Takara Bio USA, Inc.

SMART-Seq® mRNA 3' DE User Manual

Cat. Nos. 635040 & 635041
(051024)

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Table of Contents

Table of Figures

Table of Tables

I. Introduction: SMART® cDNA Synthesis for Sequencing

The **SMART-Seq mRNA 3' DE** (Cat. Nos. 635040 & 635041) is designed to generate high-quality indexed cDNA libraries directly from 1–100 cells or 10 pg–1 ng of total RNA. This kit is specialized for differential gene expression (DE) analysis and the resultant sequencing library is focused on the 3′ ends of mRNA transcripts, which are sufficient for DE analysis. This approach can reduce the number of reads required for gene quantitation, which reduces sequencing costs. This kit takes a convenient input volume of 1–10.5 µl and relies on the Illumina® Nextera® XT DNA Library Preparation Kit. For the purpose of DE studies, this kit improves on SMART-Seq mRNA by decreasing the total cost and time for sequencing while maintaining superior performance. The protocol described in this user manual has been optimized for cDNA synthesis and library preparation using the SMART-Seq mRNA 3' DE only. If you are using other SMARTer® or SMART-Seq NGS kits, please refer to the respective user manuals. In addition to focusing on the 3′ ends of transcripts, this kit also uses in-line indexes (IL1–IL12) as cell barcodes to enable you to pool your samples prior to sequencing and then demultiplex the samples after sequencing. This allows up to 12 samples to be pooled for each combination of Illumina indexes, further reducing costs and time. This allows for a maximum of up to 1,152 samples to be processed per lane. See Appendixes B, C, and D for more information.

This kit amplifies full length cDNA before selecting for the 3′ ends during the Nextera process to maximize sensitivity. The full process is described in the diagram below.

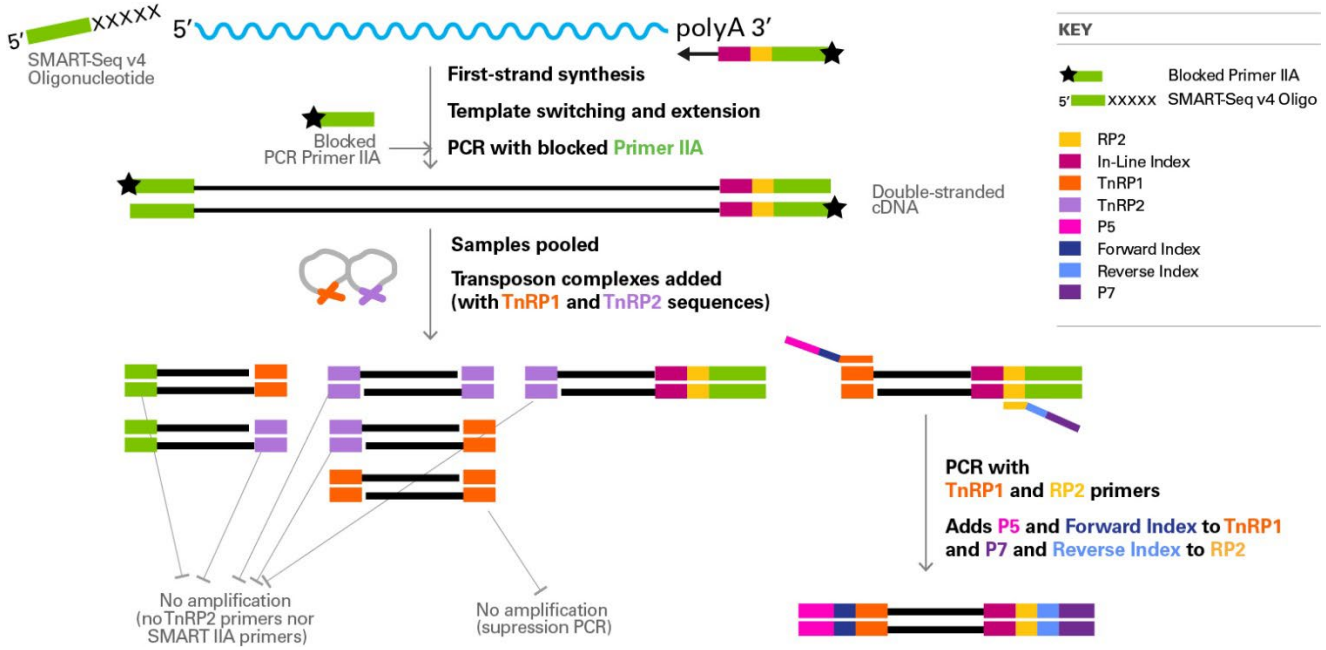

Figure 1. Flowchart of SMART cDNA synthesis and library preparation. cDNA (black) is synthesized with a blocked (black star) and modified oligo(dT) primer that adds sequences for subsequent amplification and analysis—an in-line index (magenta), part of the Illumina read primer 2 sequence (RP2, yellow), and the SMART IIA sequence (green). The SMART IIA sequence is used as a priming site during cDNA amplification, the Illumina RP2 sequence is used as a priming site during library amplification, and the in-line index is used for demultiplexing pooled samples during analysis. The process works as follows: first, the template for SMARTScribe™ reverse transcriptase switches from the mRNA (blue wavy line) to the SMART-Seq v4 Oligonucleotide (green). After reverse transcription, the full-length cDNA is amplified by PCR with blocked Primer IIA oligonucleotides. After cDNA amplification, the presence of the in-line index (magenta) allows for pooling up to 12 samples. The pooled samples are tagmented and Illumina Nextera read primer 1 and 2 sequences are added by the Nextera Tn5 transposon (TnRP1 and TnRP2, orange and purple respectively). The 3′ ends of the original mRNA are captured by selective PCR with primers for the TnRP1 and RP2 sequences. Other products of the transposon-based reaction are not amplified, either because there are no primer sites for amplification or because of suppression PCR (Siebert et al. 1995). Cluster generation (pink and dark purple) and indexing sequences (light blue and dark blue) are added during this PCR stage to generate a library ready for sequencing on Illumina platforms.

This kit has been designed and validated to prepare cDNA samples and subsequent sequencing libraries for the following Illumina sequencing platforms: HiSeq®, NextSeq®, MiSeq®, and MiniSeq™. Please see Appendix E for guidelines for the NextSeq and MiniSeq. The cDNA synthesis protocol can be completed within six hours, and the library construction protocol can be completed within three hours (Figure 2).

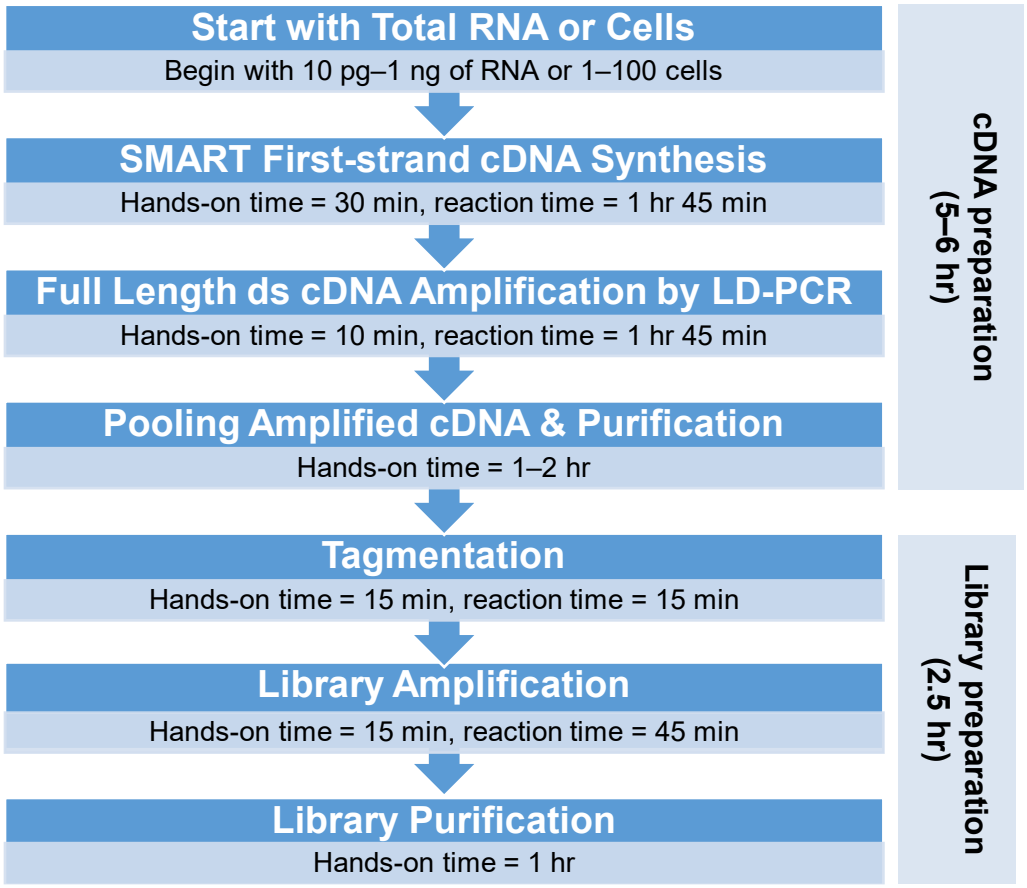

Figure 2. Protocol overview.

II. List of Components

The SMART-Seq mRNA 3' DE consists of the SMART-Seq mRNA 3' DE 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.

Table 1. SMART-Seq mRNA 3' DE components. Continued on the next page.

SMART-Seq mRNA 3' DE	Cap Color	635040 (96 rxns)	635041 (192 rxns)
SeqAmp DNA Polymerase (Store at –20°C)		**638509**	**2 x 638509**
SeqAmp DNA Polymerase	–	200 µl	2 x 200 µl
SeqAmp PCR Buffer	–	4 x 1.25 ml	8 x 1.25 ml
SMART-Seq mRNA 3' DE Components		**635042***	**635043***
Package 1 (Store at –70°C)			
Control Total RNA (1 µg/µl)	–	5 µl	5 µl
Package 2 (Store at –20°C)			
SMART-Seq v4 Oligonucleotide (48 µM)	Pink	144 µl	288 µl
PCR Primer II A (12 µM)	Green	144 µl	288 µl
5X Ultra® Low First-Strand Buffer	Red	576 µl	1.2 ml
SMARTScribe™ Reverse Transcriptase (100 U/µl)	Purple	288 µl	576 µl
Nuclease-Free Water	–	4 ml	2 x 4 ml
RNase Inhibitor (40 U/µl)	White	250 µl	500 µl
10X Lysis Buffer†	Neutral	420 µl	840 µl
Elution Buffer (10 mM Tris-Cl, pH 8.5)‡	–	3 ml	6 ml
Oligo dT In-line 1 Primer (IL1; 12 µM)	White, IL1	20 µl	40 µl
Oligo dT In-line 2 Primer (IL2; 12 µM)	White, IL2	20 µl	40 µl
Oligo dT In-line 3 Primer (IL3; 12 µM)	White, IL3	20 µl	40 µl
Oligo dT In-line 4 Primer (IL4; 12 µM)	White, IL4	20 µl	40 µl
Oligo dT In-line 5 Primer (IL5; 12 µM)	White, IL5	20 µl	40 µl
Oligo dT In-line 6 Primer (IL6; 12 µM)	White, IL6	20 µl	40 µl
Oligo dT In-line 7 Primer (IL7; 12 µM)	White, IL7	20 µl	40 µl
Oligo dT In-line 8 Primer (IL8; 12 µM)	White, IL8	20 µl	40 µl
Oligo dT In-line 9 Primer (IL9; 12 µM)	White, IL9	20 µl	40 µl
Oligo dT In-line 10 Primer (IL10; 12 µM)	White, IL10	20 µl	40 µl
Oligo dT In-line 11 Primer (IL11; 12 µM)	White, IL11	20 µl	40 µl
Oligo dT In-line 12 Primer (IL12; 12 µM)	White, IL12	20 µl	40 µl
Package 3 (Store at –20°C)			
Reverse PCR Primer HT Index 1 (R1; 10 µM)	Blue, R1	12 µl	12 µl
Reverse PCR Primer HT Index 2 (R2; 10 µM)	Blue, R2	12 µl	12 µl
Reverse PCR Primer HT Index 3 (R3; 10 µM)	Blue, R3	12 µl	12 µl
Reverse PCR Primer HT Index 4 (R4; 10 µM)	Blue, R4	12 µl	12 µl
Reverse PCR Primer HT Index 5 (R5; 10 µM)	Blue, R5	12 µl	12 µl
Reverse PCR Primer HT Index 6 (R6; 10 µM)	Blue, R6	12 µl	12 µl
Reverse PCR Primer HT Index 7 (R7; 10 µM)	Blue, R7	12 µl	12 µl
Reverse PCR Primer HT Index 8 (R8; 10 µM)	Blue, R8	12 µl	12 µl
Reverse PCR Primer HT Index 9 (R9; 10 µM)	Blue, R9	12 µl	12 µl
Reverse PCR Primer HT Index 10 (R10; 10 µM)	Blue, R10	12 µl	12 µl

SMART-Seq mRNA 3' DE	Cap Color	635040 (96 rxns)	635041 (192 rxns)
Reverse PCR Primer HT Index 11 (R11; 10 µM)	Blue, R11	12 µl	12 µl
Reverse PCR Primer HT Index 12 (R12; 10 µM)	Blue, R12	12 µl	12 µl
Tn Forward PCR Primer HT Index 1 (TnF1; 10 µM)	Red, TnF1	15 µl	15 µl
Tn Forward PCR Primer HT Index 2 (TnF2; 10 µM)	Red, TnF2	15 µl	15 µl
Tn Forward PCR Primer HT Index 3 (TnF3; 10 µM)	Red, TnF3	15 µl	15 µl
Tn Forward PCR Primer HT Index 4 (TnF4; 10 µM)	Red, TnF4	15 µl	15 µl
Tn Forward PCR Primer HT Index 5 (TnF5; 10 µM)	Red, TnF5	15 µl	15 µl
Tn Forward PCR Primer HT Index 6 (TnF6; 10 µM)	Red, TnF6	15 µl	15 µl
Tn Forward PCR Primer HT Index 7 (TnF7; 10 µM)	Red, TnF7	15 µl	15 µl
Tn Forward PCR Primer HT Index 8 (TnF8; 10 µM)	Red, TnF8	15 µl	15 µl

*Not sold separately.
†Store 10X Lysis Buffer 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 room temperature.

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
- Eight channel pipette (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1 ml
- Minicentrifuge for 1.5 ml tubes
- Minicentrifuge for 0.2 ml tubes or strips
- 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 First-Strand cDNA Synthesis:

- One dedicated thermal cycler used only for first-strand synthesis

For cDNA Amplification and Library Preparation:

- One thermal cycler
- Nextera XT DNA Sample Preparation Kit (Illumina, Cat. Nos. FC-131-1024, FC-131-1096)

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 and library purifications.

NOTE: SPRI beads need to come to room temperature before the container is opened. Therefore, we recommend aliquoting the beads into 1.5 ml Eppendorf 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 process will also decrease the chances of bead contamination. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.

- Molecular biology grade 100% ethanol
- Magnetic separation device for high volumes—used to purify amplified cDNA
  - For 1.5 ml tube: Magnetic Separator
- Magnetic separation device for small volumes—used to purify amplified libraries
  - For 8-tube strips: SMARTer-Seq™ Magnetic Separator - PCR Strip, Takara Bio, Cat. No. 635011
  - For 96-well plates: Magnetic Stand-96, Thermo Fisher, Cat. No. AM10027
- Optional, depending on the choice of magnetic separation device (96-well format):
  - 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

IV. General Considerations

NOTES:

- Please read the entire protocol before starting. This protocol is optimized for cDNA synthesis from ultra-low input amounts of total RNA (10 pg–1 ng) using the Takara Bio SMART method. The protocol is also suitable for cDNA synthesis directly from single 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 SMART cDNA Synthesis should be carried out in a PCR-clean workstation under clean room conditions.
- Because of the use of pooling to decrease overall costs and time required for sequencing, this kit is intended for at least 6 samples per experiment. If fewer than 6 samples are used in every experiment, the component volumes will not be sufficient for 96 samples.

A. Requirements for Preventing Contamination

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

- **A PCR-clean workstation** for all pre-PCR experiments that require clean room conditions, e.g. first-strand cDNA synthesis (Protocol V.A).

  NOTES:

  - The PCR-clean workstation 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 workstation located in the general laboratory** where you will perform PCR (Protocol V.B), measure cDNA concentration (Protocol V.C), and perform library preparation (Protocol VI.A, B and C).

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 SMART cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for SMART 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 SMART 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. This kit contains enough volumes to perform 16 negative and 16 positive controls.
- The SMART-Seq mRNA 3' DE requires paired-end sequencing for pooling samples and subsequent demultiplexing with the in-line indexes. Read 1 is for your samples (cDNA fragments) and Read 2 is for in-line indexes (IL1-12). In-line indexes are composed of 6 bp (Appendix C) at the beginning of Read 2. Illumina recommends sequencing at least 26 bp for each read to obtain quality score metrics. A typical 26 bp Read 2 with the SMART-Seq mRNA 3' DE is composed of 6 bp of in-line index and the following poly(T). See Appendix D for guidelines for demultiplexing your samples using the in-line indexes.

C. Sample Recommendations

1. Total RNA Extraction

The sequence complexity and the average length of SMART 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. Takara Bio 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.

2. 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.

3. 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 (Ca^{2+}/Mg^{2+}-free).

D. Sample Requirements

The SMART-Seq mRNA 3' DE works with up to 10.5 µl of cells or RNA.

1. 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 1 ng). Purified total RNA should be in nuclease-free water.

2. Cells

- This protocol has been validated to generate cDNA starting from cells; 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.
- Cells should be washed and resuspended in PBS prior to lysis. The presence of media can interfere with the first-strand synthesis. If necessary, test the effect of your media on cDNA synthesis by performing a reaction with control RNA and the estimated amount of media that you expect to accompany your cell(s).

V. Protocols for cDNA Synthesis

A. Protocol: First-Strand cDNA Synthesis

First-strand cDNA synthesis (from total RNA or lysed cells) is primed by Oligo dT In-line Primers 1–12 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 workstation, ideally in a clean room.

1. Thaw the 5X Ultra Low First-Strand Buffer at room temperature. Thaw all the remaining reagents needed for first-strand cDNA synthesis (except the enzyme) on ice. Gently vortex each reagent to mix and spin down briefly. Store all but the 5X Ultra Low First-Strand Buffer 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. Prepare a stock solution of 10X Reaction Buffer by mixing 10X Lysis Buffer with RNase Inhibitor as indicated below (scale-up as needed):

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

   Mix briefly, then spin down.

   NOTE: Lysis Buffer contains a detergent; therefore, it is critical to avoid bubbles when mixing.

3. See Table 2 (next page) for guidelines on setting up your control and test samples. Prepare each sample (11.5 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 1–10.5 µl to a 0.2 ml RNase-free PCR tube. Bring the volume to 10.5 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 10.5 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 min. See Sections IV.C and IV.D for more information on working with cells.

Table 2. Sample preparation guidelines.

Components	Negative Control	Positive Control	Test Sample
10X Reaction Buffer	1 µl	1 µl	1 µl
Nuclease-Free Water	10.5 µl	0–9.5 µl	0–9.5 µl
Diluted Control RNA*	–	1–10.5 µl	–
Sample	–	–	1–10.5 µl
Total volume	11.5 µl*	11.5 µl*	11.5 µl*

*The Control RNA is supplied at a concentration of 1 µg/µl. It should be diluted in nuclease-free water with RNase Inhibitor (1 µl RNase Inhibitor in a final volume of 50 µl of water) to match the concentration of your test sample. Perform serial dilutions on the Control RNA to obtain the appropriate concentration.

4. Place the samples on ice and add 1 µl of one of Oligo dT In-line 1–12 Primers (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:

11.5 µl	Cell/Total RNA in Reaction Buffer (from Table 1)
1 µl	Oligo dT In-line 1–12 Primer (12 µM)
12.5 µl	**Total Volume**

5. Incubate the tubes at 72°C in a preheated, hot-lid thermal cycler for 3 min.

NOTE: Prepare your Master Mix (Step 6) while your tubes are incubating. The enzyme will be added just before use (Step 9). Steps 10–11 below are critical for first-strand cDNA synthesis and should not be delayed after completing Step 7.

6. Meanwhile, prepare enough Master Mix for all the reactions, plus one extra reaction volume, by combining the following reagents in the order shown at room temperature:

4 µl	5X Ultra Low First-Strand Buffer
1 µl	SMART-Seq v4 Oligonucleotide (48 µM)
0.5 µl	RNase Inhibitor (40 U/µl)
5.5 µl	**Total Volume added per reaction**

7. Immediately after the 3 min incubation at 72°C, place the samples on ice for 2 min.
8. Preheat the thermal cycler to 42°C.
9. Add 2 µl per reaction, plus one extra reaction volume, of the SMARTScribe Reverse Transcriptase to the Master Mix. 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.

NOTE: 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 tubes.

10. Add 7.5 µl of the Master Mix to each reaction tube from Step 7. Mix the contents of the tubes by gently pipetting and spin them briefly to collect the contents at the bottom of the tubes.
11. 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

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

B. Protocol: cDNA Amplification by LD PCR

PCR Primer II A amplifies cDNA from the SMART sequences introduced by Oligo dT In-line 1–12 Primers and the SMART-Seq v4 Oligonucleotide.

IMPORTANT:

- Perform Steps 1 & 2 in a PCR-clean workstation.
- Table 2 provides guidelines for PCR optimization, depending on the amount of total RNA or cells used for the first-strand cDNA synthesis and the number of reactions that will be pooled prior to library preparation (Section VI). These guidelines were determined using the Control Mouse Brain Total RNA. The individual reaction suggestions are to be used for the positive and negative control reactions. 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 A for PCR optimization suggestions.

Table 3. Cycling guidelines based on amount of starting material.

Input amount of Total RNA	Input amount of cells	Typical number of PCR cycles		
		Individual reaction	6 reactions in pool	12 reactions in pool
1 ng	100 cells	10–11	8–9	7–8
100 pg	10 cells	14–15	12–13	11–12
10 pg	1 cell	17–18	15–16	14–15

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 one extra reaction 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 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.

3. Add 30 µl of PCR Master Mix to each tube containing 20 µl of first-strand cDNA product from Section V.A. 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 workstation 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
N cycles:*	
98°C	10 sec
65°C	30 sec
68°C	3 min
72°C	10 min
4°C	forever

*For the value of *N*, consult Table 3 for PCR cycle number guidelines.

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

C. Protocol: Pooling and Purification of Amplified cDNA Using the Agencourt AMPure XP Kit

Having an in-line index (1–12) as a cell or sample barcode, the PCR-amplified cDNA can be pooled (up to 12 reactions) prior to purification. See Table 3 for guidelines on performing individual reactions vs. performing 6 or 12 reactions simultaneously in a pool.

IMPORTANT: cDNA can be pooled (up to 12 samples per pool) when the cDNAs have different in-line indexes. DO NOT pool cDNAs that have the same in-line index.

Table 4. Purification guidelines based on number of reactions pooled.

		Individual reaction (50 µl)	6 reactions in pool (300 µl)	12 reactions in pool (600 µl)
First purification	10x Lysis Buffer	1 µl	5 µl	10 µl
	AMPure XP (X0.8)	40 µl	240 µl	480 µl
	Elution Buffer	12 µl	50 µl	
Second purification	10x Lysis Buffer	-	-	-
	AMPure XP (X0.8)	-	40 µl	40 µl
	Elution Buffer	-	12 µl	12 µl

PCR-amplified cDNA is 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 beads to room temperature for at least 30 min and mix well to disperse.
- Prepare fresh 80% ethanol for each experiment. You will need 4 ml per tube.
- Only perform a single purification (first purification) when you are not pooling reactions.

1. Combine the 50 µl of differently-indexed PCR products from step V.B.4 in a single 1.5 ml tube (up to 12 reactions).

   IMPORTANT: DO NOT pool cDNAs that have the same in-line index.

2. Add 10X Lysis Buffer to each PCR product according to Table 4.
3. Vortex AMPure XP beads until evenly mixed, then add AMPure XP Beads to each sample according to Table 4.
4. Mix by vortexing or pipetting the entire volume up and down at least 10 times to mix thoroughly.
5. 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.

6. 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 min or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
7. While the samples are on the magnetic separation device, pipette out the supernatants.
8. Keep the samples on the magnetic separation device. Add 1 ml of freshly made 80% ethanol to each sample without disturbing the beads. Wait for 30 sec and carefully pipette out the supernatant containing contaminants. DNA will remain bound to the beads during the washing process.
9. Repeat Step 8 once.
10. Briefly spin the samples to collect the liquid from the side of the wall. Place the samples on the magnetic device for 30 sec, then remove all the remaining ethanol with a pipette.
11. Allow the samples to dry at room temperature for 5 min or longer until the pellet is no longer shiny and before a crack appears.

    NOTE: It is not necessary to dry the pellets completely during the first purification for pool samples. The remaining ethanol will be removed during the second purification.

    If you over-dry the pellet, there will be cracks in the pellet. It will take longer than 2 min to rehydrate and may reduce amplified cDNA recovery and yield.

12. Once the beads are dry, add Elution Buffer (See Table 4) to cover the bead pellet. Remove the samples from the magnetic separation device and mix thoroughly to resuspend the beads.
13. Incubate at room temperature for 2 min to rehydrate.
14. 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 min 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. Pipette these unpelleted beads up and down to resuspend them with the supernatant, and then pipette 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.

15. Transfer clear supernatant containing purified cDNA from each well to a nuclease-free non-sticky tube. Skip step 16 for individual reactions.
16. Perform a second purification by repeating steps 2–15, using the volumes indicated in Table 4.

    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 min to rehydrate and may reduce amplified cDNA recovery and yield.

17. Label each elution tube with sample information and store at –20°C.

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

D. Validation Using the Agilent 2100 Bioanalyzer

Figure 3 shows example data of individual reactions. Even when the samples are pooled, the distribution and yield should be the same.

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 3) to verify whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield no product in the negative control (Figure 3, Panel B), and a distinct peak spanning 400 bp to 10,000 bp, with a maximum at ~2,000 bp for the positive control RNA sample (Figure 3, 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.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips
3. Proceed to **Library Preparation using Illumina Nextera XT DNA Sample Preparation Kit** (Section VI).

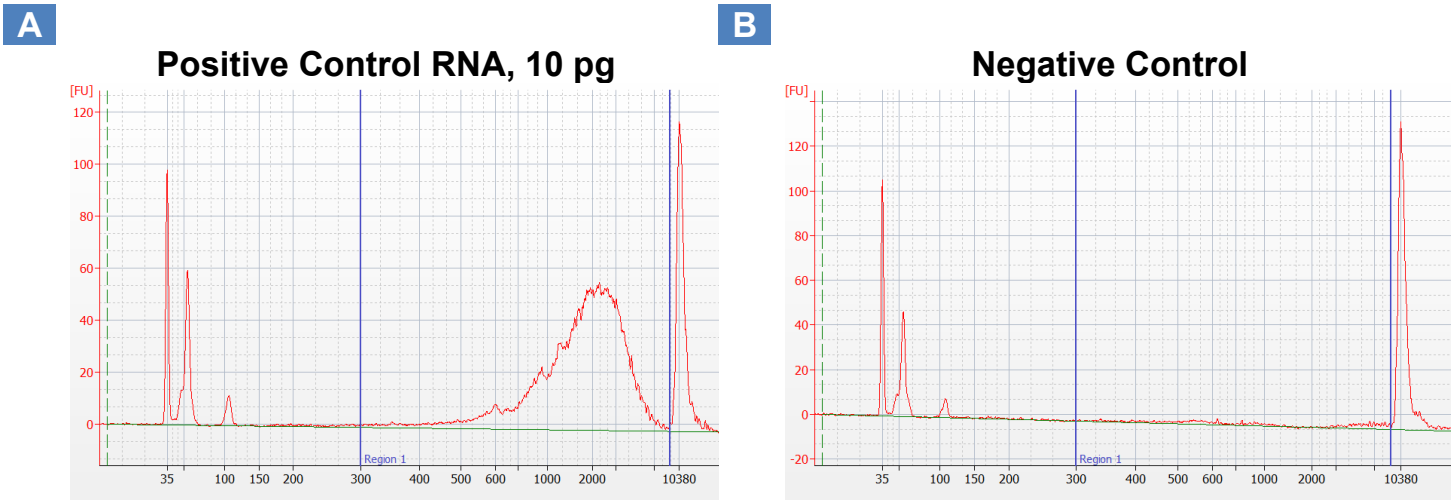

Figure 3. cDNA electropherogram example results from Agilent 2100 Bioanalyzer. All samples were subjected to SMART cDNA synthesis with Oligo dT In-line 7 Primer and amplification as described in the protocol. FU = fluorescence absorption units. **Panel A.** shows a clean product following cDNA synthesis and amplification (17 PCR cycles). **Panel B.** shows no product in the negative control following cDNA synthesis and amplification (17 PCR cycles).

VI. Protocol for Library Preparation using Illumina Nextera XT DNA Sample Preparation Kit

The following protocol captures the 3′ end of the original mRNA and generates an Illumina-ready sequencing library.

A. Protocol: Tagmentation

The protocol requires three components from the Illumina Nextera XT DNA Sample Preparation Kit: Amplicon Tagment Mix (ATM), Tagment DNA Buffer (TD) and Nextera PCR Master Mix (NPM). The following PCR primers for library amplification are included in the SMART-Seq mRNA 3' DE: Reverse PCR Primer HT Index 1–12 (R1–12) and Tn Forward PCR Primer HT Index 1–8 (TnF1–8).

1. Thaw all reagents required for tagmentation on ice. Gently vortex, spin down briefly, and store them on ice.
2. Prepare tagmentation mixtures in individual 0.2 ml RNase-free PCR tubes or in an 8-well strip on ice as shown in the table below:

Table 5. Tagmentation guidelines. Use cDNA Purified in Section V.C.

Components	Negative Control	Positive Control	Test Sample
TD Buffer (Tagment DNA Buffer)	10 µl	10 µl	10 µl
ATM (Amplicon Tagment Mix)	5 µl	5 µl	5 µl
Nuclease-free water	As needed	As needed	As needed
Synthesized Negative* Control cDNA	0.5–5 µl	–	–
Synthesized Positive Control cDNA (300-400 pg)	–	0.5–5 µl	–
Synthesized Sample cDNA (300-400 pg)	–	–	0.5–5 µl
Total Volume	20 µl	20 µl	20 µl

*Use an equal volume of synthesized negative control cDNA to that of synthesized positive control cDNA.

3. Place the reaction tube(s) in a preheated thermal cycler with a heated lid that has the following program running:

55°C	15 min
10°C	forever

4. Remove reaction tube(s) from thermal cycler.
5. Add 5 µl of NT (Neutralize Tagment Buffer) to each reaction tube. Mix the contents of the tubes by vortexing gently and spin the tubes down briefly to collect the contents at the bottom.
6. Incubate the tube(s) at RT for 5 min and then place it on ice.

B. Protocol: Library Amplification by PCR

1. Thaw NPM (Nextera PCR Master Mix) and Forward and Reverse HT Index Primers at room temperature. Gently vortex each to mix and spin down briefly. Store on ice.
2. Prepare enough PCR Master Mix for all the reactions, plus one extra reaction volume. Combine the following reagents in the order shown.

15 µl	NPM
1 µl	Reverse PCR Primer HT Index 1-12
1 µl	Tn Forward PCR Primer HT Index 1-8
8 µl	Nuclease-free water
25 µl	**Total Volume added per reaction**

3. Add 25 µl of PCR Master Mix to each tube containing 25 µl of tagmented cDNA product. Mix well and briefly spin in a minicentrifuge to collect the contents at the bottom of the tube(s).

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

72°C	3 min
95°C	30 sec
12 cycles:	
95°C	10 sec
55°C	30 sec
72°C	30 sec
72°C	5 min
10°C	Hold

C. Protocol: Purification of Amplified Library Using the Agencourt AMPure XP Kit

Amplified library 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 min and mix well to disperse.
- Prepare fresh 80% ethanol for each experiment. You will need 400 µl per sample.

1. Vortex AMPure XP beads until evenly mixed, then add 30 µl of AMPure XP Beads to each sample.
2. Mix by thoroughly vortexing or pipetting the entire volume up and down at least 10 times.
3. 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.

4. 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 min or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
5. While the samples are on the magnetic separation device, pipette out the supernatants.
6. 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 sec and carefully pipette out the supernatant containing contaminants. DNA will remain bound to the beads during the washing process.
7. Repeat Step 6 once.
8. Briefly spin the samples to collect the liquid from the side of the wall. Place the samples on the magnetic device for 30 sec, then remove all the remaining ethanol with a pipette.
9. Place the samples at room temperature for approximately 2–2.5 min 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 min to rehydrate (Step VI.C.11) and may reduce amplified cDNA recovery and yield.

10. Once the beads are dry, add 12 µl of Elution Buffer to cover the bead pellet. Remove the samples from the magnetic separation device and mix thoroughly to resuspend the beads.

11. Incubate at room temperature for 2 min to rehydrate.
12. 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 min 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. Pipette these unpelleted beads up and down to resuspend them with the supernatant, and then pipette 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.

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

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

D. Validation Using the Agilent 2100 Bioanalyzer

1. Aliquot 1 µl of the amplified library 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 sequencing. Successful library preparation should yield no product in the negative control (Figure 4, Panel B), and a distinct peak spanning 150 bp to 2,000 bp, with a maximum at 400–600 bp for the positive control RNA sample (Figure 4, Panel A), yielding approximately 20–80 ng of library (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.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

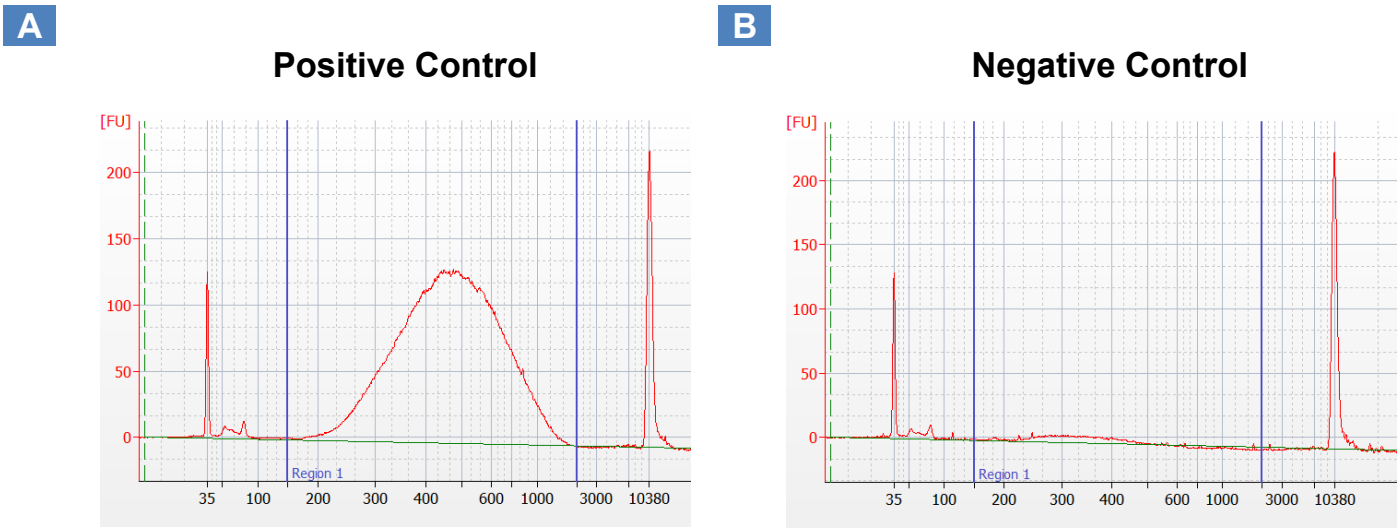

Figure 4. Library electropherogram example results from Agilent 2100 Bioanalyzer. All samples were subjected to tagmentation and amplification as described in the protocol. FU = fluorescence absorption units. **Panel A.** shows a clean product following tagmentation and amplification. **Panel B.** shows no product in the negative control following tagmentation and PCR amplification.

VII. References

Siebert, P. D., Chenchik, A., Kellogg, D. E., Lukyanov, K. A. & Lukyanov, S. A. An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res.* **23,** 1087–8 (1995).

Appendix A: 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 number of PCR cycles bracketing the recommended number of cycles. For example, if you have 1 ng of RNA, 10 cycles are recommended. You could then use 8, 10, and 12 cycles to determine which gives the best results.

1. Use the following program for thermal cycling:

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

*For the value of *N*, consult Table 3 for PCR cycle number guidelines.

2. Perform **Pooling and Purification of Amplified cDNA using the Agencourt Ampure XP Kit (Section V.C)**.
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.
5. Apply the optimal number of PCR cycles to your sample material.

Appendix B: Sequencing Indexes

Appropriate combinations of Illumina indexes are necessary to ensure enough nucleotide diversity and allow for discrimination between samples when sequencing a pool of two or more libraries on a single flow cell lane. Consult the Illumina literature (such as the TruSeq® DNA Sample Preparation Guide) for appropriate pooling guidelines. The Reverse PCR Primer HT Indexes contain the Read 2 sequence and i7 indexes. These primers are labeled sequentially, "R1"–"R12", in correspondence with Illumina indexes D701–D712. Tn Forward PCR Primer HT Indexes contain the Read 1 sequence and i5 indexes. These primers are labeled sequentially, "TnF1"–"TnF8", in correspondence with Illumina indexes S517, S502–D508.

Pairwise combination of 12 unique i7 indexes with 8 unique i5 indexes allows for multiplexing of up to 96 samples in a single flow cell lane. With this kit, you can multiplex an additional 12 samples per pairwise combination of the i7 and i5 indexes, resulting in a theoretical max of 12 x 96=1,152 samples per lane. See Appendix C and D for more information.

Table 6. PCR Primer HT Index sequences.

Reverse PCR Primer HT Index			Tn Forward PCR Primer HT Index		
Primer ID (cap label)	Illumina ID	Index Sequence	Primer ID (cap label)	Illumina ID	Index Sequence
R1	D701	ATTACTCG	TnF1	S517	GCGTAAGA
R2	D702	TCCGGAGA	TnF2	S502	CTCTCTAT
R3	D703	CGCTCATT	TnF3	S503	TATCCTCT
R4	D704	GAGATTCC	TnF4	S504	AGAGTAGA
R5	D705	ATTCAGAA	TnF5	S505	GTAAGGAG
R6	D706	GAATTCGT	TnF6	S506	ACTGCATA
R7	D707	CTGAAGCT	TnF7	S507	AAGGAGTA
R8	D708	TAATGCGC	TnF8	S508	CTAAGCCT
R9	D709	CGGCTATG			
R10	D710	TCCGCGAA			
R11	D711	TCTCGCGC			
R12	D712	AGCGATAG			

Appendix C: In-line Indexes

Appropriate combinations of Illumina indexes are necessary to ensure enough nucleotide diversity and allow for discrimination between samples when sequencing a library prepared with pooled cDNAs. The Oligo dT In-line Primers contain in-line indexes that enable additional multiplexing, which will be included at the beginning of Read 2. These primers are labeled sequentially, "IL1"–"IL12", in correspondence with Illumina indexes R738, R702–R705, R744 and R707–R712. We recommend using IL7–IL12 when you have only 6 reactions in a pool. Consult the Illumina literature (such as the TruSeq DNA Sample Preparation Guide) for appropriate pooling guidelines.

Table 7. Oligo dT In-line Primer sequences.

Primer ID (cap label)	Illumina ID	Index Sequence
IL1	R738	CTAGCT
IL2	R702	CGATGT
IL3	R703	TTAGGC
IL4	R704	TGACCA
IL5	R705	ACAGTG
IL6	R744	TATAAT
IL7	R707	CAGATC
IL8	R708	ACTTGA
IL9	R709	GATCAG
IL10	R710	TAGCTT
IL11	R711	GGCTAC
IL12	R712	CTTGTA

Appendix D: Guidelines for Demultiplexing with In-line Indexes

Read 2 has in-line indexes in the first 6 bp which are followed by poly(T). We have a simple piece of software called SMART-Seq DE3 Demultiplexer that can be used to demultiplex pooled samples. You can sign up to download the software from the link above on takarabio.com. Demultiplexing can also be achieved by using CLC Genomics Workbench.

Appendix E: Guidelines for the Use of NextSeq and MiniSeq

The resulting sequencing libraries with the SMART-Seq mRNA 3' DE can be used for NextSeq and MiniSeq. However, care must be taken to ensure that you get useful data. First, both systems use automatic adapter trimming by default. This can unexpectedly shorten your reads and cause your reads to change from the original sequence to a poly(N) sequence because of the default mask setting in Basespace. The minimum mask length is 35 cycles and any trimmed reads shorter than 35 bases will become poly(N) reads. This is especially dangerous with the Read 2 output which is of a short length and used primarily for demultiplexing samples with the in-line indexes. Therefore, we strongly recommend that you turn off the automatic adapter trimming by creating a custom library prep kit without adapter trimming. More information can be found on the Illumina website.

Secondly, because of the algorithm's sensitivity to low complexity (found in read 2 of the DE kit sequencing libraries), NextSeq and MiniSeq runs without PhiX will cause low quality sequencing reads and incorrect base calling. Therefore, we recommend that you add at least 30% by volume of spike-in PhiX for current NextSeq 500/550 v2 sequencing reagent kits and the MiniSeq Reagent Kit if the PhiX and your library are at approximately the same concentration. In order to achieve at least 20% of reads mapping to PhiX and high-quality sequencing scores, you may need to vary the amount of PhiX that you spike into your libraries. We found that we needed to spike in ~30% to have ~20% of reads mapping to PhiX.

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