Nextera® XT library prep of cDNA synthesized using the SMART-Seq® Single Cell Kit

I. Introduction

The **SMART-Seq Single Cell Kit** (Cat. Nos. 634470, 634471 & 634472) is designed to generate high-quality, full-length cDNA directly from single cells, especially those with low RNA content. This kit provides the benefit of generating cDNA that is compatible with Illumina® platform-specific library preparation kits.

This user guide provides a detailed protocol for generating libraries with the Nextera XT DNA Library Preparation Kit using the cDNA generated from the SMART-Seq Single Cell kit.

Users looking for a library generation solution from Takara Bio should purchase the SMART-Seq Library Prep Kit ((Cat. Nos. R400746 & R400747); also sold as part of the SMART-Seq Single Cell PLUS Kit (Cat. Nos. R400750 & R400751)).

II. Materials Required

- cDNA generated with the SMART-Seq Single Cell Kit (Cat. Nos. 634470, 634471 & 634472)
- Nextera XT DNA Library Preparation Kit (Illumina, Cat. Nos. FC-131-1024, FC-131-1096)
- Nextera XT Index Kit (Illumina, Cat. No. FC-131-2001) or other Nextera-compatible indexes
- NucleoMag NGS Clean-up and Size Select (available from Takara Bio; 5-ml size: Cat. No. 744970.5; 50-ml size: Cat. No. 744970.50; 500-ml size: Cat. No. 744970.500). If the NucleoMag product is not available, the AMPure XP PCR purification kit (Beckman Coulter; 5-ml size: Cat. No. A63880; 60-ml size: Cat. No. A63881) is an appropriate substitute

NOTES:

- The kit has been specifically validated with the beads listed above. Please do not make any substitutions as it may lead to unexpected results.
- Beads need to come to room temperature before the container is opened. We strongly 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 minutes). Aliquoting is also instrumental in decreasing the chances of bead contamination.
- Immediately before use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.

- 80% ethanol: freshly made for each experiment from molecular-biology-grade 100% ethanol
- Strong magnetic separation device and centrifuge appropriate for your sample tubes or plates, such as:
  - **For 12–24 samples:**
    - SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011); accommodates two 8-tube or 12-tube strips
    - Minicentrifuge for 0.2-ml tubes or strips
  - **For 24–96 or more samples:**
    - Magnetic Stand-96 (Thermo Fisher, Cat. No. AM10027); accommodates 96 samples in 96-well V-bottom plates (500 µl; VWR, Cat. No. 47743-996) sealed with adhesive PCR Plate Seals (Thermo Fisher, Cat. No. AB0558)
    - Low-speed benchtop centrifuge for a 96-well plate
  - **For 1.5-ml tubes (for pooling Nextera sequencing libraries before purification):**
    - Takara Bio USA, Inc. Magnetic Stand (Cat. No. 631964)

III. Protocol: Library Preparation for Next-Generation Sequencing

This section describes a modified protocol for the Illumina Nextera XT DNA library preparation kit to work downstream of the SMART-Seq Single-Cell Kit at one-quarter the volume originally recommended by Illumina. The Illumina Nextera XT DNA Library Preparation Guide provides detailed instructions for library preparation, and we highly recommend that you read it before proceeding. Note that the modifications included in the protocol below have been made to adapt the Nextera XT chemistry to work downstream of the SMART-Seq Single-Cell Kit.

A. Dilute and Prepare cDNA for Tagmentation

NOTES: The optimal cDNA input for Nextera XT library preparation is 100–300 pg. A larger cDNA input will generate libraries that are too large to be successfully sequenced on an Illumina instrument. The protocol below uses 125 pg of cDNA (in a volume of 1.25 µl), but any input between 100–300 pg will work.

1. Dilute each cDNA to 100 pg/µl with Nuclease-Free Water in a plate or PCR strips. Do not pool at this step. Vortex at medium speed for 20 sec and centrifuge at 350*g* for 1 min.

   NOTES:
   - Always use a minimum of 2 µl of cDNA to perform dilution.
   - Samples containing less than 100 pg/µl can still be used without dilution, but you may get fewer reads than for other samples if you pool for clean-up. Negative controls should be used without dilution.

   IMPORTANT: Warm Tagment DNA Buffer and NT Buffer to room temperature. Visually inspect NT Buffer to ensure that there is no precipitate. If there is a precipitate, vortex the buffer until all particles are resuspended.

2. After thawing, gently invert the tubes 3–5 times, followed by centrifuging the tubes briefly, to ensure all reagents are adequately mixed.

3. Label a new 96-well PCR plate "Library Prep."

4. In a 1.5-ml PCR tube, combine the components of the Tagmentation Premix as described below. Vortex gently for 20 sec and centrifuge the tube briefly.

   Tagmentation Premix:

Component	1 rxn (µl)	12 rxns (µl)	48 rxns (µl)	96 rxns (µl)
Tagment DNA Buffer	2.5	37.5	150	300
Amplification Tagment Mix	1.25	18.8	75	150
Total volume	3.75	56.3	225	450

5. Distribute 3.75 µl of the Tagmentation Premix into each well of the "Library Prep" plate.

   NOTE: If processing a large volume of samples, aliquot equal amounts of Tagmentation Premix into each tube of an 8-tube strip and then use an eight-channel pipette to distribute the Tagmentation Premix.

6. Transfer 1.25 µl of each diluted cDNA sample to the "Library Prep" plate.

7. Seal the plate and vortex at medium speed for 20 sec. Centrifuge at 2,000*g* for 2-3 min to remove bubbles.

8. Place the "Library Prep" plate in a thermal cycler with a heated lid and run the following program:

55°C	10 min
10°C	Hold

9. Once the thermal cycler reaches 10°C, pipette 1.25 µl of NT Buffer into each of the tagmented samples to neutralize the samples.

   NOTE: If processing a large volume of samples, aliquot equal amounts of NT Buffer into each tube of an 8-tube strip, then use an eight-channel pipette to distribute the NT Buffer.

10. Seal the plate and vortex at medium speed, then centrifuge at 2,000*g* for 1 min.

11. Incubate at room temperature for 5 min.

B. Amplifying the Tagmented cDNA

IMPORTANT: Consult Illumina literature (Index Adapters Pooling Guide 1000000041074) for proper index primer selection before proceeding to PCR amplification of the tagmented cDNA.

1. Pipette 3.75 µl of Nextera PCR Master Mix (NPM) into each well of the "Library Prep" plate using an eight-channel pipette.

   NOTE: If processing a large number of samples, aliquot equal amounts of NPM into each tube of an 8-tube strip, then use an eight-channel pipette to distribute the NPM.

2. Select appropriate Index 1 (N7xx) and Index 2 (S5xx) primers for the number of samples in your experiment.
   a. Pipette 1.25 µl of Index 1 Primers (N7xx) into the corresponding wells of each row of the "Library Prep" plate. As a result, each of the 12 wells in row "A" will contain different Index 1 Primers.
   b. Pipette 1.25 µl of Index 2 Primers (S5xx) to the corresponding wells of each column of the "Library Prep" plate. As a result, each of the 8 wells in column "1" will contain different Index 2 Primers.

3. Seal the plate with adhesive film and vortex at medium speed for 20 sec. Centrifuge at 2,000g for 1 min.

4. Place the "Library Prep" plate into a thermal cycler and perform PCR amplification using 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

5. Samples can be left overnight in the thermal cycler at 4°C. If not processed within the next day, freeze the PCR products at –20°C.

C. Pooling and Cleaning up the Libraries

NOTES:
- Aliquot 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 by vortexing. Use room-temperature Nuclease-Free Water for this protocol.
- Bead:sample ratio is 0.6:1.
- Prepare fresh 80% ethanol for each experiment. You need ~400 µl per sample.
- You will need a magnetic separation device for 0.2-ml tubes, 1.5-ml tubes, strip tubes, or a 96-well plate.

1. Determine the number of libraries to be pooled based on the desired sequencing depth and sequencer throughput.

   NOTE: If all samples are correctly quantified and normalized to a uniform input amount before Nextera XT library preparation, sequencing libraries can be pooled before clean-up and a relatively uniform amount of sequencing reads will be obtained.

2. Pool the libraries by pipetting a fixed volume from each sample into a PCR tube or 1.5-ml tube. Volumes between 2 to 4 µl are appropriate. See examples in the table below. Do not use less than 2 µl per sample to ensure greater accuracy.

   Table 1. Example volumes of pooled libraries and beads

Number of libraries to be pooled	Volume per library	Total pool volume	Bead volume*
8	4 µl	32 µl	19 µl
12	4 µl	48 µl	29 µl
16	2 µl	32 µl	19 µl
24	2 µl	48 µl	29 µl
32	2 µl	64 µl	38 µl
48	2 µl	96 µl	58 µl

   *The bead volume is approximately 60% of the total pool volume.

   NOTE: If pooling 96 samples or more, make sure to use a 1.5-ml tube.

3. Add a volume of AMPure XP beads representing 60% of the volume of the pooled libraries. See the table above for guidance.

   NOTE: If cleaning up libraries individually, add 40 µl of beads to each 50 µl of sample.

4. Mix well by vortexing or pipetting the entire mixture up and down 10 times.

   NOTE: The beads are viscous; pipette the entire volume and push it out slowly.

5. Incubate at room temperature for 5 min to let the cDNA libraries bind to the beads.
6. Briefly spin the sample to collect the liquid from the side of the tube. Place the tube on a magnetic stand for ~2 min or 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, remove and discard the supernatant. Take care not to disturb the beads.
8. Keep the samples on the magnetic separation device. Add 200 µl of fresh 80% ethanol to each sample without disturbing the beads. Incubate for 30 sec then, remove and discard the supernatant taking care not to disturb the beads. The cDNA remains bound to the beads during the washing process.
9. Repeat the ethanol wash (Step 8) once.
10. Briefly centrifuge the samples to collect the liquid from the side of the tube or sample well. Place the samples on the magnetic separation device for 30 sec, then remove any residual ethanol with a pipette.
11. Incubate the samples at room temperature for ~5–15 min, until the pellet is no longer shiny but before cracks appear.
12. Once the beads are dry, elute the pooled, purified libraries by adding the required volume of Nuclease-Free Water, based on the number of samples pooled.

Table 2. Example volumes of Nuclease-Free Water to resuspend libraries

Number of libraries pooled	Nuclease-Free Water*
8	48 µl
12	72 µl
16	48 µl
24	72 µl
32	96 µl
48	144 µl

*Nuclease-Free Water volume is 1.5 times the original pool volume.

NOTE: If cleaning up libraries individually, elute in 18 µl of Nuclease-Free Water.

13. Remove from the magnetic separation device and vortex the tube for 3 sec to mix thoroughly. Incubate at room temperature for ~5 min to rehydrate the beads.
14. Briefly spin to collect the liquid from the side of the tube. Place the tube back on the magnetic separation device for ~2 min or longer, until the solution is completely clear.
15. Transfer the entire volume of clear supernatant containing purified cDNA libraries to another tube.
16. Evaluate library size distribution by running samples on the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) or an equivalent microfluidic device/kit. Dilute libraries to about 1.5 ng/µl before loading the chip for a consistent library-to-library profile. See Figure 4 for an example of a successful library.
17. Refer to the Illumina Nextera XT DNA Library Preparation Guide to determine the appropriate library concentration for sequencing.

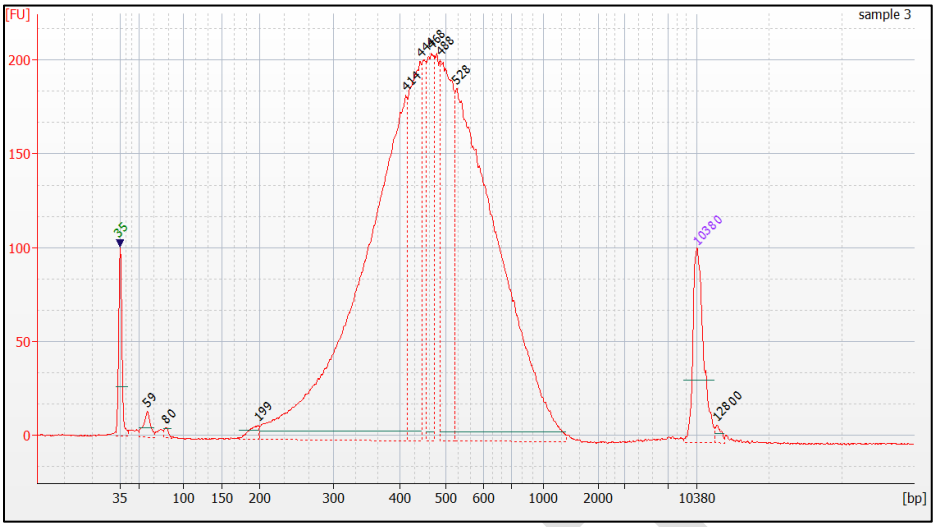

Figure 1. Example of a Nextera XT sequencing library. cDNA generated from 10 pg of Mouse Brain Total RNA control included in the kit was quantified by smear analysis after electrophoresis using Agilent's High Sensitivity DNA Kit as described in Section V.E and used as the input for Nextera XT library construction (125 pg per cell). One µl of the bead-purified library was analyzed on the Bioanalyzer using Agilent's High Sensitivity DNA Kit.

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: takarabio.com/service	web: takarabio.com/support
e-mail: ordersUS@takarabio.com	e-mail: technical_support@takarabio.com

Notice to Purchaser

Our products are to be used for **Research Use Only**. They may not be used for any other purpose, including, but not limited to, use in humans, therapeutic or diagnostic use, or commercial use of any kind. Our products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without our prior written approval.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product's web page at takarabio.com. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements.

Takara Bio USA, Inc.

United States/Canada: +1.800.662.2566 • Asia Pacific: +1.650.919.7300 • Europe: +33.(0)1.3904.6880 • Japan: +81.(0)77.565.6999

07.20 US