Clontech Laboratories, Inc.

SMARTer® Target RNA Capture for Illumina® User Manual

Cat. Nos. 635035, 635036
(090116)

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I. Introduction

SMARTer Target RNA Capture for Illumina (Cat. Nos. 635035, 635036) is designed to generate cDNA from enriched total RNA. Target-specific biotinylated DNA probes designed by the user are hybridized to transcripts of interest and the resulting RNA-DNA hybrids are captured with Capture Beads. This kit takes advantage of the sensitivity of the SMART-Seq® v4 Ultra® Low Input RNA Kit for Sequencing to generate high-quality, full-length cDNA from these enriched transcripts. The kit has been tested with 10 ng of total RNA, and has been shown to perform cDNA synthesis from enriched transcripts derived from as little as 100 pg of purified total RNA. cDNA libraries generated with this kit have been tested and confirmed for compatibility with Illumina sequencing platforms. The cDNA synthesis protocol can be completed without the need for additional rRNA removal methods or kits, and the entire library construction protocol can be completed within two working days (Figure 1).

Targeted RNA-seq can overcome several challenges inherent in whole transcriptome RNA-seq experiments, improving results while also saving costs and simplifying analysis. The first of these challenges lies in improving the sequence coverage of transcripts present in low amounts. The large dynamic range of the transcriptome often presents an issue: highly abundant transcripts (e.g., 100 copies per cell) represent only a small percentage of RNAs, but may account for more than the majority of mapped reads. In contrast, less-abundant transcripts (representing a majority of RNAs) are represented in only a small percentage of mapped reads (Jiang, *et al.*, 2011). Targeted RNA-seq provides improved sensitivity by first enriching for transcripts of interest, yielding information about transcripts that would otherwise be missed, e.g., chimeric gene fusions, transcript isoforms, and splice variants (Byron, *et al.,* 2016). By improving sensitivity, the power of analysis is also improved, as seen in a reduction in the ratio of signal to noise.

These improved results are accompanied by a reduction in experimental costs and time spent on analysis. Since it is possible to enrich for a specific sequence of interest, less sequencing depth is necessary—meaning similar information is obtained at a much lower cost. In turn, data analysis is also simpler because only sequences of interest need to be analyzed.

In brief, this enrichment protocol (Figure 1) is a combination of hybridization-based capture of transcripts and SMART-Seq v4 technology for oligo(dT)-primed cDNA synthesis. Total RNA is hybridized with user-designed biotinylated DNA probes in less than four hours, and then purified prior to capture on Capture Beads—magnetic particles coated with streptavidin. The Capture Beads have been selected because they exhibit especially low adsorption of protein and nucleic acids, and do not interfere with downstream reactions such as PCR or reverse transcription, making it possible to perform cDNA synthesis on the targeted samples while they remain attached to the beads. SMART-Seq v4 first-strand cDNA synthesis provides high sensitivity, low background, and ensures the production of full-length cDNAs. For more information on the SMART-Seq v4 technology and workflow, please visit **www.clontech.com**. A schematic outline is shown in the SMART-Seq v4 User Manual.

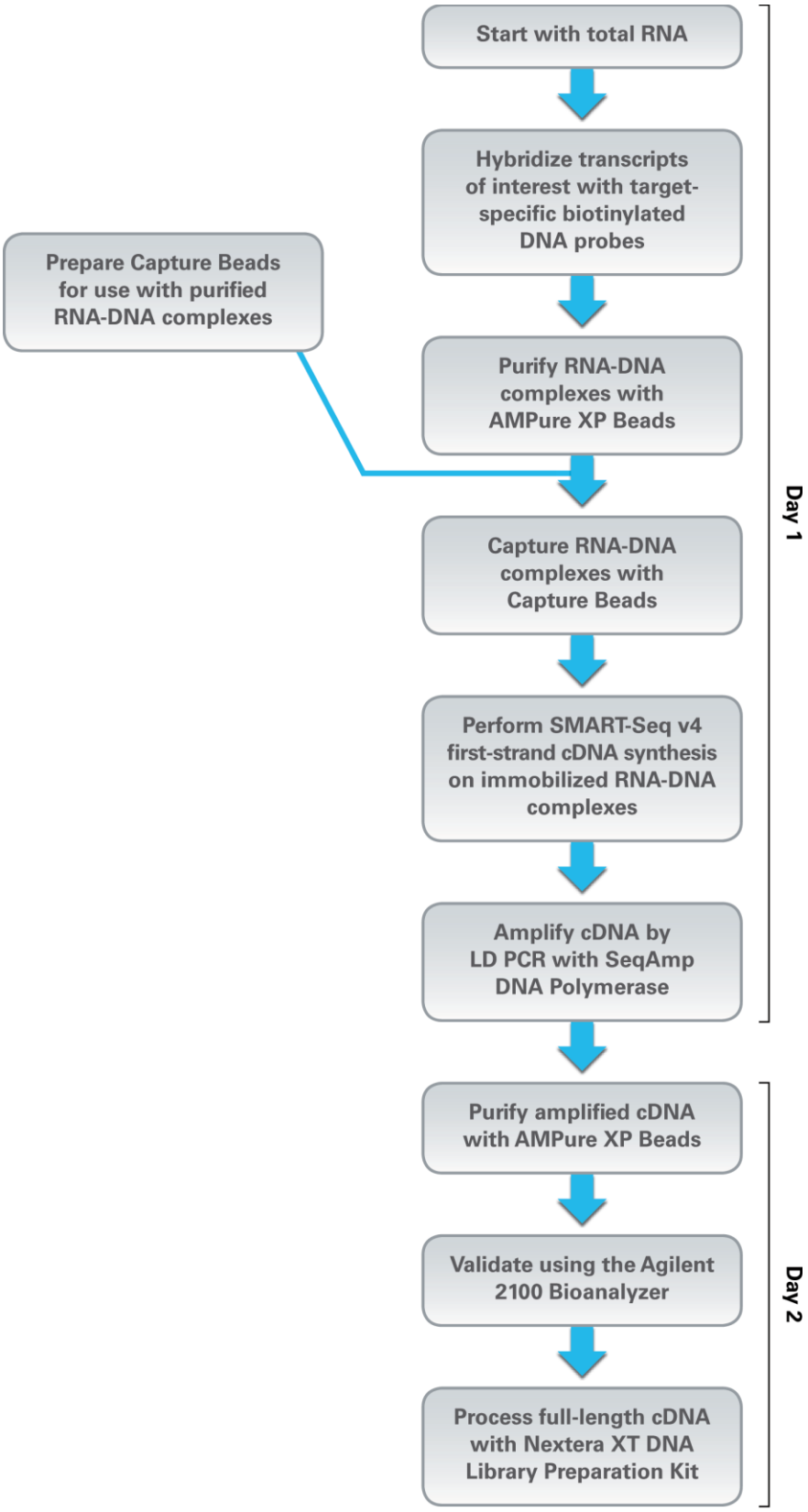

Figure 1. Protocol overview. The protocol through validation is completed over two days, with just 2.5–3 hours of hands-on time and without the need for additional rRNA removal methods or kits.

II. List of Components

SMARTer Target RNA Capture for Illumina consists of the SMARTer Target RNA Capture for Illumina 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 Target RNA Capture for Illumina	635035 (12 rxns)	635036 (48 rxns)
SeqAmp DNA Polymerase (Store at –20°C.)		
SeqAmp DNA Polymerase	50 µl	2 x 50 µl
SeqAmp PCR Buffer (2X)	1.25 ml	2 x 1.25 ml
SMARTer Target RNA Capture for Illumina Components (Not sold separately. Storage conditions are listed below for Packages 1, 2, and 3.)		
Box 1 (Store at –70°C.)		
Control Total RNA (1 µg/µl)	5 µl	5 µl
Box 2 (Store at –20°C. Once thawed, store 10X Lysis Buffer at 4°C and store Elution Buffer, 10X SSC, 1X Wash Buffer, and DMSO at room temperature. Continue to store all other reagents at –20°C.)		
SMART-Seq v4 Oligonucleotide (48 µM)	12 µl	48 µl
PCR Primer II A (12 µM)	12 µl	48 µl
5X Ultra Low First-Strand Buffer	48 µl	192 µl
SMARTScribe™ Reverse Transcriptase (100 U/µl)	24 µl	96 µl
3’ SMART-Seq CDS Primer II A (12 µM)	12 µl	48 µl
RNase Inhibitor (40 U/µl)	30 µl	120 µl
Nuclease-Free Water	1 ml	3 x 1 ml
10X Lysis Buffer	12 µl	48 µl
Elution Buffer (10 mM Tris-Cl, pH 8.5)	1.7 ml	6.8 ml
10X SSC	25 µl	100 µl
DMSO (100%)	25 µl	100 µl
GC-Melt*	200 µl	1 ml
1X Wash Buffer	10 ml	30 ml
2X Capture Buffer	120 µl	480 µl
HPRT1 Control Probes (1 pmol/µl)	5 µl	5 µl
Box 3 (Store at 4°C. Do not allow to freeze.)		
Capture Beads (1%)**	250 µl	2 x 250 µl

* At times, precipitate may be observed in the GC-Melt. This precipitate does not affect the performance of the kit. The precipitate can be dissolved rapidly by mixing at room temperature or warming at 37°C for a few min.

** Capture Beads can also be purchased separately (Cat. No. 635039).

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, 200 µl, and 1 ml
- Twelve-channel pipette (recommended): 20 µl and 200 µl
- Filter pipette tips: 10 µl, 20 µl, and 200 µl
- Minicentrifuge for 1.5-ml tubes
- Minicentrifuge for 0.2-ml tubes or strips
- Two magnetic separation devices, one for pre-PCR steps and one for post-PCR steps
    - For 24 samples ore fewer: magnetic separator - PCR Strip (Cat. No. 635011)
    - For more than 24 samples, we recommend using a 96-well magnetic separation device (Magnetic Stand-96, Thermo Fisher Scientific, Cat. No. AM10027)
- One dedicated thermal cycler used only for hybridization (Section V.A.) and first-strand cDNA synthesis (Section V.E)
- Nuclease-free thin-wall 0.2-ml PCR tubes, tube strips, or a 96-well plate

  NOTE: We recommend using strip tubes (0.2-ml PCR 8-tube strip; USA Scientific, Item No.1402-4700) or a 96-well plate (e.g., 96-well V-bottom Plate, 500 µl; VWR, Cat. No. 47743-996) wherever possible to reduce and simplify handling, especially when using the magnetic separator or other magnetic separation device.

  It is good practice to ensure that the caps remain closed while vortexing to prevent sample loss or contamination. This may require keeping pressure on the top of the caps during vortexing.

- Nuclease-free low-adhesion 1.5-ml tubes (USA Scientific, Item No. 1415-2600)

For Capture with Capture Beads:

- Tube rotator capable of maintaining a constant speed sufficient to keep the beads in solution (similar to SilentShake Revolver, Cat. No. HYQ-1130A; Sections V.B and V.D)

For PCR Amplification & Validation:

- High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626; Section V.F)

For AMPure Bead Purifications (Sections V.C and V.G):

- Agencourt AMPure XP PCR purification kit (5 ml Beckman Coulter Item No. A63880; 60 ml Beckman Coulter Item No. A63881)
    - Pre-aliquoted and labeled as “Hybridization” and “Post-PCR” samples.

  NOTE: AMPure beads need to come to room temperature before the container is opened. Therefore, we recommend aliquoting the beads into 1.5-ml tubes upon receipt, and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min). This will also decrease the chances of bead contamination. Mix well to disperse the beads before adding them to your reactions. The beads are viscous, so pipette slowly.

For Sequencing Library Generation (Section VI):

- Nextera® XT DNA Library Preparation Kit (Illumina, Cat. Nos. FC-131-1024, FC-131-1096)

IV. 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., hybridization, capture, and first-strand cDNA synthesis (Sections V.A–V.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 (Section V.F) and measure cDNA concentration (Protocol V.H).

B. General Requirements

- **The success of your experiment depends on the quality and integrity of your input RNA. Prior to hybridization, 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 or concentration of any reaction component. The amounts and concentrations have been carefully optimized for the hybridization, capture, and 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. There are several commercially available products that enable purification of total RNA preparations [e.g., Clontech offers the NucleoSpin RNA XS kit (Cat. No. 740902.10)]. 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) or Agilent RNA 6000 Nano Kit (Cat. No. 5067-1511). Refer to the manufacturer's instructions for information on how to use the Agilent RNA 6000 Pico Kit or the Agilent RNA 6000 Nano Kit.

D. Sample Requirements

SMARTer Target RNA Capture for Illumina works with up to 1 µg total RNA.

- **Total RNA**
  This protocol has been optimized for hybridization starting from 10 ng of total RNA. However, if your RNA sample is not limiting, we recommend that you start with more total RNA (up to 1 µg). Purified total RNA should be in nuclease-free water.

E. Workflow Recommendations

- **Master mixes:** Always prepare enough master mix for the number of samples, plus an extra 10% to allow for pipetting errors. Prepare and keep all master mixes on ice unless noted. While adding the master mix to the samples, keep samples on ice, or place them back on ice immediately after adding the master mix. We strongly recommend the use of a 96-well chiller rack for best results. If using an aluminum chiller rack, simply keeping it on top of ice will keep it cold.
- **Mixing:** Adequate mixing is required after the addition of components at each step. We recommend a quick, **gentle** vortexing with five 1-sec pulses (avoiding the creation of bubbles as much as possible) for mixing followed by a brief spin to gather sample at the bottom of the tube. Although gentle pipetting up and down can be used for mixing, we have found that it can lead to a lower yield.
- **Controls:**
  - If you would like to perform a positive control, the provided HPRT1 Control Probes can be used with the provided Control Total RNA. Note that the HPRT1 Control Probes should work with any human RNA.
  - If you would like to perform a no-capture control, perform the standard SMART-Seq v4 protocol with the same RNA input but amplify for the number of cycles used in the SMARTer Target RNA Capture for Illumina protocol.
  - If you would like to perform a no-probe control, perform the hybridization protocol (Step A6) with no probes and instead make up the volume with Nuclease-Free Water. Amplify your cDNA with the same number of cycles as for your sample.
  - If you would like to perform a no-RNA control, perform the hybridization protocol (Step A6) with no RNA and instead make up the volume with Nuclease-Free Water. Amplify your cDNA with the same number of cycles as for your sample.
- **Thermal cycling:** Pre-program the thermal cycler with all three programs used in this protocol before starting:
  - Hybridization (to use in Section V.A)
  - First-Strand cDNA Synthesis (to use in Section V.E)
  - cDNA Amplification by LD PCR (to use in Section V.F); may need modification based on the number of cycles to be used
- **AMPure XP Beads:**
  - Aliquot beads into 1- to 2-ml aliquots. You will need 66 µl per sample.
  - Before use, beads should be brought to room temperature for at least 30 min.
  - Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogenous. Confirm that there is no remaining pellet of beads at the bottom of the tube.
  - After adding the beads to the reaction, mix the solution thoroughly by vortexing with five 1-sec pulses.
  - Change the tips for each sample; or when using a multi-channel pipette, change the tips after each column.

- **Capture Beads:**
  - Capture Beads are large and settle quickly. Therefore, every effort should be made to keep them in suspension.
  - For Cat. No. 635036 (48 rxns), aliquot the Capture Beads into an 8-tube strip. You will need 10 µl per sample. Use a multi-channel pipette to prepare the beads as described in Section V.B.
  - Prior to any incubation step, vortex with five 1- sec pulses to be sure the beads are in suspension.
  - If any beads are on the side of the tube after vortexing, spin down briefly—but be sure the beads are still in suspension following the spin.
  - We do not recommend pipetting up and down to mix, as this could result in the loss of beads.

V. Protocols

NOTE: Please read the entire protocol before starting. This protocol is optimized for cDNA synthesis from ultra-low input amounts of total RNA. Due to the sensitivity of the protocol, the input material (total RNA) needs to be collected and purified under clean-room conditions to avoid contamination. The whole process of SMART-Seq v4 cDNA Synthesis should be carried out in a PCR Clean Work Station under clean-room conditions.

The protocol described in this user manual has been optimized for cDNA synthesis using SMARTer Target RNA Capture for Illumina only.

A. Protocol: Hybridization (Perform in dedicated PCR Clean Work Station)

Target-specific biotinylated DNA probes are hybridized to transcripts of interest.

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

1. Make sure the 10X SSC and DMSO are at room temperature. Gently vortex each tube to mix, and spin down briefly.
2. Thaw the Nuclease-Free Water, GC-Melt, and probe mixture (pooled to a concentration of 1 pmol of total probes per µl; see Appendix A) at room temperature. Gently vortex each tube to mix and spin down briefly. After thawing, store all on ice.
3. Thaw the RNA on ice. Gently vortex each tube to mix and spin down briefly, then store on ice.
4. If you need to dilute your RNA and will use it immediately, dilute it in Nuclease-Free Water. If you will be storing your RNA for later use, first mix it Nuclease-Free Water with 5 µl of RNase Inhibitor at a ratio of 50:1, and then dilute your RNA appropriately.
5. Prepare Master Mix by combining the following reagents at room temperature, in the order shown below. If you are running 3 or fewer reactions, prepare enough Master Mix for 4 reactions, otherwise, prepare enough Master Mix for the total number of samples + 1. Mix by vortexing with five 1-sec pulses and spin down briefly.

Reagent	Amount (µl)
GC-Melt	3
10X SSC	0.5
DMSO	0.5

NOTE: If the same probe set will be used in each reaction, it can be included in the Master Mix at 1 µl per reaction. This will alter the amount of Master Mix to add in Step 6 accordingly.

6. Aliquot 4 µl of Master Mix for each sample and add probes and RNA in the amounts shown below, and add enough Nuclease-Free Water to bring the total reaction volume to 10 µl. Mix by vortexing with five 1-sec pulses and spin down briefly.

Reagent	Test Sample	Positive Control
Master Mix	4 µl	4 µl
Probe Mix (1 pmol total probes)	1 µl	1 µl
RNA sample	1 to 5 µl	1–5 µl*
Nuclease-Free Water	0–4 µl	0–4 µl
Total volume	10 µl	10 µl

*We recommend using the same amount of Control Total RNA as used in your test sample.

7. Place reactions in a preheated thermal cycler with a heated lid and run the following Hybridization program:

NOTE: Depending on the capabilities of your thermal cycler, this can be set up as a ramping program, with the ramp set at 1 degree per 10 min. However, we recommend setting it up as a stepwise program, as shown below.

Under certain conditions, a much faster hybridization protocol may be used. See Appendix B.

75°C	2 min
65°C	10 min
64°C	10 min
63°C	10 min
62°C	10 min
61°C	10 min
60°C	10 min
59°C	10 min
58°C	10 min
57°C	10 min
56°C	10 min
55°C	10 min
54°C	10 min
53°C	10 min
52°C	10 min
51°C	10 min
50°C	10 min
20°C	forever

While the program is running, complete Protocol B and Steps C1–C2 below.
When the program is complete, immediately go to Step C3 below.

B. Protocol: Preparation of Capture Beads (Perform in PCR Clean Work Station)

Capture Beads are prepared for Protocol D, where the hybridized RNA-DNA complexes are immobilized on the beads. It is important that the Capture Beads be ready for immediate use at the start of Protocol D.

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

1. Prepare reagents and Capture Beads:
    a. Warm up the Capture Beads and 1X Wash Buffer at room temperature for 30 min before use.
    b. Thaw the 2X Capture Buffer at room temperature. Vortex and spin down. Store on ice.
    c. Preparation for Protocol D: Thaw all the reagents needed for first-strand cDNA synthesis (except the SMARTScribe Reverse Transcriptase) on ice. Vortex each reagent to mix, and spin down briefly. Store on ice.

NOTE: The 5X Ultra Low First-Strand Buffer may form precipitates. Thaw this buffer at room temperature and vortex before using to ensure all components are completely in solution

2. Label fresh tubes. Vortex the tube of Capture Beads for 20 sec and immediately transfer 10 µl to each well of the tubes.

   NOTE: The Capture Beads settle to the bottom of the tube quickly, so make sure to mix the beads by vortexing the tube with two 1-sec pulses prior to each transfer.

3. Place the tube strips on the magnetic separator until the liquid appears completely clear and there are no beads left in the supernatant, and remove the supernatant.
4. Add 50 µl of 1X Wash Buffer and mix by vortexing with five 1-sec pulses. Spin down for 5 sec.
5. Place the tube strips on the magnetic separator until the liquid appears completely clear and there are no beads left in suspension. Remove the supernatant.
6. Spin down briefly to collect the liquid at the bottom of the tubes. Place the tube strips on the magnetic separator until the liquid appears completely clear and there are no beads left in suspension and remove any remaining supernatant.
7. Immediately add 10 µl of 2X Capture Buffer to the bead pellet. Mix by vortexing with five 1-sec pulses followed by rotating for 10–15 min at room temperature. The exact time is not critical, but excessive incubation may be detrimental. Rotate at a speed sufficient to keep the Capture Beads in solution.

C. Protocol: Purification of Hybridized Sample (Perform in PCR Clean Work Station)

Hybridized RNA-DNA complexes are separated from free, unhybridized probes by immobilization on AMPure XP Beads. The beads are then washed with 80% ethanol and the complexes are eluted with Nuclease-Free Water.

1. Warm up the AMPure XP Beads (pre-aliquoted AMPure XP Beads labeled “Hybridization”) at room temperature for ~30 min.

   NOTES:

   - Aliquot AMPure XP beads into 1.5-ml tubes upon receipt in the laboratory.
   - Before each use, bring bead aliquots to room temperature for at least 30 min and mix well to disperse.
   - Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.

2. Prepare 80% ethanol using Nuclease-Free Water.
3. Prepare a mixture of 1 µl of RNase Inhibitor and 9 µl of Nuclease-Free Water per reaction + 10%. Mix and spin down briefly. Add 10 µl of this mixture to each reaction.
4. Vortex the AMPure XP Beads for 20 sec to make sure the beads are well resuspended and homogeneous before use.
5. Add 16 µl of AMPure XP Beads to each reaction.

   NOTE: The beads are viscous; suck up the entire volume **SLOWLY**, and push it out **SLOWLY**.

6. Tightly close the lids, vortex the samples with five 1-sec pulses, and incubate at room temperature for 8 min to allow the RNA-DNA complexes to bind to the beads.

7. After the 8-min incubation, spin down the samples for 5 sec and place them on the magnetic separator for ~5 min or longer, until the liquid appears completely clear and there are no beads left in the supernatant.
8. While leaving the samples on the magnetic separator, open the lids, and carefully pipette off the supernatant, being careful not to remove any beads.
9. Keep the samples on the magnetic separator. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads. Wait for 30 sec and carefully pipette out the supernatant, again being careful not to remove any beads. The RNA-DNA complex will remain bound to the beads and the free probes washed away.
10. Repeat Step 8–9 once more.
11. Close the lids and briefly spin down for 10 sec to collect the liquid at the bottom of the well.
12. Place the samples on the magnetic separator for 30 sec, then remove all remaining ethanol by carefully pipetting.

    NOTE: It is important to make sure all ethanol is removed so that the samples will elute well from the beads, and recovery will be efficient.

13. Remove the samples from the magnetic separator and place the samples (with lids open) at room temperature for 2 min, until the pellet appears dry. Only let dry until the ethanol has just evaporated, and the pellet is no longer glossy.

    NOTE: If you over-dry the beads, you may see cracks in the pellet. If this occurs, the complex may not elute well from the beads, and recovery may be lowered. We recommend having Nuclease-Free Water ready (e.g., in strip tubes for convenient multi-channel pipetting) to add to the samples immediately after the pellet has dried.

14. Add 10 µl of Nuclease-Free Water from to each sample to cover the beads. Incubate at room temperature for 2 min to rehydrate the pellet.
15. Mix the bead pellet by pipetting up and down 10 times to elute the complex from the beads. Put the samples back on the magnetic stand.
16. Transfer all of the clear supernatant containing the purified RNA-DNA complexes to the corresponding tubes containing the prepared Capture Beads (from Step B7).

D. Protocol: RNA Capture with Capture Beads (Perform in PCR Clean Work Station)

Hybridized RNA-DNA complexes are immobilized on streptavidin-coated Capture Beads, where downstream cDNA synthesis will take place.

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

1. Place the tube strips containing the Capture Beads in solution and the RNA-DNA complexes (Step C16) on a rotator and rotate end-over-end for 10 min.
2. Place the tubes on the magnetic separator for 1 min and carefully remove the supernatant without removing any Capture Beads.

3. Wash the samples by adding 100 µl 1X Wash Buffer per tube and tightly close the lid. Resuspend the beads by vortexing with five 1-sec pulses, and leave the tube at room temperature for 2 min. Spin down for 5 sec, place the tube strips on the magnetic separator for 1 min, and carefully remove the supernatant without touching the beads.

   NOTE: Some excess Wash Buffer is provided in your kit. We recommend using a disposable reservoir and multi-channel pipette in order to add the Wash Buffer to your samples. Use enough Wash Buffer in the reservoir to ensure that each sample will receive the entire 100 µl for each wash. You will need 300 µl per sample. Discard any unused Wash Buffer remaining in the reservoir.

4. Repeat Step 2 two more times, for a total of three washes. Prepare the mixture in Step E1 while performing these wash steps, in order to be prepared to immediately move to Protocol E following the third wash.

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

First-strand cDNA synthesis on immobilized, targeted transcripts is primed by the 3' SMART-Seq CDS Primer II A and uses the SMART-Seq v4 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 requires the use of a PCR clean work station, ideally in a clean room.

1. While the samples incubate during the wash steps (Steps D3 and D4), prepare a mixture of 11.5 µl of Nuclease-Free Water and 1 µl of 3' SMART-Seq CDS Primer IIA for each reaction, + 10%. Vortex with five 1-sec pulses, spin down briefly, and place on ice.
2. Immediately following the third wash (Step D3), spin down the tube for 3 sec, place the tube on the magnetic separator, and remove any remaining liquid to ensure all the Wash Buffer has been removed. Residual Wash Buffer may inhibit downstream reactions. Add 12.5 µl of the 3' SMART-Seq CDS Primer IIA mixture from Step E1 to the bead pellet.
3. Mix by vortexing with five 1-sec pulses. Make sure all the beads are resuspended well. If needed, spin down the tube for 1 sec to collect all the contents at the bottom of the tube. Proceed immediately to the next step.
4. Incubate all samples at 72°C in a thermal cycler with a heated lid for 3 min, then immediately place the samples on ice for 2 min.
5. Make enough Master Mix for your samples + 10% on ice, as shown in the table below. Mix by vortexing with five 1-sec pulses, and spin down for 2 sec.

Component	Amount (µl)
5X Ultra Low First-Strand Buffer	4
SMART-Seq v4 Oligonucleotide	1
RNase Inhibitor	0.5
SMARTScribe Reverse Transcriptase	2

6. Transfer 7.5 µl of each mixture to each of the sample tubes for a total of 20 µl in each tube. Vortex with ten 1-sec pulses. Make sure all the beads are resuspended. If there are beads on the walls of the tubes, spin down for 1–2 sec to collect all the beads at the bottom of the tubes, but be sure that the beads remain in suspension.

7. Immediately place the tubes in a thermal cycler with a heated lid, preheated to 42°C. Run the following First-Strand cDNA Synthesis 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 1–4 in a PCR Clean Work Station)

PCR Primer II A amplifies cDNA from the sequences introduced by 3' SMART-Seq CDS Primer II A and the SMART-Seq v4 Oligonucleotide. This reaction is performed in the presence of the Capture Beads, however the cDNA template is in solution.

IMPORTANT: Table 1 below provides guidelines for PCR optimization, depending on the amount of total RNA used for the hybridization and how many genes are being captured. These guidelines were determined using the Control Total RNA and the HPRT1 Control Probes. Optimal parameters may vary for different templates, different cell types, different numbers of genes targeted, 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.

Table 1. Cycling Guidelines Based on Amount of Starting Material

Input Amount of Total RNA	Cycles for Control RNA with HPRT1 Control Probes	Typical Number of Cycles for Multi-Gene Capture
1 µg	14	11
100 ng	17	14
10 ng	20	17
1 ng	23	20

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:

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

NOTE: Remove the SeqAmp DNA polymerase from the freezer, gently but thoroughly mix the tube without vortexing, and add to the Master Mix just before use.

3. Mix well by vortexing gently with five 1-sec pulses. If possible, turn down the speed of the vortex for gentle but thorough mixing. Spin the tube down briefly.

4. Add 30 µl of PCR Master Mix to each tube containing 20 µl of first-strand cDNA product from Section V.E. Gently vortex all samples with five 1-sec pulses. If possible, turn down the speed of the vortex for gentle but thorough mixing. Spin down for 2 sec.

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

5. Immediately place the tube(s) in a preheated thermal cycler with a heated lid and run the following cDNA Amplification by LD PCR 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 1 for PCR cycle number guidelines.

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

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

PCR-amplified cDNA is separated from the Capture Beads and then purified by immobilization on 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 bead aliquots to room temperature for at least 30 min and mix well to disperse.
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample. Add 1 µl of 10X Lysis Buffer to each PCR product from Section V.F.

1. Warm up the AMPure XP Beads (pre-aliquoted AMPure XP Beads labeled "Post-PCR") at room temperature for ~30 min.
2. Prepare 80% ethanol using Nuclease-Free Water.
3. Vortex the AMPure XP Beads for 20 sec to make sure the beads are well resuspended before use.
4. Place the tubes from Step F6 on the magnetic separator for 2 min or until the supernatant is clear. Transfer the clear supernatant to fresh tube strips.
5. Add 1 µl of 10X Lysis Buffer and 50 µl of AMPure XP Beads to each PCR sample.

   NOTE: Make sure only 1 µl of Lysis Buffer is added. You may need to set a 2-µl pipette to 0.7 µl in order to take up only 1 µl of Lysis Buffer.

6. Mix by vortexing with five 1-sec pulses, and incubate at room temperature for 8 min to let the cDNA bind to the beads.
7. After the 8-min incubation, spin down the samples for 5 sec and place them on the magnetic separator for ~5 min or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.

8. While the samples are on the magnetic separator, open the lids and carefully pipette out the supernatant without removing any beads.
9. Spin down the sample for 5 sec to collect any liquid in the bottom of the tubes. Places the samples back on the magnetic separator for 1 min. Completely remove the liquid without removing any beads using a multi-channel pipette set to 20 µl.
10. Keep the samples on the magnetic separator. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads. Wait for 30 sec and carefully pipette out the supernatant. cDNA will remain bound to the beads during the washing process.
11. Repeat the ethanol wash (Step 10) once.
12. Close the lids and spin down for 5 sec to collect the liquid at the bottom of the tubes.
13. Place the samples on the magnetic separator for 30 sec, then remove all the remaining ethanol with a pipette.

    NOTE: It is important to make sure all ethanol is removed so that the samples will elute well from the beads, and recovery will be efficient.

14. Remove the samples from the magnetic separator and place them at room temperature for approximately 2–2.5 min until the pellet is no longer shiny, but 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.
    - We recommend having the Elution Buffer ready (e.g., in strip tubes for convenient multi-channel pipetting) to add to the samples immediately after the pellet has dried.
    - 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 min to rehydrate (Step 15) and may reduce amplified cDNA recovery and yield.

15. Once the beads are dry, add 17 µl of Elution Buffer to cover the bead pellet. If you are concerned about having too low a concentration of your cDNA, you may add 12 µl instead. Incubate at room temperature for 2 min to rehydrate.
16. Mix the bead pellet by pipetting up and down 10 times to elute the cDNA from the beads, then place the samples back on the magnetic separator.
17. Transfer all the clear supernatant containing purified cDNA from each well to a nuclease-free, low-adhesion 1.5-ml tube. Label each tube with sample information and store at –20°C.

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

H. Protocol: Validation Using the Agilent 2100 Bioanalyzer

1. Aliquot 1 µl of the amplified cDNA for validation using the Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). See the Agilent High Sensitivity DNA Kit User Manual for instructions.

   NOTE: Be careful not to transfer beads with your sample.

2. Compare the results for your samples and controls (see Figure 2) to verify whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield no product in the negative controls (Figure 2, Panels B and C), and a single distinct peak spanning 1,400 bp to 1,650 bp for the positive control RNA sample (Figure 2, Panel A), yielding approximately 1.7–5.1 ng of cDNA (depending on the input type and amount). Depending on the number and characteristics of the genes captured, you may see a broad size distribution spanning ~400 to 10,000 bp as shown in Figure 2, Panel D. You also may see distinct peaks representing specific cDNAs generated from your targeted transcripts, as in the positive control RNA sample (Figure 3, Panel A).

   NOTES:
   We have occasionally observed a small amount of product in the no-probe negative control, but this does not affect results obtained in the test sample. However, if excessive product is observed in the no-probe negative control, this could indicate that enrichment did work as well as expected.

   For more information on how an electropherogram of a positive control reaction should look and compare to that of a negative control, please visit **www.clontech.com**

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

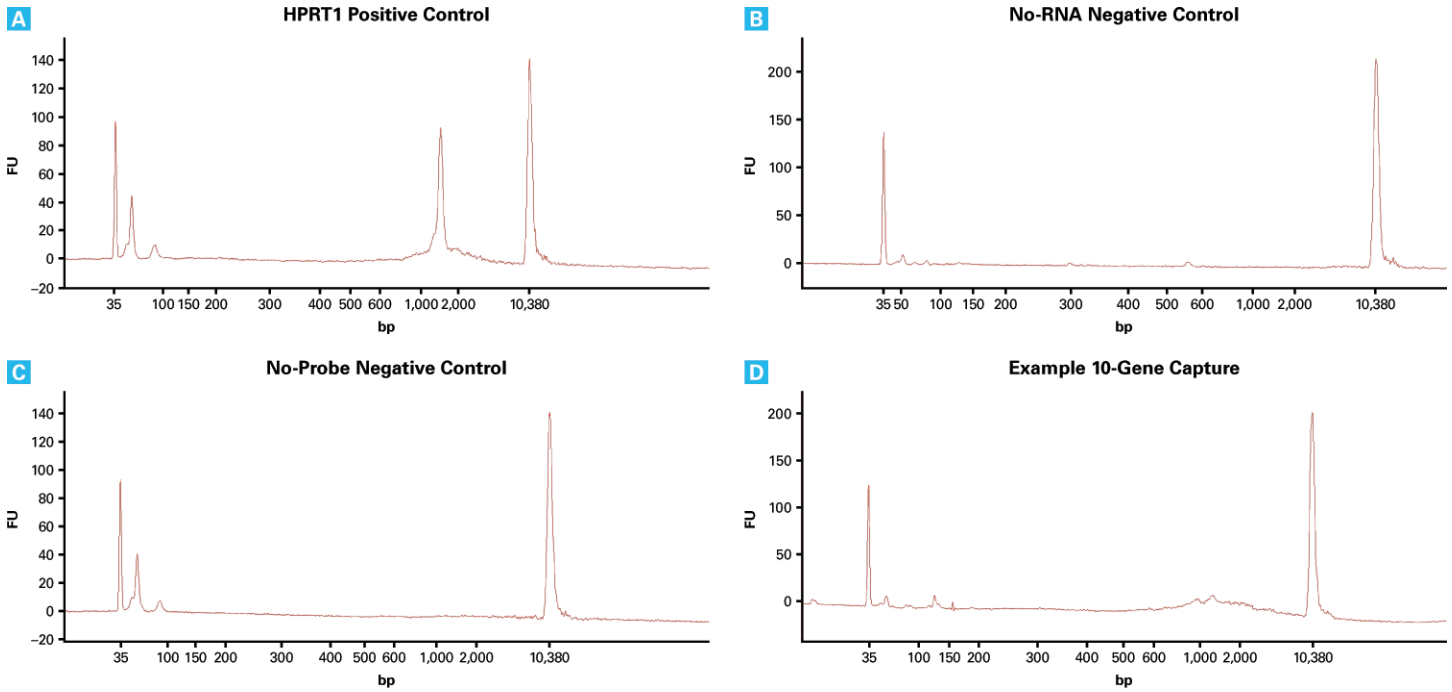

Figure 2. Example electropherogram results from Agilent 2100 Bioanalyzer. Unless indicated, 10 ng of Control Total RNA was subjected to hybridization with HPRT1 Control Probes, capture with Capture Beads, SMART-Seq v4 cDNA synthesis, and amplification for 20 cycles as described in the protocol. **Panel A** shows a single distinct peak between 1,400–1,650 bp. **Panels B and C** show no product in the negative controls (no RNA and no probes, respectively). **Panel D** shows the cDNA profile resulting from 10 ng of Control Total RNA hybridized to probes for 10 different genes, following amplification for 17 cycles.

VI. Library Preparation for Sequencing on Illumina Sequencing Platforms

Process the full-length cDNA output of the SMARTer Target RNA Capture for Illumina with the Nextera XT DNA Library Preparation Kits (Illumina, Cat. Nos. FC-131-1024 and FC-131-1096). We recommend using 100–150 pg of amplified cDNA in the input volume recommended in the Nextera XT Sample Preparation Guide. Follow the rest of the protocol as written.

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Appendix A: Pooling Probes

1. If receiving individual probes, resuspend each to a concentration of 10 pmol/µl (10 µM).
    a. If receiving pooled probes, dilute to a final concentration of 1 pmol/µl as stated in Step 3.
2. Pool 2 µl of each probe to make a solution at 10 pmol/µl.
3. Dilute 1 in 10 for a final concentration of 1 pmol/µl.
4. The final solution is your working solution for hybridization (Protocol A).

Appendix B: What Affects Results?

- **RNA type:** Different genes are expressed differently in different RNAs, therefore affecting enrichment metrics.
- **RNA input:** With lower inputs, the ability to capture a gene of interest becomes more stochastic, e.g., at 100 pg and 1 ng, medium-to-high expressers may still be enriched, but some low expressers may be lost.
- **Number of sequencing reads:** With more reads, the coverage of your target region will improve.
- **Number of targeted genes:** Given limited sequence space, the overall number of reads available for each gene decreases as the number of targeted genes increases. As the number of targeted genes increase, you may also find you need fewer cycles of PCR to generate sufficient cDNA. (For example, compare cycling conditions for Figure 2, Panels A and D.)
- **Number of probes (correlated with number of genes):** The quality of probes is more important than the number of probes (see Appendix C). We have found that 1 pmol of total probe is optimal for hybridization independent of the number of probes.
- **Hybridization parameters:** In some cases, we have found that a fast 10-minute hybridization protocol may be sufficient to capture targeted genes. These cases include: capture of very few genes or of medium to highly expressed genes, or when only minimal enrichment of the target gene is required. In this protocol, the sample is heated to 75°C for 2 min, then ramped to 25°C at a rate of –0.1°C per sec.
- **Volume of Capture Beads used:** As the number of targeted genes increases, greater volumes of Capture Beads may be necessary, but there is a limit beyond which additional beads will produce no significant improvement in the ability to recover the targeted genes (see Appendix D).

Appendix C: Probe Design

- For optimal enrichment, design one probe per exon for each transcript or transcript isoform of interest, if possible. Since the starting material is high-quality RNA, each probe should be capable of capturing the entire RNA transcript that contains the corresponding exon. However, since each probe may have different hybridization efficiency, we recommend multiple probes per transcript.
- Probes should be 60mers, with a 5'-Biotin.
    - Probes smaller than 60 nt may work, but specificity becomes a concern.
    - Desalted probes are of sufficient purity. We have not found a need for PAGE or HPLC purification.
- If any of the following is true for a given exon, then its corresponding probe may be removed from the pool:
    - It is particularly short (such that specificity becomes a concern)
    - It has unusually extreme GC-content (such that hybridization efficiency, in either direction, becomes a concern)
    - It has extremely high homology with other exons
- Tiled probes that completely cover each exon have been observed to decrease on-target rates and enrichment efficiency while substantially increasing cost.
- We recommend that housekeeping genes, or genes known to be highly expressed, are not targeted. Highly expressed genes can dominate the sequencing reads, limiting reads to other genes of interest that may be expressed at very low levels.

Appendix D: Capture Bead Optimization

As the number of targeted genes increases, greater volumes of Capture Beads may be necessary, but there is a limit beyond which additional beads make no difference. In our own experiments, we have observed that a 10-gene panel (~130 probes) can be captured efficiently with 10 µl of Capture Beads, and shows no improvement when higher volumes of beads are used. A 60-gene panel (1,048 probes) requires an increased volume of Capture Beads (20 µl), and further increasing the bead volume has little to no effect on capture efficiency.

Note that a 10-gene panel with ~1,000 probes can use 10 µl of Capture Beads and show no improvement with higher volumes of beads. Therefore, the volume of Capture Beads scales up with the number of genes targeted, **NOT** the number of probes used.

We recommend titrating beads in 2X increments. Do not change the final volume of 2X Capture Buffer listed in the protocol (10 µl) used to resuspend the Capture Beads (Step B7).

cDNA traces will not give an accurate indication of successful capture; it is necessary to sequence and analyze for enrichment metrics.

Appendix E: Library Sequencing and Informatics Analysis

A. Low complexity

You may need to include a 5–10% PhiX Control v3 (Illumina, Cat. No. FC-110-3001) spike-in to overcome low complexity on Illumina sequencing platforms. This is particularly important if you are targeting few genes.

B. Read data quality control

FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), or a similar application, should be used to parse the read data for quality issues prior to read alignment.

C. Read alignment

Use an RNA splicing-aware read mapper to map the reads against the reference genome. The choice in RNA-seq aligner is driven by project design and available computational resources. For a discussion regarding spliced aligner choices, see Engström, *et al.*, (2013). We achieved similar results in terms of mapping rates using either STAR (Dobin, *et al.*, 2013) or TopHat (Trapnell, *et al.*, 2009) aligners, running each with the default parameter set.

D. Target enrichment quality control

It is often informative to get a sense of how well the experiment performed with regard to target enrichment, sequence coverage, and duplication rate. A number of software tools can be used to accomplish this task, including TEQC (an R package; https://www.bioconductor.org/packages/release/bioc/html/TEQC.html) and BEDTools (a collection of command line utilities; Quinlan, *et al.*, 2010). Regardless of the chosen tool, be sure that the analysis is relevant to RNA-seq. For sample-to-sample comparisons, it is often useful to compare experiments with a similar number of reads.

E. Gene expression quantification

There is currently a plethora of gene quantification approaches available to researchers. Read counts can be obtained either as part of the STAR output (by using the quantMode GeneCounts option) or by running HTSeq (Anders, *et al.*, 2015) on an alignment file. If gene quantification is required, these gene counts can be imported into any number of gene quantification routines, including DESeq2 (Love, *et al.*, 2014).

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