optional [depending on the choice of magnetic stand for amplified cDNA purifications (Section V.A.)]:
  - Magnetic Stand-96 (Life Technologies, Part No. AM10027)
  - 96-well V-bottom Plate (500 µl; VWR, Cat. No. 47743-996)
  - MicroAmp Clean Adhesive Seal (Life Technologies, Part No. 4306311)
  - Low speed benchtop centrifuge for spinning a 96-well plate

For Sequencing Library Generation:

The components you need are dictated by which library preparation protocol you follow (Section VI).

- Ion Xpress Plus Fragment Library Kit (Life Technologies, Cat. No. 4471269) for enzymatic shearing
- Nextera XT DNA Sample Preparation Kit (Illumina, Cat. Nos. FC-131-1024, FC-131-1096)
- Covaris Instrument and related materials for DNA shearing
- Low Input Library Prep Kit (Cat. No. 634947)

IV. SMARTer cDNA Synthesis

NOTE: Please read the entire protocol before starting. This protocol is optimized for cDNA synthesis from ultra-low input amounts of total RNA using Clontech's SMARTer method. The protocol is also suitable for cDNA synthesis directly from whole cells. Due to the sensitivity of the protocol, the input material (total RNA or cells) needs to be collected and purified under clean room conditions to avoid contamination. The whole process of SMARTer cDNA Synthesis should be carried out in a PCR Clean Work Station under clean room conditions.

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.E).

  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.F) and measure cDNA concentration (Protocol V.B).

B. General Requirements

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

C. Sample Recommendations

- **Total RNA Extraction**
  The sequence complexity and the average length of SMARTer cDNA are noticeably dependent on the quality of starting RNA material. Due to the limiting sample size, most traditional RNA isolation methods may not be applicable. There are several commercially available products that enable purification of total RNA preparations from extremely small samples [e.g. Clontech offers the NucleoSpin RNA XS Kit (Cat. No. 740902.10) for purification of RNA from 10^2 cells]. When choosing a purification method (kit), ensure that it is appropriate for your sample amount. Input RNA should be free from poly(A) carrier DNA that will interfere with oligo(dT)-primed cDNA synthesis.

- **Evaluation of RNA Quality**
  After RNA extraction, if your sample size is not limiting, we recommend evaluating total RNA quality using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513). Refer to the manufacturer's instructions for information on how to use the Agilent RNA 6000 Pico Kit.

- **Cell Culture Media**
  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 cells suspended in cell culture-grade PBS.

D. Sample Requirements

The SMARTer Ultra Low Input RNA Kit for Sequencing - v3 works with up to 9 µl of cells or RNA.

- **Total RNA**
  This protocol has been optimized for cDNA synthesis starting from 10 pg of total RNA. However, if your RNA sample is not limiting, we recommend that you start with more total RNA (up to 10 ng). Purified total RNA should be in nuclease-free water.

- **Cells**
  This protocol has been validated to generate cDNA starting from cells. Cells that have been frozen prior to starting cDNA synthesis will show a reduced yield of cDNA relative to those that have never been frozen. However, it is possible to use this protocol with previously-frozen cells. The cDNA synthesis protocol has been tested with suspension cells without internal labeling. It cannot be used with cells that have undergone fixation.

E. Protocol: First-Strand cDNA Synthesis (Perform in PCR Clean Work Station)

First-strand cDNA synthesis (from total RNA or lysed cells) is primed by the 3' SMART CDS Primer II A and uses the SMARTer II A Oligonucleotide for template switching at the 5' end of the transcript.

IMPORTANT: To avoid introducing contaminants into your RNA sample, the first part of the cDNA synthesis protocol (Section E) requires the use of a PCR work station, ideally in a clean room.

1. Thaw all the reagents needed for first-strand cDNA synthesis (except the enzyme) on ice. Gently vortex each reagent to mix and spin down briefly. Store on ice.

2. Prepare a stock solution of 10X Reaction Buffer by mixing the 10X Lysis Buffer - v3 with the RNase Inhibitor as indicated below (scale-up as needed):

19 µl	10X Lysis Buffer - v3
1 µl	RNase Inhibitor
20 µl	Total Volume

   Vortex briefly to mix, then spin down.

3. See Table 1 for guidelines on setting up your control and test samples. Prepare each sample (10 µl total volume) in individual 0.2 ml RNase-free PCR tubes or in an 8-well strip.

   a. If you are working with purified total RNA, transfer 1–9 µl to a 0.2 ml RNase-free PCR tube. Bring the volume to 9 µl with nuclease-free water. Add 1 µl of 10X Reaction Buffer.

   b. If you are working with cells, isolate cells in validated media and transfer to a 0.2 ml RNase-free PCR tube. Bring the volume to 9 µl with nuclease-free water. Add 1 µl 10X Reaction Buffer. Gently vortex or pipette to mix the sample. Incubate at room temperature for 5 minutes. See Sections IV.C and IV.D for more information on working with cells.

Table 1. Sample Preparation Guidelines

Components	Negative Control	Positive Control	Test Sample
10X Reaction Buffer	1 µl	1 µl	1 µl
Nuclease-free water	9 µl	0–8 µl	0–8 µl
Diluted Control RNA*	–	1–9 µl	–
Sample	–	–	1–9 µl
Total Volume	10 µl	10 µl	10 µl

*The Control RNA is supplied at a concentration of 1 µg/µl. It should be diluted in nuclease-free water to match the concentration of your test sample. Perform serial dilutions on the Control RNA until you obtain the appropriate concentration.

4. Place the samples on ice, and add 1 µl of 3' SMART CDS Primer II A (12 µM). Mix well by gently vortexing and then spin the tube(s) briefly in a minicentrifuge to collect the contents at the bottom of the tube:

10 µl	Cell/Total RNA in Reaction Buffer (from Table 1)
1 µl	3' SMART CDS Primer II A (12 µM)
11 µl	Total Volume

5. Place the tubes into a preheated thermal cycler and run the following program:

72°C	3 min
4°C	forever

NOTE: Steps 9–10 below are critical for first-strand cDNA synthesis and should not be delayed after completing Step 6. It is therefore recommended that you prepare your Master Mix in Step 8 with all components (except the enzyme) while your tubes are incubating in Step 5.

6. When the thermal cycler reaches 4°C, remove the tubes from the thermal cycler and put them on ice.
7. Preheat the thermal cycler to 42°C.
8. Meanwhile, prepare enough Master Mix for all the reactions, plus 10% of the total reaction mix volume, by combining the following reagents in the order shown at room temperature:

4 µl	5X First-Strand Buffer
0.5 µl	DTT (100 mM)
1 µl	dNTP Mix (20 mM)
1 µl	SMARTer IIA Oligonucleotide (12 µM)
0.5 µl	RNase Inhibitor (40 U/µl)
2 µl	SMARTScribe Reverse Transcriptase (100 U/µl)*
9 µl	Total Volume added per reaction

* Add the reverse transcriptase to the Master Mix just prior to use, making sure to gently mix the reverse transcriptase tube without vortexing before adding it. Mix the Master Mix well by gently vortexing and then spin the tube(s) briefly in a minicentrifuge to collect the contents at the bottom of the tube.

9. Add 9 µl of the Master Mix to each reaction tube from Step 6. Mix the contents of the tubes by gently pipetting, and spin them briefly to collect the contents at the bottom.
10. Place the tubes in a thermal cycler with a heated lid, preheated to 42°C. Run the following program:

42°C	90 min
70°C	10 min
4°C	forever

STOPPING POINT: The tubes can be stored at 4°C overnight.

F. Protocol: cDNA Amplification by LD PCR (Perform Steps 1 & 2 in PCR Clean Work Station)

PCR Primer II A - v3 amplifies cDNA from the SMART sequences introduced by 3' SMART CDS Primer II A and the SMARTer II A oligonucleotide.

IMPORTANT: Table 2 provides guidelines for PCR optimization, depending on the amount of total RNA or cells used for the first-strand cDNA synthesis. These guidelines were determined using the Control Mouse Brain Total RNA. Typical cycle numbers are provided as a rough guide for working with small amounts of RNA. Optimal parameters may vary for different templates, different cell types, and different thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles. See Appendix B for PCR optimization suggestions.

Table 2. Cycling Guidelines Based on Amount of Starting Material

Input Amount of Total RNA	Input Amount of Cells	Typical Number of PCR Cycles
10 ng	1,000 cells	9
1 ng	100 cells	12
100 pg	10 cells	15
10 pg	1 cell	18

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

   NOTE: Remove the DNA polymerase from the freezer, gently mix the tube without vortexing, and add to the Master Mix just before use. Mix the Master Mix well by vortexing gently and spin the tube briefly in a microcentrifuge to collect the contents at the bottom of the tube:

25 µl	2X SeqAmp PCR Buffer
1 µl	PCR Primer II A - v3 (12 µM)
1 µl	SeqAmp DNA Polymerase
3 µl	Nuclease-free water
30 µl	Total Volume added per reaction

3. Add 30 µl of PCR Master Mix to each tube containing 20 µl first-strand cDNA product from Section IV.E. Mix well and briefly spin in a minicentrifuge to collect the contents at the bottom of the tube(s).

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

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

95°C	1 min
X cycles:[a]	
98°C	10 sec
65°C	30 sec
68°C	3 min
72°C	10 min
4°C	forever

[a]Consult Table 2 for PCR cycle number guidelines.

STOPPING POINT: The tubes may be stored at 4°C overnight.

V. Amplified cDNA Purification & Validation

A. Protocol: Purification of Amplified cDNA using the Agencourt AMPure XP Kit

PCR-amplified cDNA is purified by immobilization onto AMPure XP beads. The beads are then washed with 80% ethanol and cDNA is eluted with Elution Buffer.

NOTES:

- Aliquot AMPure XP beads into 1.5 ml tubes upon receipt in the laboratory.
- Before each use, bring beads to room temperature for at least 30 minutes and mix well to disperse.
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.
- You will need a magnetic separation device for 0.2 ml tubes, strip tubes, or a 96-well plate. If you do not have such a device, we recommend constructing one using the instructions in Appendix A.

1. Add 1 µl of 10X Lysis Buffer - v3 to each PCR product from Section IV.F.
2. Vortex AMPure XP beads until evenly mixed, then add 50 µl of Ampure XP Beads to each sample.
3. Mix by vortexing or pipetting the entire volume up and down at least 10 times to mix thoroughly.
4. 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.

5. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples on the magnetic separation device for ~5 minutes or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
6. While the samples are on the magnetic separation device, pipette out the supernatants.
7. Keep the samples on the magnetic separation device. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads. Wait for 30 seconds and carefully pipette out the supernatant containing contaminants. DNA will remain bound to the beads during the washing process.
8. Repeat Step 7 once.
9. Briefly spin the samples to collect the liquid from the side of the wall. Place the samples on the magnetic device for 30 seconds, then remove all the remaining ethanol with a pipette.
10. Place the samples at room temperature for approximately 2.5–3 minutes until the pellet is no longer shiny, and before a crack appears.

    NOTE: Be sure to dry the pellet only until it is just dry. The pellet will look matte with no shine.

    - If you under-dry the pellet, ethanol will remain in the sample wells. The ethanol will reduce your amplified cDNA recovery rate and ultimately your yield. Allow the plate to sit at room temperature until the pellet is dry.
    - If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 2 minutes to rehydrate (Step V.A.12) and may reduce amplified cDNA recovery and yield.

11. Once the beads are dry, add 17 µl of Elution Buffer to cover the bead pellet. Remove the samples from the magnetic separation device and mix thoroughly to resuspend the beads.
12. Incubate at room temperature for 2 minutes to rehydrate.
13. Briefly spin the samples to collect the liquid from the side of the tube or sample well. Place the samples back on the magnetic device for 1 minute or longer until the solution is completely clear.

    NOTE: There may be a small population of beads that do not pellet against the magnet during incubation. Pipet these unpelleted beads up and down to resuspend them with the supernatant, and then pipet them towards the magnet where the rest of the beads have already pelleted (without disrupting the existing pellet). Continue the incubation until there are no beads left in the supernatant.

14. Transfer clear supernatant containing purified cDNA from each well to a nuclease-free nonsticky tube. Label each tube with sample information and store at –20°C.

    STOPPING POINT: The samples may be stored at -20°C indefinitely.

B. Validation Using the Agilent 2100 BioAnalyzer

1. Aliquot 1 µl of the amplified cDNA for validation using the Agilent 2100 BioAnalyzer and the High Sensitivity DNA Chip from Agilent's High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). See the Agilent High Sensitivity DNA Kit User Manual for instructions.
2. Compare the results for your samples and controls (see Figure 4) to verify whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield no product in the negative control (Figure 4, Panel B), and a distinct peak spanning 400 bp to 10,000 bp, peaked at ~2,000 bp for the positive control RNA sample (Figure 4, Panel A), yielding approximately 2–10 ng of cDNA (depending on the input).

   NOTE: For more information on how a trace of a positive reaction/control should look and compare to that of a negative control, please visit **www.clontech.com/rna-seq-tips**

3. Proceed to **Library Preparation for Sequencing on Next-Generation Sequencing Platforms** (Section VI).

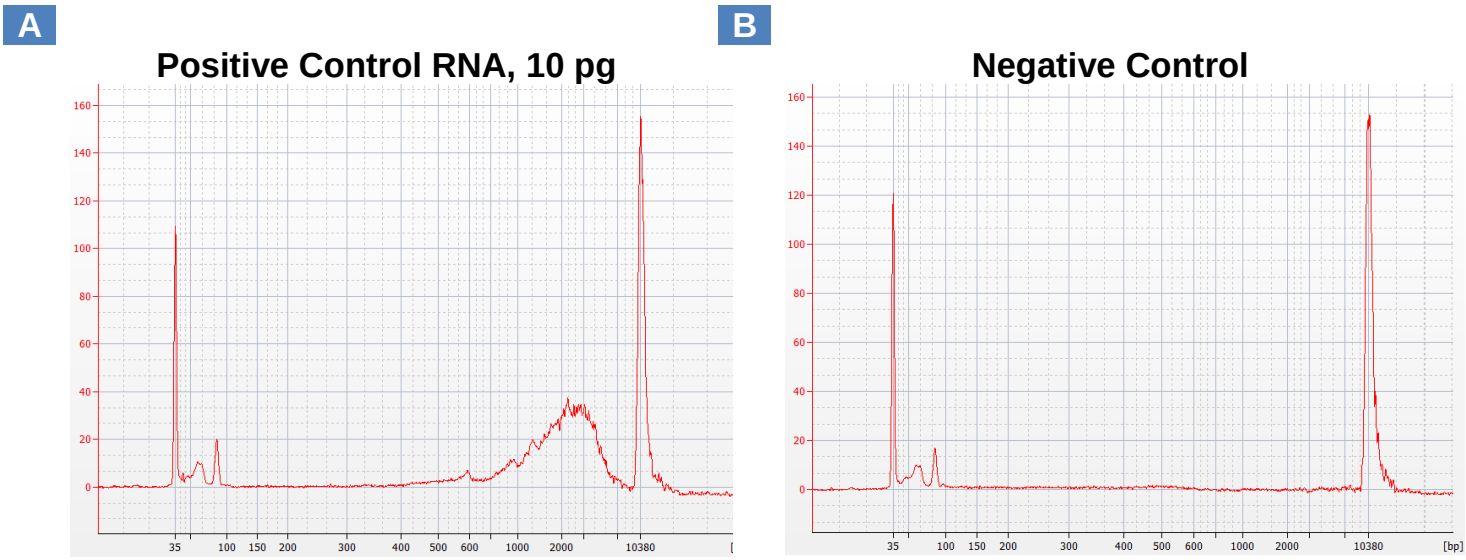

Figure 4. Electropherogram example results from Agilent 2100 Bioanalyzer. All samples were subjected to SMARTer cDNA synthesis and amplification as described in the protocol. FU = fluorescence absorption units. **Panel A** shows a clean product following cDNA synthesis and amplification (18 PCR cycles). **Panel B** shows no product in the negative control following cDNA synthesis and amplification (18 PCR cycles).

VI. Library Preparation for Sequencing on Next-Generation Sequencing Platforms

If you are preparing your library for Illumina sequencing as described below, please see Section I.B. of the Introduction, "SMART Adapter in Illumina Primer 2 Read" for more information on how to avoid sequencing the SMART adapter.

A. Protocol: Illumina Library Preparation Using Nextera DNA Sample Preparation Kits

The full-length cDNA output of the SMARTer Ultra Low Input RNA Kit for Sequencing - v3 can be processed with the Nextera XT DNA Sample Preparation Kits from Illumina. We recommend using an input amount of 100–150 pg amplified DNA in the input volume recommended in the Nextera XT Sample Preparation Guide. Follow the rest of the protocol as written.

B. Protocol: Illumina Library Preparation Using Covaris Shearing and the Low Input Library Prep Kit

Prior to generating the final library for Illumina sequencing, the Covaris AFA system is used for controlled DNA shearing. The resulting DNA will be in the 200–500 bp size range.

1. Turn power ON for the Covaris system and the main cooler. Add about 1.9 L of distilled or deionized water to the water bath. The water level in the cooler should be within +/- 3 mm of the "FULL" waterline when the transducer is submerged. If needed, add distilled or deionized water to the water bath until the "FULL" line is reached.

   Important: Never run a process without the water bath. This will permanently damage the transducer.

2. Close the door and open the Sonolab software. Click "ON" for the degassed button, and degas the water bath for ½ hour (30 minutes).

3. Add 65 µl of Elution Buffer to the DNA from Section V.A. Transfer 75 µl of the Elution Buffer + DNA mixture into the 100 µl Covaris tube. Put the sample tubes into the appropriate location on the Sample holder.

   Set up the process configuration panel as shown in Table 3:

   Table 3. Process Configuration Panel Setup

Peak Power*	Duty %	Burst Cycle	Time (min)	Mode
175	10	200	5 min	Frequency Sweeping

   *Starting with SonoLab version 7 software, Peak Incident Power replaced Intensity as a parameter. For previous versions of SonoLab, set Intensity to 5.

4. Save the file and click return to go back to the main page.

5. Open the door. Place the tube holder with sample tubes on the transducer positioning system.

6. Close the door.

7. Click "START" on the main page to run the process.

8. After shearing is complete, transfer 75 µl of sheared DNA to 1.5 ml tubes.

9. Proceed to generate an Illumina Sequencing Library with the Low Input Library Prep Kit (Cat. No. 634947). Dispose of all tubes and pipettes that have been exposed to amplicons in a sealed trash bag.

C. Protocol: Ion Torrent Sequencing Platforms

Prior to generating the final library for Ion Torrent sequencing, the cDNA is simultaneously digested with AfaI to remove SMART adapters and enzymatically sheared using reagents from the Ion Xpress Plus Fragment Library Preparation Kit. Just 1 ng of amplified cDNA is sufficient for this protocol, but it is recommended that you use as much of your cDNA as possible for best results.

The resulting sheared cDNA from the protocol will be in the 80–600 bp size range. The conditions have been optimized for library sizes yielding 200 base reads on the Ion Proton platform. Shearing conditions may need to be modified for different sized libraries. Refer to the Ion Xpress Plus Fragment Library Preparation User Guide for guidelines on optimizing the Ion Shear reaction time, but note that the reaction time will be affected by the presence of AfaI enzyme.

1. Vortex the Ion Shear Plus 10X Reaction Buffer and the Ion Shear Plus Enzyme Mix II for 5 seconds each and spin briefly to collect the contents at the bottom of the tubes. Store on ice.
2. Add the following reagents, in the order shown, to individual 0.2 ml nuclease-free low adhesion tubes or an 8-well strip. Mix vigorously by vortexing for 5 seconds. Spin the tube(s) briefly in a minicentrifuge to collect the contents at the bottom of the tube.

   NOTE: Do not make a master mix.

15 µl	Purified ds cDNA (from Section V.A., Step 14)
5 µl	Ion Shear Plus 10X Reaction Buffer
19 µl	Nuclease-free water
39 µl	Total Volume each reaction

3. Add 1 µl of AfaI (10 U/µl) to each tube containing reaction mix. Proceed immediately to the next step to add the Ion Shear Plus Enzyme Mix II.
4. Add 10 µl Ion Shear Plus Enzyme Mix II to each tube containing reaction mix and AfaI. Proceed immediately to the next step to mix the enzyme mix with the DNA and buffer. The total reaction volume is 50 µl.
5. Set a pipettor to a 40 µl volume and mix the reaction by rapidly pipetting up and down 8–10 times.

   NOTE: Do not mix by vortexing. Avoid creating bubbles.
6. Incubate the tube(s) in a preheated thermal cycler set to 37°C for 25 minutes.

   NOTE: The Ion Shear reaction is very sensitive to the quality of the starting sample and operator handling method. The reaction time may need to be optimized under your laboratory conditions and for different median fragment sizes.
7. Add 5 µl of Ion Shear Stop Buffer immediately after incubation, and mix thoroughly by vortexing for at least 5 seconds. Store the reaction tube(s) on ice.
8. Note that you may optionally confirm the shear profile of the purified DNA with 1 µl of eluted DNA as described in Step V.B.1.
9. Proceed to generate an Ion Torrent sequencing library with the Ion Xpress Plus Fragment Library Preparation User Guide starting with “Purify the fragmented DNA” following the Ion Shear reaction. There is no need to end-repair the Ion Shear fragmented cDNA. See Table 4 for cycling guidelines.

Table 4. Cycling Guidelines Based on Amount of Amplified cDNA (Section V.B. Step 2)

Input Amount of Amplified cDNA	Typical Number of PCR Cycles
10 ng	11
1 ng	14

VII. References

Chenchik, A., Zhu, Y., Diatchenko, L., Li., R., Hill, J. & Siebert, P. (1998) Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In *RT-PCR Methods for Gene Cloning and Analysis*. Eds. Siebert, P. & Larrick, J. (BioTechniques Books, MA), pp. 305–319.

Ramsköld, D., Luo, S., Wang, Y.-C., Li, R., Deng, Q., Faridani, O.R., Daniels, G.A., Khrebtukova, I., Loring, J.F., Laurent, L.C., Schroth, G.P. & Sandberg, R. (2012) Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. *Nature Biotechnology* **30**(8): 777-782.

Appendix A: Constructing an Effective Magnetic Separation Device for use with SPRI Bead Separation from a Small Volume Supernatant

Using an optimized magnetic separation device is essential for efficient SPRI bead separation from a small volume supernatant. It can be difficult to find strong magnetic separation devices designed specifically to handle small volumes or 0.2 ml PCR strip tubes. One can place strip tubes in a column/row of a magnetic separation device designed for use with 96-well plates, or use a plate and a 96-well magnet. The latter can be useful with a large number of samples. Alternatively, one can construct a suitable low-cost separation device from common laboratory materials. Please visit **www.clontech.com/rna-seq-tips** for a video tutorial on how to construct your own efficient magnetic separation device.

Example 1: Using a 96-well Axygen V-bottom plate and Life Technologies Magnetic Stand-96

You may use a 96-well Axygen V-bottom plate in combination with the Life Technologies Magnetic Stand-96 to purify your PCR-amplified cDNA. Below are modifications to the protocol described in Section V.A.

Before purification (Section V.A. Step 1), cover all the wells of a 96-well Axygen V-bottom plate with a MicroAmp Clean Adhesive Seal. You may use a razor blade or scalpel to score the seal and uncover only the wells that you want to use. Add AMPure XP beads to the wells and then transfer the entire PCR product to the wells containing beads.

In order to magnetically pellet the beads, place the 96-well plate on the Life Technologies Magnetic Stand-96.

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

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

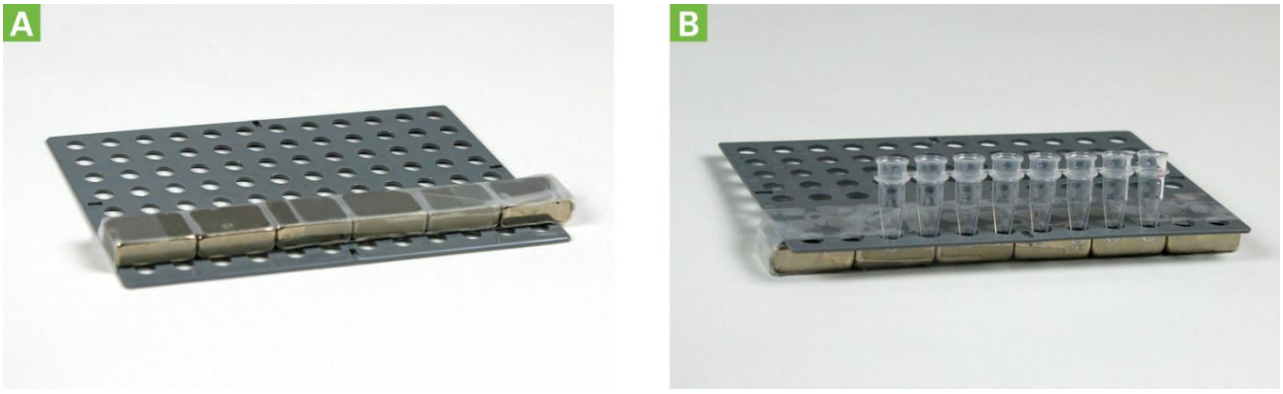

Figure 5. Constructing a magnetic separation device for 0.2 ml tubes from rare earth magnets. Panel A shows six 0.75" x 0.25" x 0.5" neodymium bar magnets (Applied Magnets Model# NB026) taped together on the underside of the top section of a 20 µl tip rack. Panel B shows the upright rack, holding an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

Appendix B: PCR Optimization

If you have a sufficient amount of starting material (>1 ng total RNA), we recommend optimizing the PCR cycling parameters for your experiment. If you have a very limited amount of material or your sample is unique, use a similar source of RNA or cells to perform PCR cycle optimization prior to using the actual sample. Choosing the optimal number of PCR cycles ensures that the double-stranded cDNA will remain in the exponential phase of amplification. When the yield of PCR products stops increasing with more cycles, the reaction has reached its plateau. Overcycled cDNA can result in a less representative sample. Undercycling, on the other hand, results in a lower yield of cDNA. The optimal number of cycles for your experiment is one cycle fewer than is needed to reach the plateau. Be conservative: when in doubt, it is better to use fewer cycles than too many.

To perform PCR cycle optimization, prepare several tubes containing an amount of RNA equal to your sample amount. Subject each tube to a different range of PCR cycles. For example if you have 1 ng of RNA, subject one tube to the recommended a number of cycles. Subject the other two tubes to 2–3 cycles fewer or more than the first tube, i.e. 15, 12 (recommended for 1 ng), and 10 cycles.

1. Use the following program for thermal cycling:

95°C	1 min
X cycles:	
98°C	10 sec
65°C	30 sec
68°C	3 min
72°C	10 min
4°C	forever

2. Perform **Purification of Amplified cDNA using the Agencourt Ampure XP Kit** (Section V.A).
3. Run samples on an Agilent High Sensitivity DNA Chip using the Agilent 2100 Bioanalyzer to evaluate DNA output. See the user manual for the Agilent High Sensitivity DNA Kit for instructions.
4. Determine the optimal number of PCR cycles required for each experimental and control sample. We recommend using the lowest PCR cycle number that generates enough material for Ion library construction.
5. Apply the optimal number of PCR cycles to your sample material.

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