Clontech Laboratories, Inc.

SMARTer® Ultra® Low Input RNA for Illumina® Sequencing - HV User Manual

Cat. Nos. 634820, 634823, 634826, 634828 & 634830
(010616)

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Avenue, Mountain View, CA 94043, USA
U.S. Technical Support: tech@clontech.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.543.6116

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I. Introduction

SMARTer cDNA Synthesis for the Illumina Sequencing Platform

The **SMARTer Ultra Low Input RNA for Illumina Sequencing - HV** kits (Cat. Nos. 634820, 634823, 634826, 634828 & 634830) allow high-quality cDNA synthesis starting from as little as 10 pg of total RNA or cells, in an input volume of up to 9 µl. (Our original SMARTer Ultra Low Input RNA Kit for Illumina Sequencing accommodates input volumes of up to 1 µl.)

The kits have been designed and validated to prepare cDNA samples for sequencing and quantitation with the Illumina HiSeq®, MiSeq®, and Genome Analyzer™ sequencing instruments. The entire library construction protocol can be completed within two days (Figure 1). SMART® technology offers unparalleled sensitivity and unbiased amplification of cDNA transcripts, enabling 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.

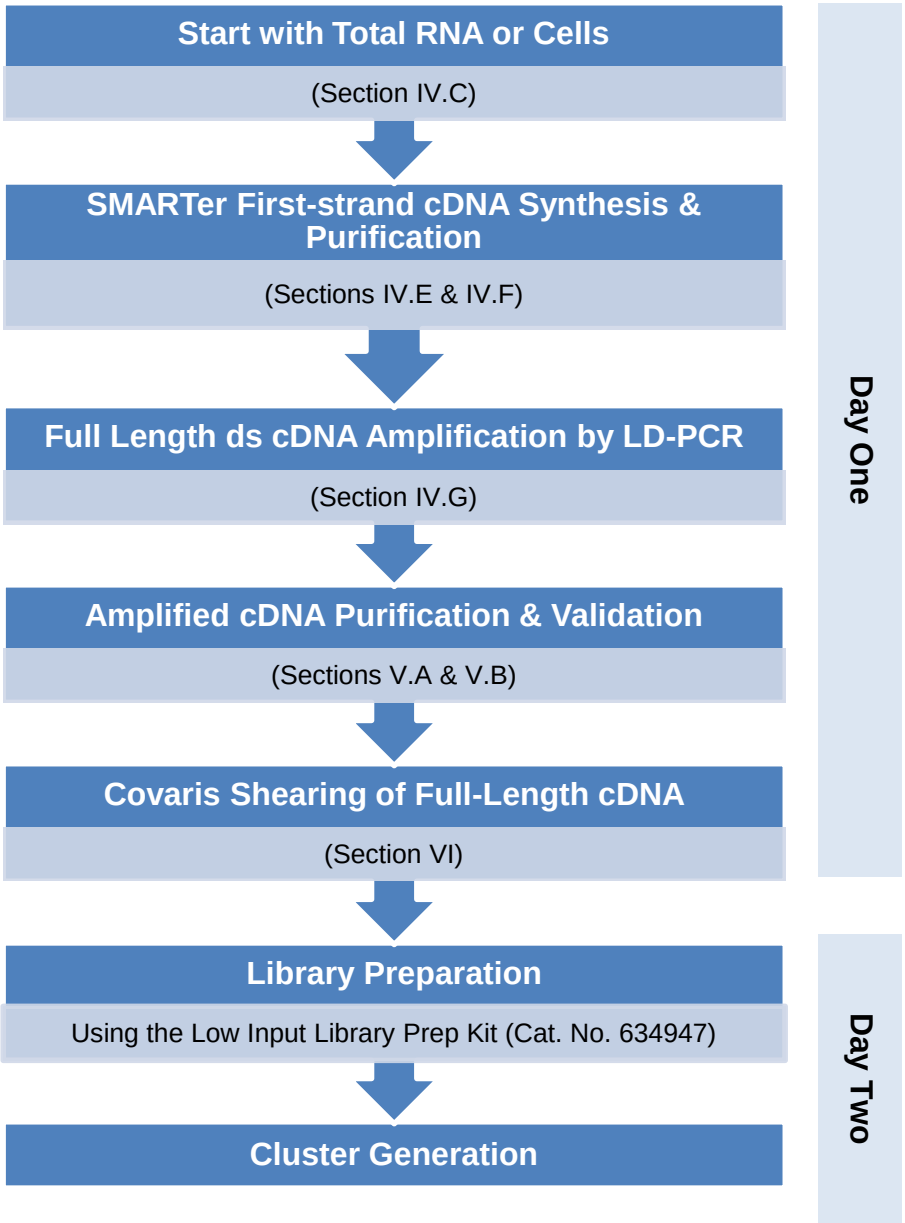

Figure 1. Protocol Overview.

SMARTer cDNA synthesis starts with picogram amounts of total RNA. A modified oligo(dT) primer (the SMART CDS Primer) primes the first-strand synthesis reaction (Figure 2). When SMARTScribe™ Reverse Transcriptase reaches the 5' end of the mRNA, 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 continue replicating to the end of the oligonucleotide (Chenchik *et al*., 1998). The resulting full-length, single-stranded (ss) cDNA contains the complete 5' end of the mRNA, as well as sequences that are complementary to the SMARTer Oligonucleotide. In cases where the RT pauses before the end of the template, the addition of non-template nucleotides is much less efficient than with full-length cDNA/RNA hybrids, thus the overhang needed for base-pairing with the SMARTer Oligonucleotide is absent. The SMARTer anchor sequence and the poly A sequence serve as universal priming sites for end-to-end cDNA amplification. In contrast, cDNA without these sequences, such as prematurely terminated cDNAs, contaminating genomic DNA, or cDNA transcribed from poly-A RNA, will not be exponentially amplified. However, truncated RNAs with poly A tails that are present in poor quality RNA starting material will be amplified yielding shorter cDNA fragments.

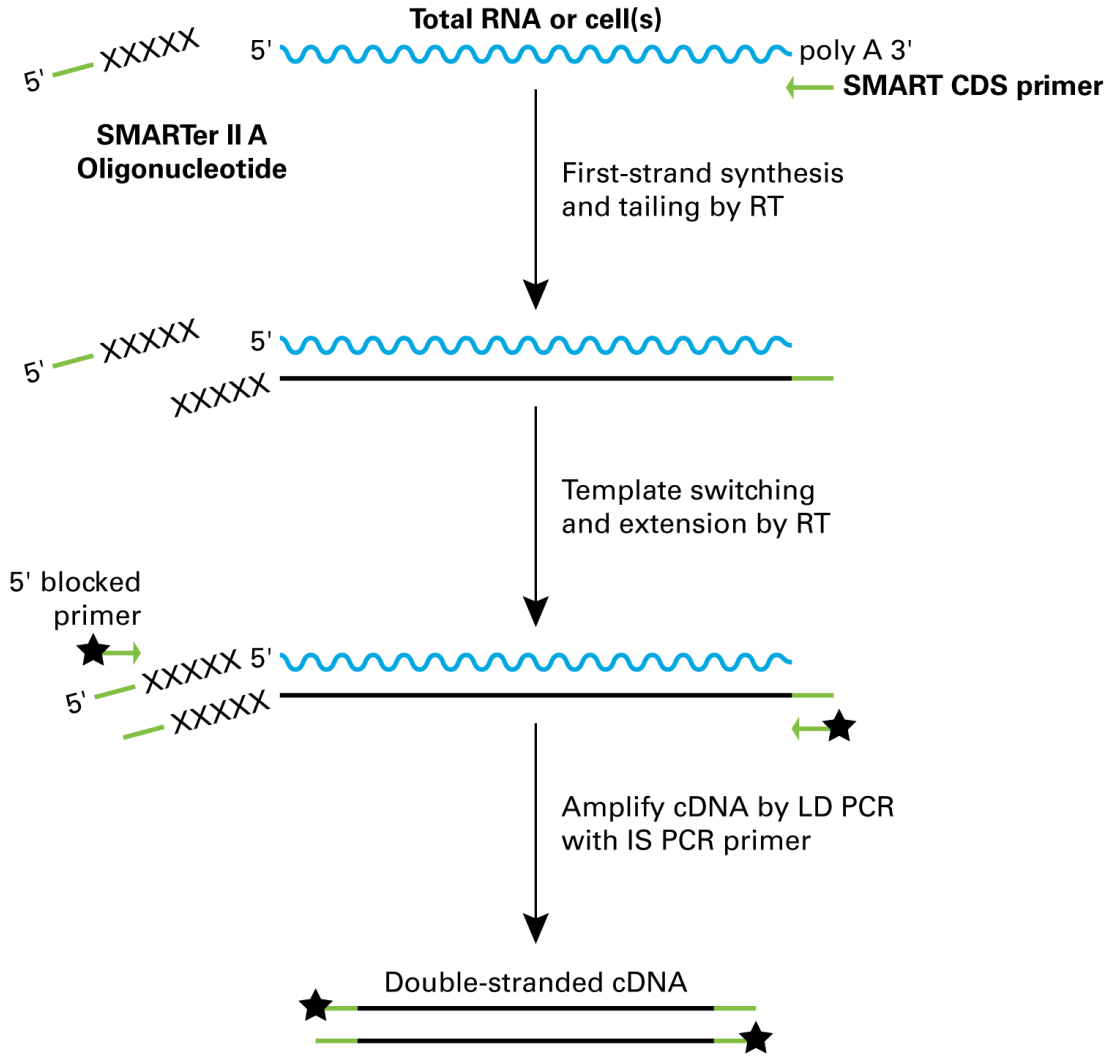

Figure 2. Flowchart of SMARTer cDNA synthesis. The SMARTer II A Oligonucleotide, 3' SMART CDS Primer II A, and IS PCR Primer all contain a stretch of identical sequence.

SMART Adapter in Primer 2 Read

The IS primer used for amplification of the double-stranded cDNA is blocked, which stops Illumina sequencing adapter ligation at the 5' ends of the ds cDNA fragments containing the SMART sequence. In many library preparation methods, the double-stranded Illumina adapters are added to the cDNA fragments through ligation. Unfortunately, in these reactions, ligation takes 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 CDS primer 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 are interested in avoiding sequencing the SMART adapter, there are three options:

1. Sequence only from Read Primer 1.
2. Use the Nextera® or Nextera XT library preparation method.
3. Use the Low Input Library Prep Kit (Cat. No. 634947). This unique adapter addition method does not allow for erroneous ligation.

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

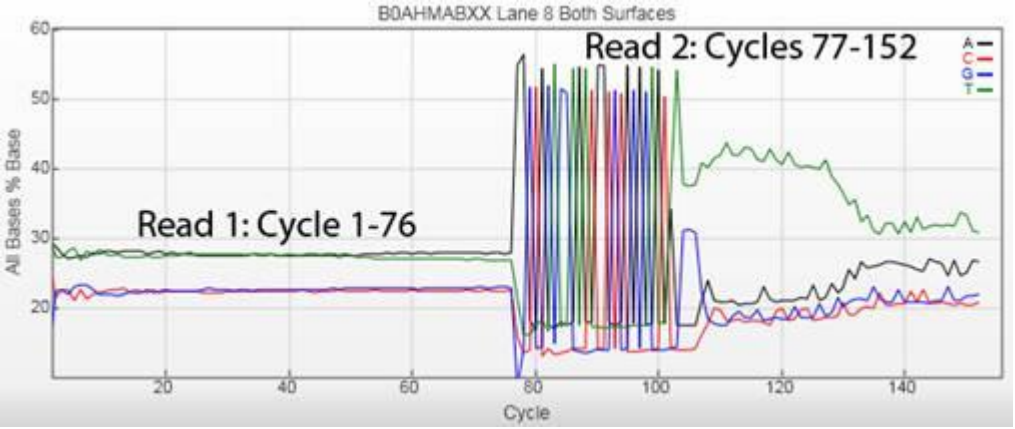

Figure 3. SMART Adapter in Primer 2 Read. The presence of the SMART adapter in Read 2 commonly occurs at a high enough rate to be observed in the base distribution by cycle graph generated by the run analysis (cycles 77-106), as does the dT30 sequence (cycles 107-136).

II. List of Components

The following 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.

The **SMARTer Ultra Low Input RNA for Illumina Sequencing - HV** kits (Cat. Nos. 634820, 634823, 634826, 634828 & 634830) consist of:

- **The Advantage® 2 PCR Kit** (Cat. No. 639206 or 639207), which has been specially formulated to provide automatic hot-start PCR (Kellogg *et al*., 1994) and can efficiently amplify full-length cDNAs with a significantly lower error rate than that of conventional PCR (Barnes, 1994).
- **The SMARTer Ultra Low RNA Kit for Illumina Sequencing Components** (Cat. No. 634822, 634825, 634827, or 634831)

NOTES:

- The SMARTer Ultra Low Input RNA for Illumina Sequencing Components - HV (Cat. Nos. 634822, 634825, 634827 & 634831) and the Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207) both include dNTP mixes.
  - Use the SMARTer dNTP Mix (20 mM each dNTP) for first-strand cDNA synthesis (Step IV.E.5).
  - Use the Advantage 2 dNTP Mix (10 mM each dNTP) for double-stranded cDNA amplification (Step IV.G.1)
- Do not use the Advantage 2 SA Buffer supplied with the Advantage 2 PCR Kit with this SMARTer cDNA synthesis protocol.

The specific composition of each kit is as follows:

SMARTer Ultra Low Input RNA for Illumina Sequencing - HV (12 rxns, Cat. No. 634820)

- **12 rxns SMARTer Ultra Low Input RNA for Illumina Sequencing Components - HV** (Cat. No. 634822; Not sold separately)

Box 1:

12 µl	SMARTer II A Oligonucleotide (24 µM)
5 µl	Control Total RNA (1 µg/µl)

Box 2:

12 µl	3' SMART CDS Primer II A (24 µM)
24 µl	IS PCR Primer (12 µM)
48 µl	5X First-Strand Buffer (RNase-Free)
12 µl	SMARTer dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 20 mM)
12 µl	Dithiothreitol (DTT; 100 mM)
24 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
1 ml	Nuclease-Free Water
66 µl	RNase Inhibitor (40 U/µl)
1 ml	Dilution Buffer
1 ml	Purification Buffer (10 mM Tris-Cl, pH 8.5)

- **30 rxns Advantage 2 PCR Kit** (Cat. No. 639207)

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

SMARTer Ultra Low Input RNA for Illumina Sequencing - HV (24 rxns, Cat. No. 634823)

- **24 rxns The SMARTer Ultra Low Input RNA for Illumina Sequencing Components - HV** (Cat. No. 634825; Not sold separately)

Box 1:

24 µl	SMARTer II A Oligonucleotide (24 µM)
5 µl	Control Total RNA (1 µg/µl)

Box 2:

24 µl	3' SMART CDS Primer II A (24 µM)
48 µl	IS PCR Primer (12 µM)
96 µl	5X First-Strand Buffer (RNase-Free)
24 µl	SMARTer dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 20 mM)
24 µl	Dithiothreitol (DTT; 100 mM)
48 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
2 ml	Nuclease-Free Water
132 µl	RNase Inhibitor (40 U/µl)
2 ml	Dilution Buffer
2 ml	Purification Buffer (10 mM Tris-Cl, pH 8.5)

- **2 x 30 rxns Advantage 2 PCR Kit** (Cat. No. 639207)

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

SMARTer Ultra Low Input RNA for Illumina Sequencing - HV (48 rxns, Cat. No. 634826)

- **48 rxns The SMARTer Ultra Low Input RNA for Illumina Sequencing Components - HV** (Cat. No. 634827; Not sold separately)

Box 1:

48 µl	SMARTer II A Oligonucleotide (24 µM)
5 µl	Control Total RNA (1 µg/µl)

Box 2:

48 µl	3' SMART CDS Primer II A (24 µM)
96 µl	IS PCR Primer (12 µM)
192 µl	5X First-Strand Buffer (RNase-Free)
48 µl	SMARTer dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 20 mM)
48 µl	Dithiothreitol (DTT; 100 mM)
96 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
5 ml	Nuclease-Free Water
264 µl	RNase Inhibitor (40 U/µl)
5 ml	Dilution Buffer
5 ml	Purification Buffer (10 mM Tris-Cl, pH 8.5)

- **100 rxns Advantage 2 PCR Kit** (Cat. No. 639206)

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

SMARTer Ultra Low Input RNA for Illumina Sequencing - HV (96 rxns, Cat. No. 634828)

- **96 rxns The SMARTer Ultra Low Input RNA for Illumina Sequencing Components - HV** (Cat. No. 634831; Not sold separately)

Box 1:

96 µl	SMARTer II A Oligonucleotide (24 µM)
5 µl	Control Total RNA (1 µg/µl)

Box 2:

96 µl	3' SMART CDS Primer II A (24 µM)
192 µl	IS PCR Primer (12 µM)
384 µl	5X First-Strand Buffer (RNase-Free)
96 µl	SMARTer dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 20 mM)
96 µl	Dithiothreitol (DTT; 100 mM)
192 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
10 ml	Nuclease-Free Water
528 µl	RNase Inhibitor (40 U/µl)
10 ml	Dilution Buffer
10 ml	Purification Buffer (10 mM Tris-Cl, pH 8.5)

- **2 x 100 rxns Advantage 2 PCR Kit** (Cat. No. 639206)

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

SMARTer Ultra Low Input RNA for Illumina Sequencing - HV (480 rxns, Cat. No. 634830)

- **480 rxns The SMARTer Ultra Low Input RNA for Illumina Sequencing Components - HV** [Cat. No. 634829 (contains 5 x Cat. No. 634831); Not sold separately]

Box 1:

5 x 96 µl	SMARTer II A Oligonucleotide (24 µM)
5 x 5 µl	Control Total RNA (1 µg/µl)

Box 2:

5 x 96 µl	3' SMART CDS Primer II A (24 µM)
5 x 192 µl	IS PCR Primer (12 µM)
5 x 384 µl	5X First-Strand Buffer (RNase-Free)
5 x 96 µl	SMARTer dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 20 mM)
5 x 96 µl	Dithiothreitol (DTT; 100 mM)
5 x 192 µl	SMARTScribe Reverse Transcriptase (100 U/µl)
5 x 10 ml	Nuclease-Free Water
5 x 528 µl	RNase Inhibitor (40 U/µl)
5 x 10 ml	Dilution Buffer
5 x 10 ml	Purification Buffer (10 mM Tris-Cl, pH 8.5)

- **10 x 100 rxns Advantage 2 PCR Kit** (Cat. No. 639206)

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

Storage Conditions

- Store Control Total RNA and SMARTer IIA Oligonucleotide at –70°C.
- Store Dilution Buffer at –20°C. Once thawed, the buffer can be stored at 4°C.
- Store Purification Buffer at –20°C. Once thawed, the buffer can be stored at Room Temperature.
- Store all other reagents at –20° C.

III. Additional Materials Required

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

- Single channel pipette: 10 µl, 20 µl and 200 µl, one each
- Eight channel pipette: 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:

- One 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 Beckman Coulter Part No. A63880; 60 ml Beckman Coulter Part No. A63881)
  Use this kit for the SPRI Purifications (Sections IV.F & V.A).
- MagnaBot II Magnetic Separation Device (Promega Part No. V8351)
  Use this stand for the first purification (Section IV.F).
- Magnetic Stand-96 (Ambion Part No. AM10027)
  Use this stand for the second purification (Section V.A).
- 96-well V-bottom Plate (500 µl; VWR Cat. No. 47743-996)
- MicroAmp Clean Adhesive Seal (AB Part No. 4306311)
- 80% Ethanol

For Sequencing Library Generation:

- Low Input Library Prep Kit (Cat. No. 634947)
- Covaris Instrument and Related Materials for DNA Shearing

IV. SMARTer cDNA Synthesis

NOTE: Please read the entire protocol before starting. This protocol is optimized for the generation of cDNA starting from ultra-low amounts of total RNA using Clontech's SMART technology. The protocol also works starting from 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) and first-strand cDNA purification (Protocol IV.F).

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

B. General Requirements

- **The success of your experiment depends on the quality of your starting sample of 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

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.

- Although SMART Technology is sensitive enough to generate cDNA from as little as 10 pg of total RNA, the use of a higher amount of starting material (100 pg to 10 ng) is recommended for reproducible amplification of low abundance mRNA transcripts.
- 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).

D. Sample Requirements

The original SMARTer Ultra Low RNA Kit for Illumina Sequencing (Cat. No. 634936) works with a 1 µl sample containing your cells or RNA. We have optimized SMARTer Ultra Low Input RNA for Illumina Sequencing – HV (Cat. Nos. 634820, 634823, 634826, 634828, and 634830) to work 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**
  Although this protocol was optimized for cDNA synthesis starting from total RNA, the protocol has also been validated to work starting from cells.

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

IMPORTANT: To avoid introducing contaminants into your RNA sample, the first part of the cDNA synthesis protocol (Sections E–G) requires use of a PCR work station in a clean room. Standard clean room procedure should be followed. If no clean room is available, you may work with just a PCR Clean Work Station on a temporary basis. We strongly recommend putting the PCR work station in a clean room to avoid contamination. It is critical to have an air blower in the PCR work station turned "on" during the whole process.

1. Prepare a stock solution of Reaction Buffer by mixing the Dilution Buffer with the RNase Inhibitor as indicated below (scale up as needed):

19 µl	Dilution Buffer
1 µl	RNase Inhibitor
20 µl	Total Volume

2. 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 in an 8-well strip. Add 1–5 µl of Reaction Buffer and 1–9 µl of cells or RNA. Use the same volume of Reaction Buffer in the negative and positive controls as in your sample.

Table 1. Sample Preparation Guidelines

Components	Negative Control	Positive Control	Test Sample
Reaction Buffer	1–5 µl	1–5 µl	1–5 µl
Nuclease-free water	1–9 µl	0–4 µl	0–4 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. The Control RNA 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.

3. Place the samples on a –20°C prechilled IsoFreeze PCR rack in a PCR clean station, and add 1 µl of 3' SMART CDS Primer II A (24 µM). Mix the contents and spin the tube(s) briefly in a microcentrifuge:

10 µl	Cell/Total RNA in Reaction Buffer (from Table 1)
1 µl	3' SMART CDS Primer II A (24 µM)
11 µl	Total Volume

4. Place the tubes into a preheated thermocycler and incubate the tube(s) at 72°C in a hot-lid thermal cycler for 3 min, then put the samples on the IsoFreeze PCR rack.

NOTE: The initial reaction steps (Step 6–8) are critical for first-strand synthesis and should not be delayed after completing Step 4. You can prepare your master mix (for Step 5) while your tubes are incubating (Step 4) in order to jump start the cDNA synthesis.

5. 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 (24 µM)
0.5 µl	RNase Inhibitor
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. Mix well by gently vortexing and spin the tube(s) briefly in a microcentrifuge.

NOTE: The SMARTer Ultra Low Input RNA for Illumina Sequencing Components - HV (Cat. Nos. 634822, 634825, 634827 & 634831) and the Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207) both include dNTP mixes. **Use the SMARTer dNTP Mix (20 mM each dNTP) for first-strand cDNA synthesis.**

6. Add 9 µl of the Master Mix to each reaction tube from Step 4. Mix the contents of the tubes by gently pipetting, and spin them briefly to collect the contents at the bottom.
7. Place the tubes in a preheated thermocycler and incubate at 42°C for 90 min.
8. Terminate the reaction by heating the tube(s) at 70°C for 10 min.

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

F. Protocol: Purification of First-Strand cDNA using SPRI Ampure Beads (Perform in 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 are then directly used for PCR amplification.

NOTES:

- Aliquot SPRI beads prior to use.
- Before use, bring beads to room temperature and mix well to disperse.
- In order to ensure proper and steady positioning of the tubes containing first-strand cDNA (from Protocol E), you may place the tubes in the top part of an inverted P20 or P200 Rainin Tip Holder which is taped to the MagnaBot II Magnetic Separator.

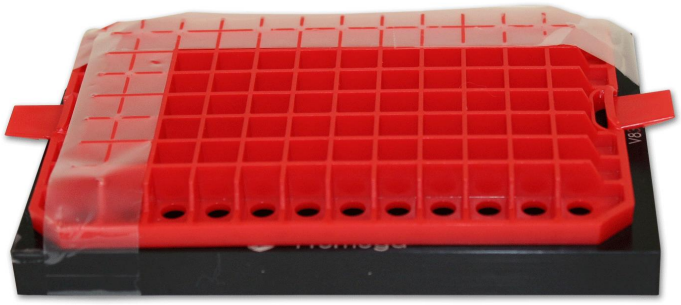

Figure 4. Optional setup to ensure proper and steady positioning of tubes containing first-strand cDNA.

To purify the SMART cDNA from unincorporated nucleotides and small (<0.1 kb) cDNA fragments, follow this procedure for each reaction tube:

1. Add 36 µl of SPRI Ampure XP beads to each sample using a 200 µl pipetter. Adjust the pipetter to 56 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. Incubate at room temperature for 8 minutes to let DNA bind to the beads.
2. 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 min or longer, until the solution is completely clear.
3. While samples are still on the Magnetic Separation Device pipette out the solution and discard. Briefly spin the tubes to collect the liquid at the bottom.
4. Place the tubes back in the Promega MagnaBot II Magnetic Separation Device for 2 min or longer to let beads separate from the liquid completely. Pipette out the residual liquid from the beads using a 10 µl pipetter and discard. Make sure that there is no supernatant remaining in the tube. **Be careful not to remove any beads with the supernatant.**

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

We strongly recommend using the **Advantage 2 PCR Kit** (included in this kit; also sold separately as Cat. Nos. 639206 & 639207) for PCR amplification. The Advantage 2 Polymerase Mix has been specially formulated for efficient and accurate amplification of cDNA templates by long-distance PCR (LD PCR; Barnes, 1994). Table 2 provides guidelines for optimizing your PCR, depending on the amount of total RNA used in the first-strand synthesis.

IMPORTANT: Optimal parameters may vary for 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. Do not exceed the recommended cycle numbers in the table below for different starting amounts of material.

Table 2. Cycling Guidelines Based on Amount of Starting Material

Input Amount, Total RNA	Input Amount, Cells	Typical No. of PCR Cycles
10 ng	1,000 cells	12
1 ng	100 cells	12
500 pg	50 cells	13
100 pg	10 cells	15
10 pg	1 cell	18

1. 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, then mix well by vortexing and spin the tube briefly in a microcentrifuge:

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

NOTE: The SMARTer Ultra Low Input RNA for Illumina Sequencing Components - HV (Cat. Nos. 634822, 634825, 634827 & 634831) and the Advantage 2 PCR Kit (Cat. Nos. 639206 & 639207) both include dNTP mixes. **Use the Advantage 2 dNTP Mix (10 mM each dNTP) for ds cDNA amplification.**

2. Add 50 µl of PCR Master Mix to each tube containing DNA bound to the beads from Section IV.F., Step 4. Mix well and briefly spin down.

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

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

95°C		1 min
X[a] cycles:		
	95°C	15 sec
	65°C	30 sec
	68°C	6 min
72°C		10 min
4°C		forever

[a]Consult Table 2 for guidelines.

V. Amplified cDNA Purification & Validation

A. Protocol: Purification of ds cDNA using SPRI Ampure Beads

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

1. Take out a 96-well Axygen V-bottom plate and cover all the wells with a MicroAmp Clean Adhesive Seal. Uncover only the wells that you want to use. Vortex SPRI beads till even, and then add 90 µl of SPRI Ampure XP Beads to the wells of the 96-well plate.
2. Transfer the entire PCR product including the SPRI beads (from Section IV.G, Step 3) to the wells of the plate containing the SPRI beads (from Step 1 above). Pipette the entire volume up and down 10 times to mix thoroughly. Incubate at room temperature for 8 min 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 min or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
4. While the plate is sitting on the magnetic stand, 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. DNA will remain bound to the beads during the washing process.
6. Repeat Step 5 one more time.
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. Place the plate at room temperature for ~3–5 min until the pellet appears dry. You may see a tiny crack in the pellet when it is dry.

   NOTE: Be sure to dry the pellet enough.
   - If you under-dry the pellet, ethanol will remain in the sample wells. The ethanol will reduce your ds 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, it will take longer than 2 min to rehydrate (Step V.A.10).

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 min to rehydrate.
11. Mix the pellet by pipetting up and down 10 times to elute DNA 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 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.

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 (Cat. No. 5067-4626). See the user manual for the Agilent High Sensitivity DNA Kit for instructions.
2. Compare the results for your samples & controls (see Figure 5) to verify whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield no product in the negative control (Figure 5, Panel B), and a distinct peak spanning 400 bp to 9,000 bp, peaked at ~2,000 bp for the positive control RNA sample (Figure 5, Panel A), yielding approximately 2–7 ng of cDNA (depending on the input). Contaminated samples will have a broader peak, and an abnormally high yield (Figure 5, Panel C).
3. Proceed to **Section VI: Covaris Shearing**.

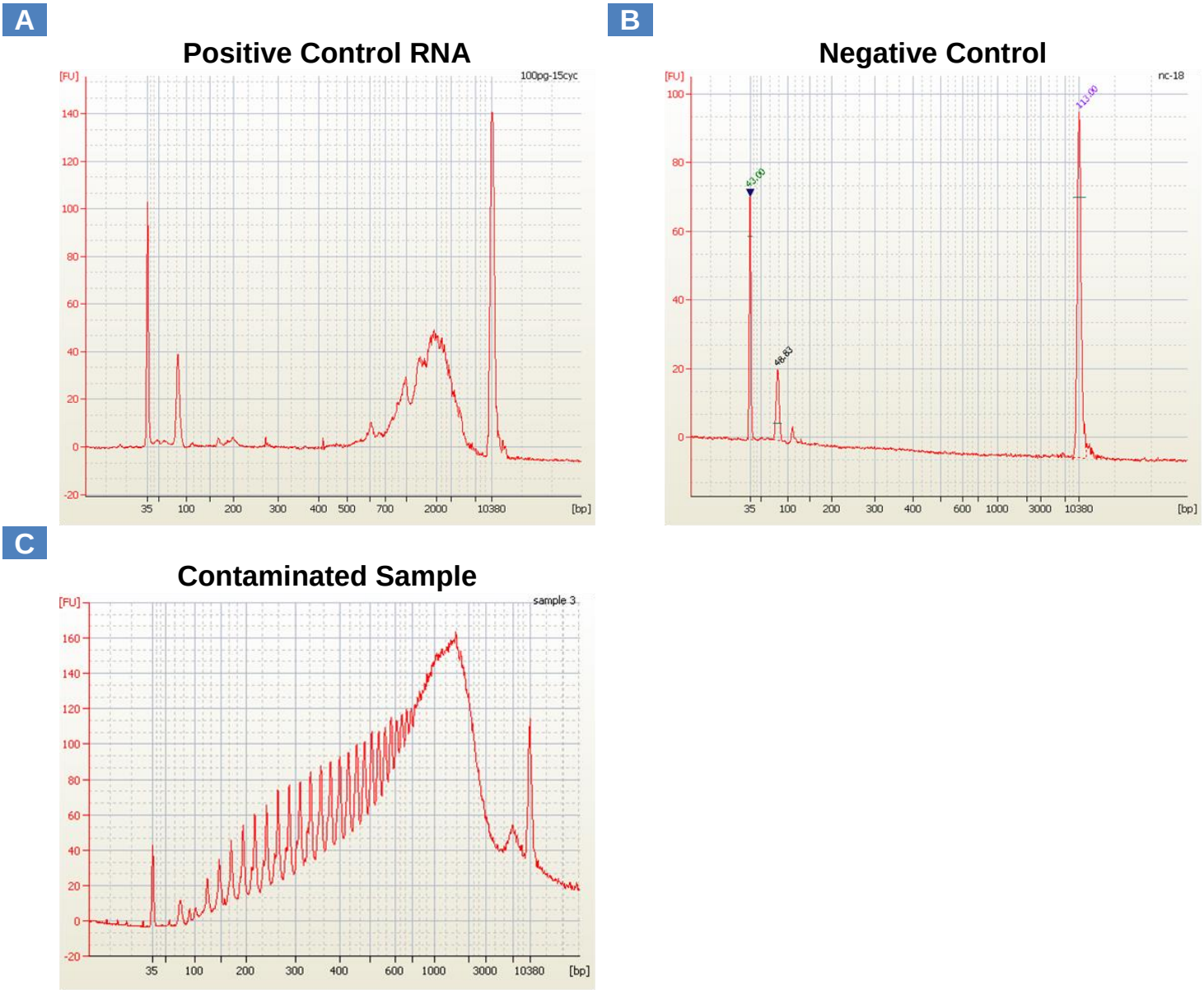

Figure 5. Electropherogram example results from Agilent 2100 Bioanalyzer. All samples were subjected to SMARTer cDNA synthesis and amplification as described in the protocol. FU = fluorescence absorption units. **Panel A (top left).** Clean SMARTer Amplification Product (15 PCR cycles) **Panel B (top right).** Clean SMART Negative Control (18 PCR cycles). **Panel C (bottom).** Example of Contaminated SMARTer Amplification Product.

VI. Covaris Shearing

A. Protocol: Covaris Shearing of Full-length cDNA

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.

NOTE: The full-length cDNA output of the SMARTer Ultra Low Input RNA for Illumina Sequencing - HV can be processed using either the following protocol or our modified protocol for the Nextera DNA Sample Preparation kits from Illumina.

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 Purification Buffer to the DNA from Section V.A., Step 12. Transfer 75 µl of the Purification Buffer + DNA mixture into the 130-µl Covaris tube. Put the sample tubes into the appropriate location on the Sample holder.
4. Set up the process configuration panel based on the following table:

   Table 3. Process Configuration Panel Setup

Duty %	Intensity	Burst Cycle	Time (min)	Mode
10	5	200	5 min	Frequency Sweeping

5. Save the file and click return to go back to the main page.
6. Open the door. Place the tube holder with sample tubes on the transducer positioning system.
7. Close the door.
8. Click "START" on the main page to run the process.
9. After shearing is complete, transfer 75 µl of sheared DNA to 1.5 ml tubes.
10. Proceed to generate an Illumina Sequencing Library with the Low Input Library Prep Kit (Cat. No. 634947). Dispose all tubes and pipettes that have been exposed to amplicons in a sealed trash bag.

VII. References

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