Takara Bio USA, Inc.

SMART-Seq® Pro Application Kit User Manual

Cat. No. 640257
for ICELL8® cx CELLSTUDIO™ v2.5 Software
(100824)

Takara Bio USA, Inc.
2560 Orchard Parkway, San Jose, CA 95131, USA
U.S. Technical Support: technical_support@takarabio.com

Table of Contents

Table of Figures

Table of Tables

I. Introduction

High-throughput full-length analysis of single cells

The **SMART-Seq® Pro** application protocol enables Illumina® sequencing and full-length transcriptome analysis of single cells isolated on the **ICELL8® cx Single-Cell System**. By leveraging the capabilities of the ICELL8 cx Single-Cell System along with SMART® (**S**witching **M**echanism **A**t 5′ end of **R**NA **T**emplate) technology, this protocol provides an efficient, cost-effective solution for high-throughput full-length transcriptome analysis of single cells.

The workflow (Figure 1, below) begins with staining and dilution of cell samples and the preparation of positive and negative controls. The cells and controls are then dispensed into the 5,184 nanowells of the ICELL8 350v Chip using the ICELL8 cx system and ICELL8 cx CELLSTUDIO™ v2.5 Software. Up to eight different samples can be dispensed in a single run and is completed in approximately 15 min. During this time, cells are maintained in the humidity- and temperature-controlled environment provided by the ICELL8 cx instrument.

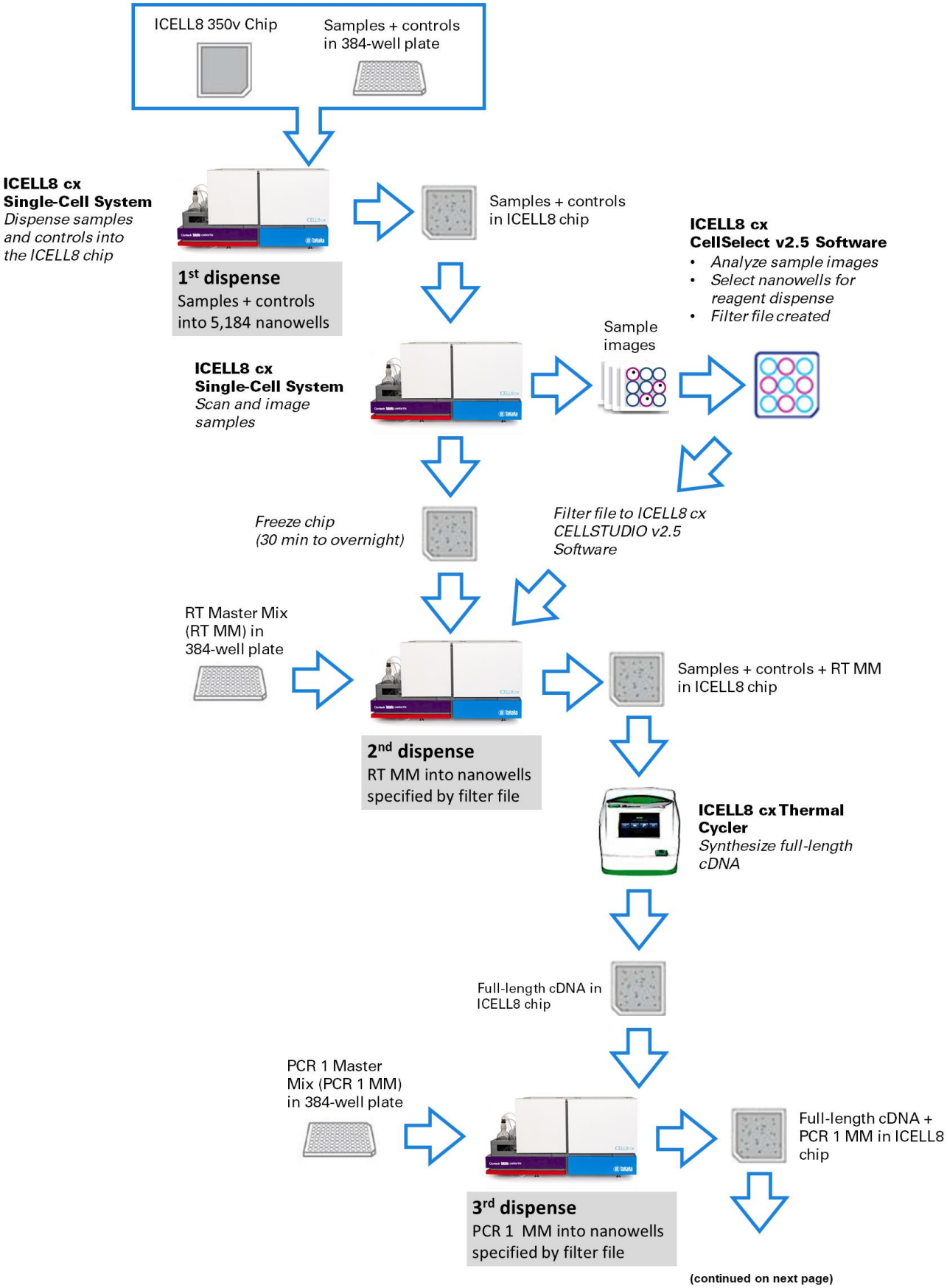
After sample and control dispense, the 5,184 nanowells are imaged by the ICELL8 cx system with both blue and red wavelength filters, and the ICELL8 cx CellSelect® v2.5 Software is used to analyze the resulting images. Initial candidate identification is performed automatically by the software, based on the size and morphology of the cells and nuclei stained with Hoechst 33342 (blue) with additional differentiation by propidium iodide (red). Users may further refine candidate identification by configuration adjustments to the automated threshold detection (auto-tune) or by visual triage of the images. After selection of candidate wells, CellSelect software generates a filter file to be imported into CELLSTUDIO software to control downstream reagent dispenses.

For library construction, the ICELL8 cx Single-Cell System dispenses RT reagents into the candidate wells of the ICELL8 chip designated by the filter file. The chip is run through first-strand cDNA synthesis on the ICELL8 cx Thermal Cycler (referred to as thermal cycler throughout this manual) initiated by SMART-Seq Pro CDS (an oligo-dT primer). Following first-strand cDNA synthesis, the SMART-Seq Pro Oligonucleotide hybridizes to the 3′ end of the full-length cDNA, mediates template switching, and serves as a priming site for second-strand cDNA synthesis. Once synthesized, the second-strand cDNA is amplified through PCR, resulting in copies of unbiased, full-length cDNA.

After amplification, the full-length cDNA is tagmented by Illumina Bead-Linked Transposome (BLT). The tagmented cDNA is then amplified using forward and reverse indexing primers, generating the final library construct. The resulting libraries are extracted from the chip, purified, amplified, and purified again.

After validation steps, the libraries are ready for sequencing on Illumina platforms. The kit contains two sets of primer indexing plates (Cat. Nos. 640258 and 640260); if each primer set is used per provided chip, two SMART-Seq Pro libraries can be multiplexed in a single sequencing run.

Figure 1 (over the next two pages) shows a workflow of the application starting from samples to creation of sequencing-ready libraries.



(continued on next page)

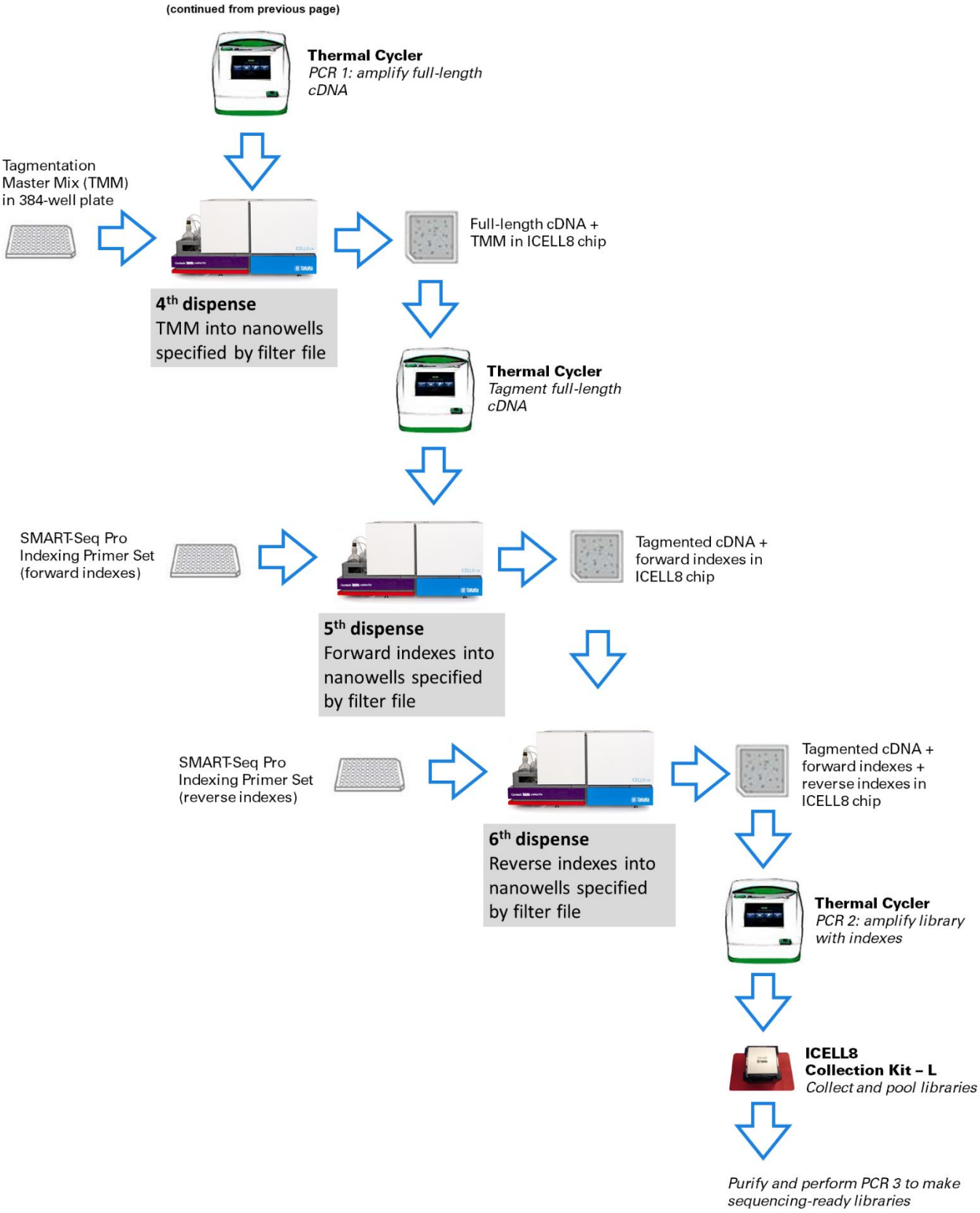


Figure 1. SMART-Seq Pro application workflow.

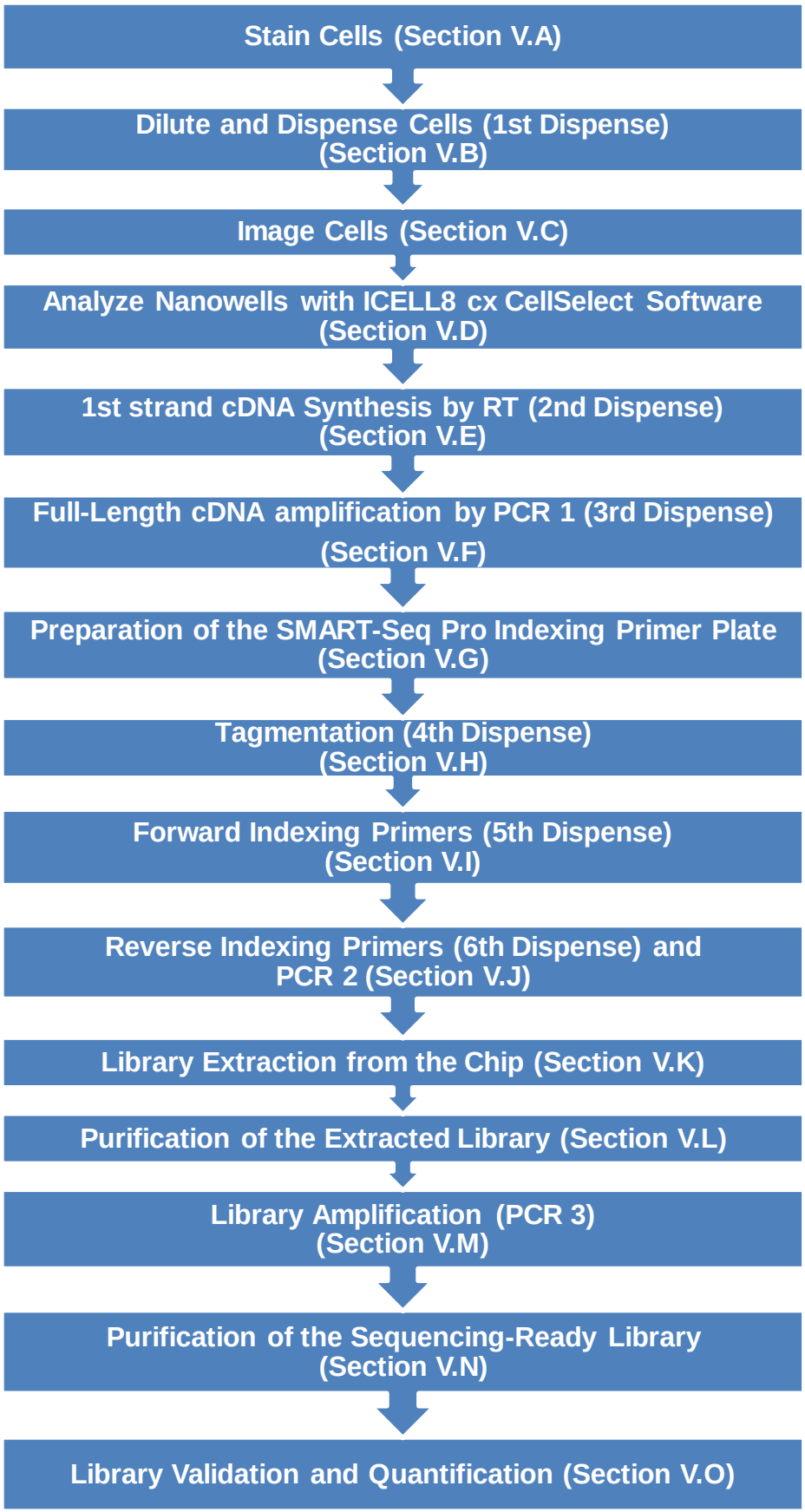


Figure 2. Protocols in the SMART-Seq Pro workflow. Click any box to jump to that section in the manual.

II. List of Components

The SMART-Seq Pro application workflow requires use of an ICELL8 cx Single-Cell System (Cat. No. 640188 or 640189) and the SMART-Seq Pro Application Kit - 2 Chip (Cat. No. 640257).

Table 1. SMART-Seq Pro Application Kit - 2 Chip components.

SMART-Seq Pro Application Kit - 2 Chip	640257 (2 chip)
SMART-Seq Pro Reagent Kit (Cat. No. 640259)*	
Box 1 (Store at –70°C)	
Control K-562 RNA (1 µg/µl)	10 µl
Box 2 (Store at –20°C)	
Second Diluent (100X)	25 µl
BSA (1%)	25 µl
RNase Inhibitor (40 U/µl)	50 µl
SMART-Seq Pro CDS (100 µM)	5 µl
SMART-Seq Pro Oligonucleotide (100 µM)	20 µl
SMART-Seq Pro RT Buffer	200 µl
SMART-Seq Pro Lysis Buffer	40 µl
SMARTScribe™ Reverse Transcriptase (200 U/µl)	80 µl
SMART-Seq Pro PCR 1 Buffer	60 µl
Terra™ Direct PCR Polymerase Mix (1.25 U/µl)	90 µl
$MgCl_2$	10 µl
SeqAmp™ CB PCR Buffer (2X)	100 µl
5X Primer Mix	40 µl
Elution Buffer (10 mM)	350 µl
Nuclease-Free Water	500 µl
SMART-Seq Pro Index Resuspension Buffer	20 ml
Box 3 (Store at room temperature)	
SMART-Seq Pro Tagmentation Buffer	220 µl
SMART-Seq Pro Indexing Primer Set - A (Cat. No. 640258)	1
SMART-Seq Pro Indexing Primer Plate - A	0.05 nmol/well†
Plate Sealing Film	
SMART-Seq Pro Indexing Primer Set - B (Cat. No. 640260)	1
SMART-Seq Pro Indexing Primer Plate - B	0.05 nmol/well†
Plate Sealing Film	
ICELL8 350v Chip (Cat. No. 640019)	2
ICELL8 Collection Kit – L (Cat. No. 640212)	2
ICELL8 Loading Kit – B (Cat. No. 640206)	2

*Enough reagents are included in Cat. No. 640259 to dispense to 2,000 wells per ICELL8 350v Chip (4,000 for the 2-chip kit). For more details, refer to Section V.D “Analyze Nanowells with ICELL8 cx CellSelect Software”.

†Each well contains a specific forward or reverse primer. See Appendix A for the well map and complete list of index barcodes.

III. Additional Materials Required

Required general lab supplies

- Personal protective equipment (PPE): powder-free gloves, safety glasses, lab coat, sleeve protectors, etc.
- Takara Bio 384-well plates and seals (choose one of the following):
  - 2 x ICELL8 384-Well Source Plate and Seal, 5/pack (Cat. No. 640192)
  - 1 x MSND 384-Well Source Plate and Seals, 20 or 120/pack (Cat. Nos. 640018 or 640037)
- Plate Seal Applicator, included with the ICELL8 cx instrument
- Film Sealing Roller for PCR Plates ("film sealing roller") (Bio-Rad, Cat. No. MSR0001)
- PCR thermal cycler compatible with 0.2-ml tubes
- Minicentrifuges for 1.5-ml tubes and 0.2-ml tubes or strips
- 384-well plate orbital shaker with 3 mm mixing orbit
- Vortex mixer
- Centrifuges, rotors, and adapters.
  Recommended:
  - Eppendorf 5810R with Microplate Buckets (VWR, Cat. No. 53513-874), ≥2,600*g*, room temperature & 4ºC operation
  - Kubota 3740 with rotor SF-240 for cell preparation
- Nuclease-free, non-stick 0.2-ml PCR tubes
- Nuclease-free LoBind 1.5-ml microcentrifuge tubes (Eppendorf)
- Conical tubes, 50-ml and 15-ml sizes
- 5-ml flip-cap tubes
- Single-channel pipettes: 2 µl, 10 µl, 20 µl, 200 µl, and 1,000 µl
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1,000 µl
- Wide-bore pipette tips: 200 µl and 1,000 µl
- Serological pipettes and controller
- Nuclease-decontamination solution
- Exhaust hood system with UV

For staining and dispensing cells

- ICELL8 Chip Holder (Takara Bio, Cat. No. 640008); two chip holders are included with the instrument, additional chip holders can be ordered separately
- 1X PBS (no Ca^{2+}, Mg^{2+}, phenol red, or serum, pH 7.0–7.3; Thermo Fisher Scientific, Cat. No. 14190144 or an equivalent PBS)
- ReadyProbes Cell Viability Imaging Kit, Blue/Red (Thermo Fisher Scientific, Cat. No. R37610); contains Hoechst 33342 and propidium iodide

For dissociating adherent cells:

- TrypLE Express (Thermo Fisher Scientific, Cat. No. 12604021)
- Appropriate cell culture medium

For tagmentation

- ILMN DNA LP (M) Tagmentation (24 Samples, IPB) (Illumina, Cat. No. 20060060) or ILMN DNA LP (M) Tagmentation (96 Samples, IPB) (Illumina, Cat. No. 20060059)

NOTE: The SMART-Seq Pro application only requires the BLT (Bead-Linked Transposome). Do not use other components of either of these kits in this workflow.

For library purification and validation

- SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)
- Magnetic Separator compatible with 1.5-ml tubes.
- NucleoMag NGS Clean-up and Size Select (Takara Bio, Cat. No. 744970.50 or 744970.500)

NOTES:

- Beads need to come to room temperature before the container is opened. We therefore recommend aliquoting the beads into 1.5-ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be used for each experiment, allowing them to come to room temperature more quickly (~30 min). This aliquoting process also minimizes the chances of bead contamination.
- Immediately prior to 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: prepared fresh from anhydrous ethanol for each experiment
- Bioanalyzer instrument (Agilent Technologies) or similar
- Agilent High Sensitivity DNA Kit (110 samples; Agilent Technologies, Cat. No. 5067-4626)
- Library Quantification Kit (Takara Bio, Cat No. 638324).
- Qubit fluorometer (Thermo Fisher Scientific) or similar
- Qubit dsDNA HS Assay Kit (100 assays; Thermo Fisher Scientific, Cat. No. Q32851)

For cell counting

- Recommended: Moxi Z Mini Automated Cell Counter Kit, U.S. Version (ORFLO, Cat. No. MXZ001).

NOTES:

- Refer to a Moxi Z user guide for guidance in selecting an appropriate cassette size for the cells being analyzed
- Alternatively, you may use a hemocytometer or any preferred cell counter with demonstrated, accurate cell counting

IV. General Considerations

A. Protocol Best Practices

- Perform all experimental procedures in sterile environments with the proper personal protective equipment (PPE). Use designated UV hoods with proper ventilation for manipulating cells and setting up molecular biology reactions. Decontaminate gloves with nuclease decontamination solution, water, and ethanol. Change gloves routinely.
- Minimize the exposure time of unsealed chips, reservoirs, reagents, and other consumables to the open air. Wearing laboratory sleeve protectors may reduce the likelihood of introducing contaminants from exposed hands and arms.
- The assay is very sensitive to variations in pipette volume. Please make sure that all pipettes are calibrated for reliable reagent delivery and that nothing adheres to the outsides of the tips when dispensing liquids.
- All lab supplies related to cDNA synthesis need to be stored in a DNA-free, closed cabinet. Ideally, reagents for cDNA synthesis should be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.

- Use nuclease-free, molecular biology- or PCR-grade reagents to set up all molecular biology reactions.
- Add enzymes to reaction mixtures last and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- DO NOT change the amount or concentration of any of the components in the reactions; they have been carefully optimized for the SMART-Seq Pro application.
- Because of the large volume or viscosity of mixtures subject to purification using NucleoMag NGS Clean-up and Size Select beads, each round of purification requires a very strong magnet. 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.
- UV-treat reagent reservoirs, seals, pipettes, filter tips, and compatible reagents prior to use.

B. Safety

Refer to safety guidelines in the user manuals for all equipment used in this protocol.

WARNING: Perform all experimental procedures in sterile environments with the proper personal protective equipment (PPE). Use designated UV hoods with proper ventilation for manipulating cells and setting up molecular biology reactions. Decontaminate gloves with nuclease decontamination solution, water, and ethanol. Change gloves routinely.

WARNING: Use of equipment and reagents for cell preparation and isolation with the ICELL8 cx Single-Cell System may cause exposure to toxic or biohazardous chemicals, thereby presenting a hazard. Always wear appropriate personal protective equipment (PPE), which should at minimum include gloves, eye protection, and a lab coat, when handling equipment and reagents and operating instruments.

Note and heed all warning labels on the instruments used in this protocol.

C. ICELL8 cx System Application Notes

Refer to the ICELL8 cx Single-Cell System User Manual for full details. Included below are general reminders.

- All dispensing steps in the ICELL8 cx Single-Cell System Stage Module should be performed with a 384-Well Source Plate oriented with the A1 well positioned at the top-right corner of the 384-well plate nest (Figures 3 and 4, below). The source plate must be fully seated. This may be accomplished by pushing the source plate down after it has been placed on the plate nest.
- All dispensing steps in the ICELL8 cx Single-Cell System Stage Module should be performed with the chip oriented with the chamfered (beveled) corner positioned towards the bottom-right corner of the chip nest (Figure 3, next page).

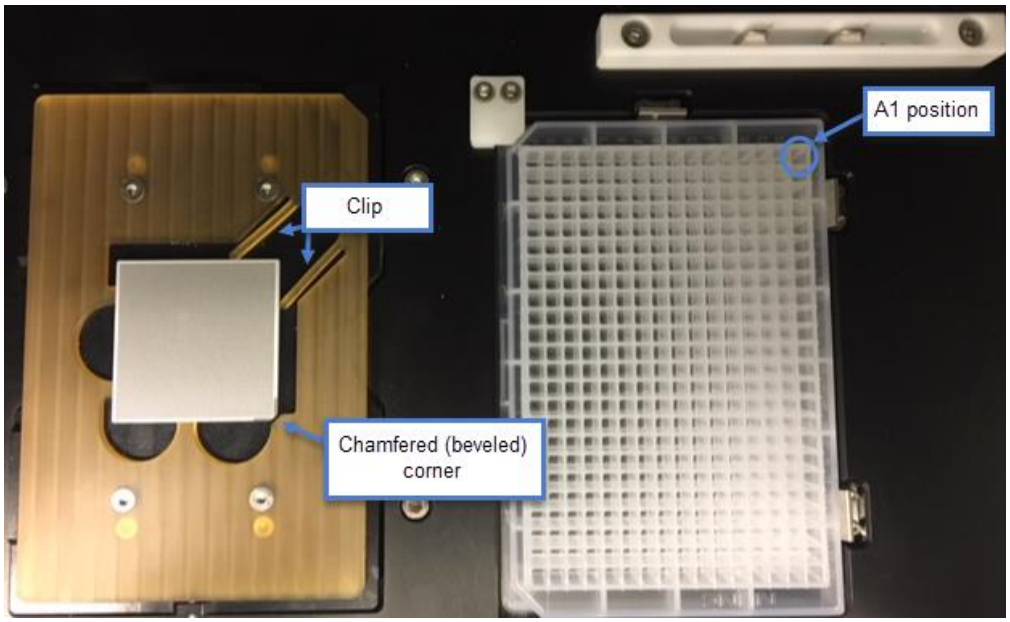


Figure 3. ICELL8 cx Single-Cell System Stage Module. **(Left)** chip nest. **(Right)** source plate nest.

D. ICELL8 350v Chip

Each ICELL8 350v Chip is engraved with a unique number (Figure 4). You can use this number to link your chip images and other experimental record files.

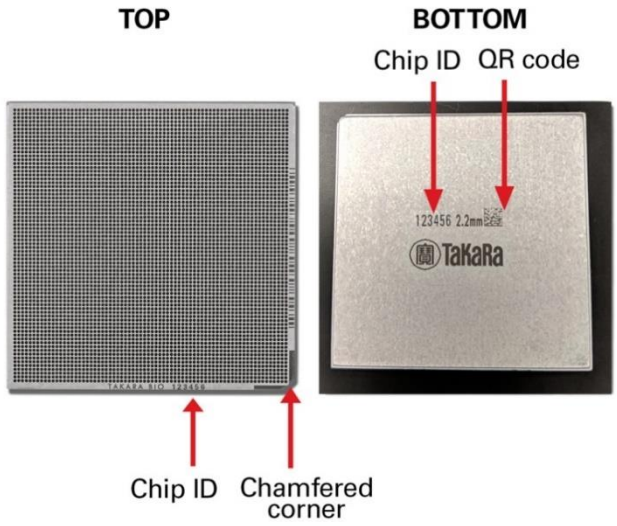


Figure 4. ICELL8 350v Chip features. **(Left)** Top view of the chip. Note the chamfered (beveled) corner at the bottom right. The "TaKaRa" logo and the chip ID (unique to each chip) are engraved on the chip border, near the chamfered corner. (**Right)** Bottom view of the chip. The chip ID is also engraved on the other side of the chip with a corresponding QR code that can be scanned by a barcode reader, allowing for the chip ID to be easily entered into the software.

E. Software

The instructions in this manual are written for use with CELLSTUDIO (v2.5.6 or later) and CellSelect (v2.5.11 or later) Software.

If you do not see the *SMART-Seq Pro* tab as an option in your CELLSTUDIO user interface:

- If you are running version **2.5.21 or later**, you may need to mark the workflow as 'Visible' in the Application Manager. Refer to the ICELL8 cx Single-Cell System User Manual , Section V.C "Application Manager" for detailed instructions on how to do this.
- If you are running a version prior to 2.5.21, please contact technical support to upgrade your CELLSTUDIO software.

  NOTE: If you are running version 2.5.6 and you do not wish to upgrade at this time, use the SMART-Seq Pro Application Installation Protocol-At-A-Glance to install the workflow.

V. Procedure

A. Protocol: Stain Cells

In this protocol, sample cells are stained with Hoechst 33342 and propidium iodide dyes. These dyes enable imaging, analysis, and selection of candidate wells suitable for downstream analysis following cell dispense into ICELL8 350v chips.

- If starting from a nonadherent suspension culture, such as K-562 cells, use Procedure 1 (Section V.A.3).
- If starting from an adherent culture, such as 3T3 cells, use Procedure 2 (Section V.A.4), and then stain the trypsinized cell suspension using Procedure 1.

1. Cell and Chip Handling Notes

- This protocol requires healthy cell culture suspension(s). Some cell lines may require trypsinization to achieve a single-cell suspension. As a general guideline, a minimum of 100,000 cells should be stained per ICELL8 chip.
- Keep cells at 37°C with 5% CO_2 in a cell culture incubator when not performing manipulations.
- Perform all wash steps in an exhaust UV hood. Avoid exposing the cell culture to ambient air to reduce the likelihood of contamination.
- Treat cells gently: do not vortex; minimize bubble formation and frothing.
- Wear nitrile or powder-free gloves to reduce imaging artifacts.
- Centrifugation speed and time may need to be modified for different cell types.

2. Before You Start

- Perform a once-a-day warmup of the ICELL8 cx system. Refer to the ICELL8 cx Single-Cell System User Manual (Section VIII) for more details.
- Pre-freeze ICELL8 Chip Holder(s) (Figure 5) at –80°C.

Figure 5. ICELL8 Chip Holder.

- Chill some of the 1X PBS to 4°C.
- If using adherent cells (Procedure 2), prewarm to 37°C:
  - 11 ml 1X PBS
  - 4 ml TrypLE Express
  - 8 ml of cell culture medium for your sample
- Set the chip centrifuge to 4°C.
- Dilute Control K-562 RNA (1 µg/µl) to 1 ng/µl for use in the next protocol (Section V.B) as indicated in the following steps. Keep the dilution on ice:

  a) Dilute Control K-562 RNA to 100 ng/µl by mixing 18 µl of Nuclease-Free Water with 2 µl of Control K-562 RNA (1 µg/µl) in a sterile nuclease-free microcentrifuge tube.

  b) Dilute Control K-562 RNA to 10 ng/µl by mixing 18 µl of Nuclease-Free Water with 2 µl of Control K-562 RNA (100 ng/µl) in a sterile nuclease-free microcentrifuge tube.

  c) Dilute Control K-562 RNA to 1 ng/µl by mixing 18 µl of Nuclease-Free Water with 2 µl of Control K-562 RNA (10 ng/µl) in a sterile nuclease-free microcentrifuge tube.

3. Procedure 1: Staining Cells in Suspension

1. Prepare a 1:1 mixture of Hoechst 33342 and propidium iodide by combining 80 µl of each dye per 1 ml of cells to be stained. Depending on your cell density and sample volume, scale as needed. An example using 2 ml of cells is described below (e.g., prepare 320 µl of premixed dye solution).

   NOTE: Protect this mixture from light until ready for use.

2. Transfer ~2.1 ml of suspension cells to a fresh 5-ml tube.
3. Determine the cell concentration using your preferred method or a Moxi automated cell counter and an appropriate Moxi cassette (refer to the Moxi user manual for guidance in selecting an appropriate cassette size for the cells being analyzed).
4. Add 320 µl of the premixed Hoechst 33342 and propidium iodide dye mix to the 2.1 ml of cells. Mix gently by inverting the tube 5 times. DO NOT vortex or overagitate the cells.
5. Incubate cells at 37°C for 20 min.
6. Add an equal volume of prechilled 1X PBS to stained cells. For the example described here, 2 ml of cold 1X PBS is added to the 5-ml tube containing the stained cell suspension.
7. Mix the stained cell suspension and PBS by gently inverting the tube 5 times. DO NOT vortex or overagitate the cells.
8. Pellet the cells by centrifugation at 4°C. Avoid over-centrifugation or pelleting into a firm pellet or clump.

   NOTE: Optimal centrifugation speed and time may vary depending on the cell type being analyzed. Examples:

   - K-562 or 3T3 cells: 100*g* for 3 min.
   - PBMCs or similarly sized cells: 500*g* for 3 min.
9. Gently remove the tube from the centrifuge without disturbing the cell pellet.
10. Carefully decant the supernatant without disturbing the cell pellet.

11. Wipe remaining fluid from the top of the tube using a fresh Kimwipe such that it is gently removed.
12. Gently add 1 ml of prechilled 1X PBS to the side wall of the tube.
13. Use a wide-bore 1-ml pipette tip to gently mix the cell suspension by slowly pipetting up and down ~5 times. DO NOT vortex or overagitate the cells.
14. Count the cells using your preferred method. Take two readings for each stained cell sample and average the results.

    The recommended concentration range of stained cell suspension is 0.5–5.0 x 10^5 cells/ml.

    - If the concentration is lower than 0.5 x 10^5 cells/ml, pellet the stained cell suspension and resuspend in a lower volume of 1X PBS to achieve a concentration in the recommended range.
    - If the concentration is higher than 5.0 x 10^5 cells/ml, dilute with additional 1X PBS until the concentration is within the recommended range.
15. Proceed to the next protocol (Section V.B). Keep the prepared cell suspension on ice.

4. Procedure 2: Preparing Adherent Cell Types from a 75-cm Culture Flask*

*Adjust volumes accordingly for different-sized flasks.

1. Carefully remove culture media from a 75-cm flask containing adherent cells using a serological pipette.
2. Add 10 ml of 1X PBS prewarmed to 37°C by dispensing the PBS on the side walls of the flask. DO NOT pour PBS directly onto cells.
3. Wash the cells by tilting the flask gently. DO NOT mix by pipetting.
4. Remove the PBS from the cells using a serological pipette.
5. Add 3 ml of prewarmed TrypLE Express to the flask to dissociate the cells.
6. The efficiency of cell dissociation from the flask surface may vary with cell type. Monitor the process visually using a microscope.
7. Once cell dissociation has occurred, neutralize the trypsinization reaction by gently adding 7 ml of prewarmed cell culture medium. Do not vortex or overagitate cells.
8. Pellet the cells by centrifugation at 100*g* for 3 min at room temperature

   NOTES:
   - Optimal centrifugation speed and time may vary depending on the cell type being analyzed. For K-562 or 3T3 cells, centrifuge at 100*g*. For smaller cells or cells that are not pelleting at 100*g*, centrifuge speed can be increased up to 500*g*.
   - Avoid over-centrifugation or pelleting into a firm pellet or clump.
9. Gently add 10 ml of prewarmed 1X PBS to the side wall of the tube.
10. Use a wide-bore 1-ml pipette tip to gently mix the cell suspension by slowly pipetting up and down ~5 times. Do not vortex or overagitate the cells.
11. Follow Procedure 1: Staining Cells in Suspension (Section V.A.3, above) starting from Step 1.

B. Protocol: Dilute and Dispense Cells (1st Dispense)

In this protocol, sample cells and controls are diluted and aliquoted into a 384-Well Source Plate and dispensed into the ICELL8 350v Chip using the ICELL8 cx Single-Cell System. Refer to the ICELL8 cx Single-Cell System User Manual for detailed information about instrument setup (Section VIII) and operation (Section X).

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Reagent Kit
    - Control K-562 Total RNA (diluted to 1 ng/µl in the previous protocol, Section V.A.2), Second Diluent (100X), BSA (1%), RNase Inhibitor
  - ICELL8 Loading Kit – B
    - Blotting Paper, RC Film
- From the ICELL8 384-Well Source Plate and Seal: a 384-well source plate and plate seal
- A plate seal applicator and film sealing roller

2. Before You Start

- Confirm that initialization and setup procedures for the ICELL8 cx Single-Cell System have been completed (see "Before You Start" in Section V.A, above).
- Confirm the empty ICELL8 Chip Holder (Figure 5, above) is frozen at –80°C.
- Aliquot 300–500 µl of 1X PBS (Ca^{2+} and Mg^{2+} free, pH 7.4) on ice for positive and negative controls.
- Thaw Second Diluent (100X) and BSA (1%) on ice. Once thawed, keep on ice for the remainder of the protocol.
- Use the concentration of stained cell suspension measured at the end of the previous protocol (Section V.A.3) and the information in Table 2 (below) to calculate the volumes of stained cell suspension and 1X PBS that should be combined for a final concentration of 1.4 cells/35 nl (40,000 cells/ml) in a total volume of 1 ml.

  NOTE: If starting from a small sample size, refer to the instructions in Appendix D to calculate the volumes of reagents to combine.

3. Procedure

Prepare the ICELL8 chip

1. Open the packet containing the sealed ICELL8 350v Chip.

   NOTE: Do NOT remove the chip seal at this time.

2. Place the chip on the chip nest in the ICELL8 cx Single-Cell System. The chamfered (beveled) corner of the chip should align with the chamfered corner of the chip nest (refer to Figure 3, above, or the ICELL8 cx Single-Cell System User Manual, Section X.A).

Prepare diluted stained cell suspension

3. Briefly vortex each component: Second Diluent, RNase Inhibitor, and BSA (1%). Spin the tubes briefly to collect contents at the bottom.

4. In a 1.5-ml microcentrifuge tube, combine the volumes of Second Diluent, BSA (1%), RNase Inhibitor, and cold 1X PBS indicated in the corresponding column of Table 2 (below). Mix the combined reagents by vortexing, then spin the tubes briefly to collect contents at the bottom.

   NOTE: The amount of 1X PBS added will depend on the starting concentration of stained cell suspension.

5. To the 1.5-ml microcentrifuge tube from the previous step (containing Second Diluent, BSA (1%), RNase Inhibitor, and 1X PBS), add the calculated volume of stained cell suspension that will yield a final concentration of 1.4 cells/35 nl in a total volume of 1 ml (refer to the ICELL8 Cell Dilution Tool spreadsheet provided by the Field Application Specialist during training). DO NOT use a pipette to mix at this step.

   NOTES:
   - Before obtaining the sample, mix the stained cell suspension gently by inverting the tube several times.
   - Take the required volume of stained cell suspension from the center of the tube using a 20 µl, 200 µl, or 1 ml pipette tip and add it slowly to the tube containing the other reagents.
   - Work quickly to avoid settling of cells.

Prepare positive and negative controls

6. Prepare positive and negative controls in separate 1.5-ml microcentrifuge tubes using the volumes indicated in Table 2 (below).

   NOTES:
   - Keep the positive control sample on ice.
   - Mix well, but do not vortex the positive and negative control samples.
   - The final concentration of Control K-562 Total RNA should be 2 pg/35 nl (equivalent to the total RNA content from 1 cell).

Table 2. Sample preparation guidelines.*

Components	Negative control	Positive control	Diluted stained cell suspension	Volume per source well (for each sample)†
Second Diluent (100X)	1.0 µl	1.0 µl	10 µl	1 µl
BSA (1%)	1.0 µl	1.0 µl	10 µl	1 µl
RNase Inhibitor (40 U/µl)	1.0 µl	1.0 µl	10 µl	1 µl
Control K-562 RNA (1 ng/µl)	–	5.7 µl	–	–
Stained cell suspension	–	–	Dilute to 1.4 cells/35 nl†	Dilute to 1.4 cells/35 nl†
1X PBS (Ca^{2+} and Mg^{2+} free)	97.0 µl	91.3 µl	Up to 1,000 µl	Up to 100 µl
Total	100 µl	100 µl	1,000 µl‡	100 µl§

* This table is the primary recommended guideline for making diluted stained cell suspensions. However, in cases where cells are scarce, reduced suspension volumes may be prepared. See Appendix D.

†Sufficient stained-cell suspension should be included such that the final concentration in the 1,000-µl volume of diluted stained cell suspension is 1.4 cells/35 nl (i.e., 40,000 cells/ml).

‡The 1,000-µl total volume of diluted stained cell suspension is sufficient for distributing 80 µl of cell suspension in each of eight source wells (see Figure 6, below).

§The 100-µl total volume of diluted stained cell suspension is sufficient for distributing 80 µl of cell suspension into a single source well. Multiply appropriately for the number of source wells used for each sample type.

Prepare sample dispense source plate

7. Add positive control and negative control to the 384-Well Source Plate as indicated in the following steps and in Figure 6 (below):

   - Add 25 µl of prepared negative control to well A24.
   - Add 25 µl of prepared positive control to well P24.

8. Using a wide-bore 1-ml pipette tip, gently mix the diluted stained cell suspension prepared in step 5 of this protocol (above) by slowly pipetting up and down ~5 times. DO NOT vortex or overagitate the cells.

   IMPORTANT: Proceed to the next step quickly to avoid settling of cells.

9. Using a 200-µl pipette tip, slowly and carefully load 80 µl of cell suspension into wells A1, A2, B1, B2, C1, C2, D1, and D2 of a 384-Well Source Plate as indicated in Figure 6 (below).

   NOTES:
   - Make sure to take each aliquot from the center of the tube containing the diluted stained cell suspension.
   - Be careful not to splash liquid into neighboring wells.
   - Make sure not to introduce bubbles when adding the cell suspension to the 384-Well Source Plate.
   - DO NOT vortex or spin down the 384-Well Source Plate.
   - DO NOT tap the plate. If any bubbles are present, remove gently using a pipette tip.
   - Proceed immediately to the next step to avoid settling of cells.

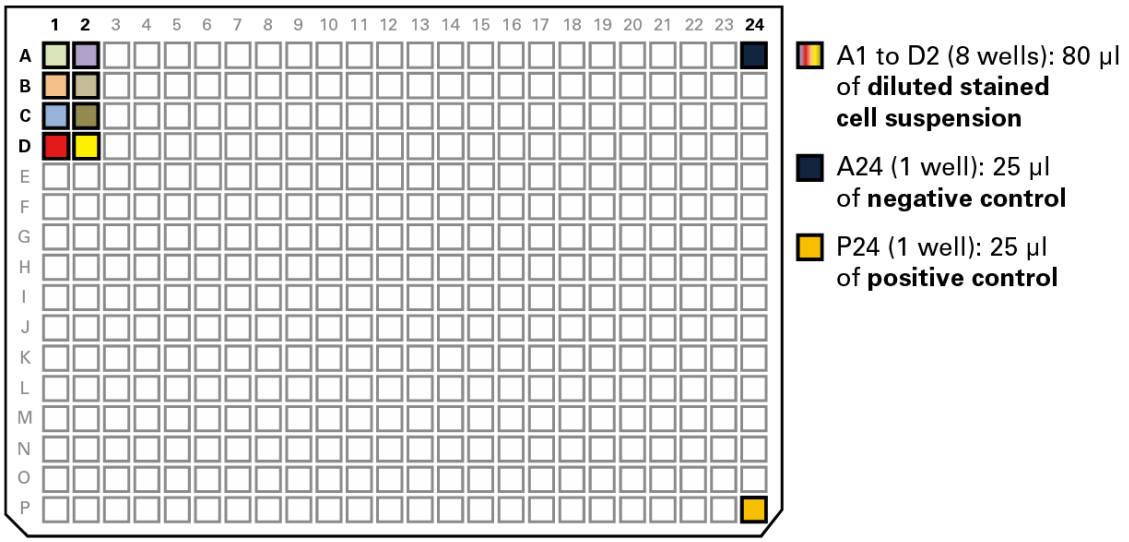


Figure 6. Setting up the 384-Well Source Plate for dispensing cell samples and controls.

10. Place the 384-Well Source Plate in the ICELL8 cx Single-Cell System with the A1 corner positioned at the top-right corner of the plate nest. The beveled corners of the 384-Well Source Plate should be on the left side (refer to the ICELL8 cx Single-Cell System User Manual, Section X.B).

11. In CELLSTUDIO software, click the [Dispense cells and Controls (35 nl)] button (Figure 7).

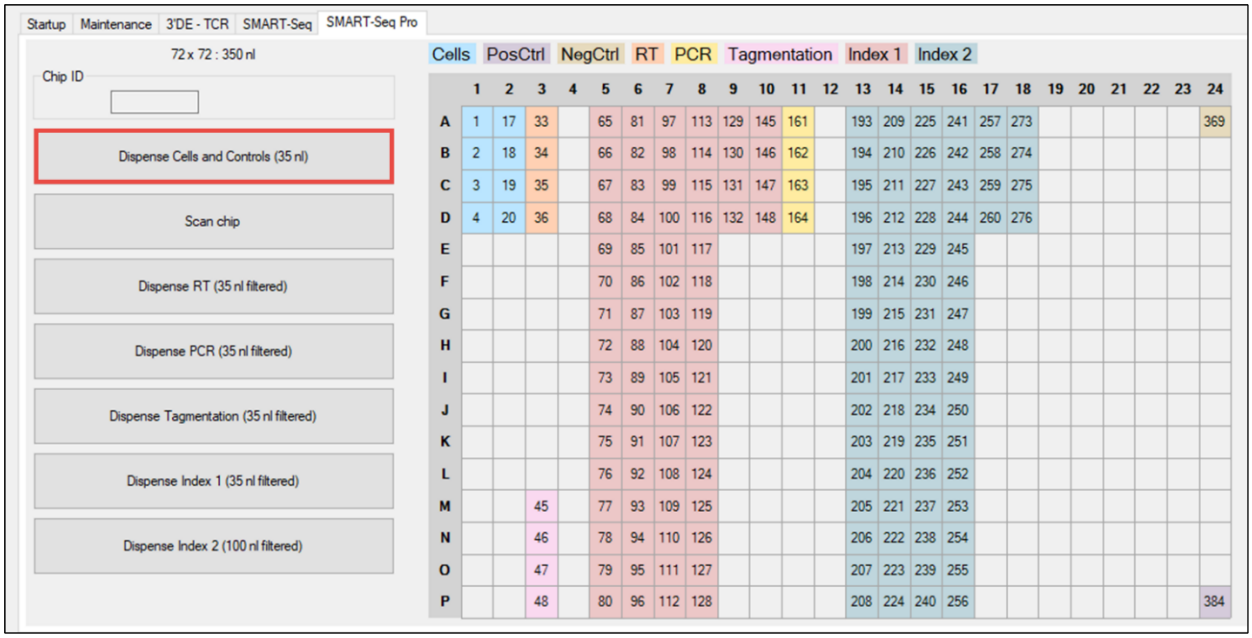


Figure 7. Using CELLSTUDIO software to dispense cells into the ICELL8 350v Chip.

A "Select index set" *Workflow* window will display asking you to indicate which SMART-Seq Pro Indexing Primer Plate you are using (Figure 8). Choose the one you are using for this run from the drop-down menu and then click [Done].

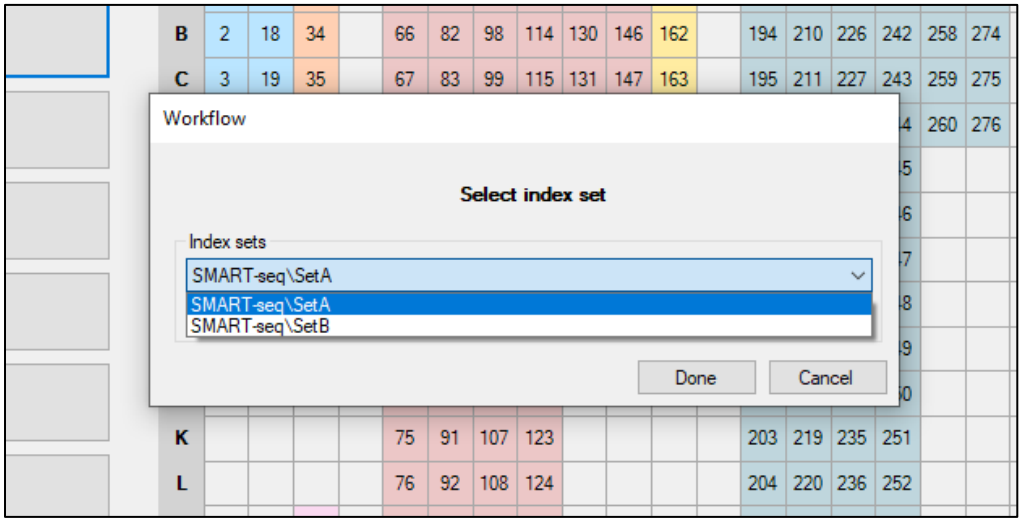


Figure 8. *Select index set* workflow prompt. The display defaults to using Set A. Ensure the correct indexing primer set is selected before clicking [Done].

12. Follow the subsequent software prompts and check the orientation of the source plate and ICELL8 350v Chip to ensure they are correctly loaded on the Dispensing Platform.

    During these checks, remove the seal on the ICELL8 350v Chip and, if a seal was used, from the 384-well plate when prompted.

13. Click [Done] to proceed.

NOTE: Refer to the ICELL8 cx Single-Cell System User Manual, Section X.C "Dispense the Sample Cells and Experimental Controls into the Chip" for details about the dispense step.

14. After the sample and control dispense is completed, remove the chip from the chip nest and blot with blotting paper. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.D "Blot and Centrifuge the Chip" for instructions to do that.

15. Seal the loaded chip with the RC Film (Figure 9, right)

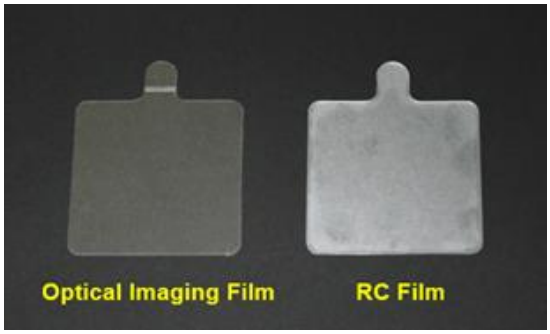


Figure 9. Imaging and RC Films required for the dispenses. The Optical Imaging Film (left) has three layers. The RC Sealing Film (right) has a translucent backing. Please follow the instructions in each subprotocol regarding preparation and handling of the films.

16. Remove the liner from the RC Film and apply the exposed side of the film to the blotted chip (Figure 10, left, below).

17. Seal the blotted chip with the film using a film sealing roller (Figure 10, right, below).

NOTE: The RC Film is nonadhesive and can easily peel off. Be careful when handling.

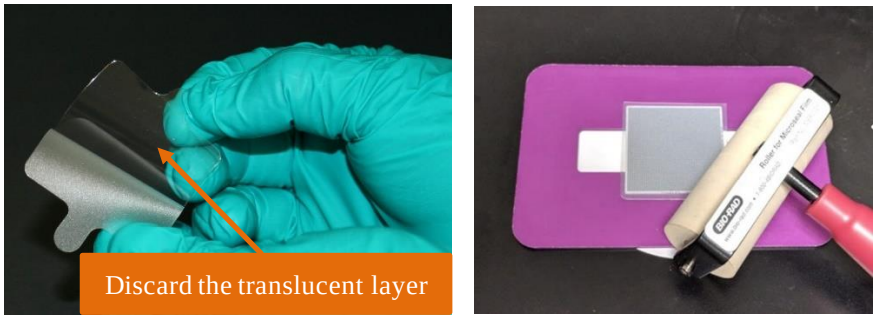


Figure 10. Preparing and adhering the RC Film. RC Film is composed of two layers: one clear and the other translucent. **(Left)** When sealing a chip, remove the translucent layer and discard it. Put the remaining layer on the chip. **(Right)** Tightly adhere the RC Film using the film sealing roller. Make sure that the chip is securely sealed to avoid well-to-well contamination and evaporation.

18. Place the sealed chip on a centrifuge spin plate and centrifuge the sealed chip at 300*g* for 5 min at 4°C with full acceleration and full brake (Figure 11, below). If you have one chip, balance the centrifuge with the supplied Balance Chip or a blank chip. Proceed to the next protocol (Section V.C) once the centrifugation is complete.

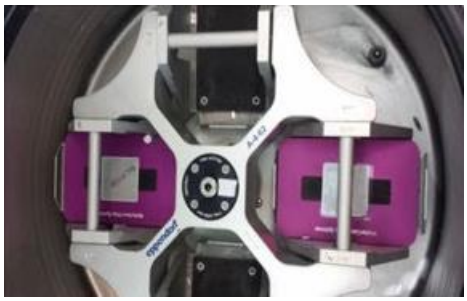


Figure 11. ICELL8 cx Centrifuge Chip Spinner.

19. On the ICELL8 cx Single-Cell System, perform the [Tip Clean] procedure 3–4 times.

C. Protocol: Image Wells

In this protocol, images of all 5,184 nanowells of the ICELL8 350v Chip are acquired. Below is the general imaging workflow. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.E "Scan Chip for Single Cells and Freeze the Chip" for more detailed information if necessary.

1. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.E "Scan Chip for Single Cells and Freeze the Chip" for detailed information about this protocol, with the following guidelines:
   a. Follow Steps 1–5.
   b. For Step 6, the *New stack info* dialog window will display (Figure 12, below). The "Barcodes" field will be grayed out because the barcode file is pre-configured during the Dispense (Section V.B, Figure 8).

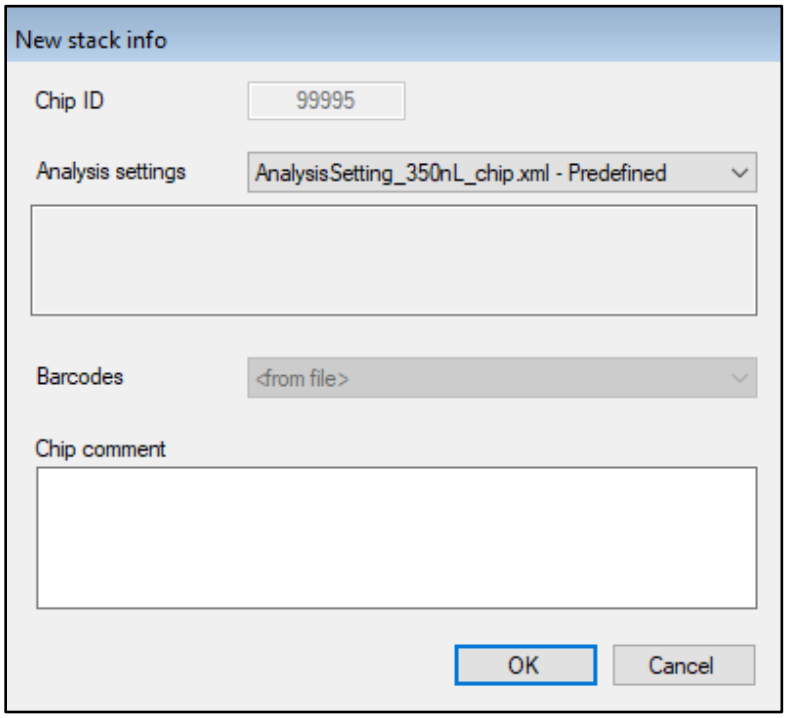


Figure 12. *New stack info* dialog window.

NOTE: If an incorrect barcode file was selected during the Dispense step, it can be corrected during the next step. Continue with the imaging procedure and then proceed to Section V.D.

   c. Continue with the Section X.E procedure from Steps 7–12.
   d. At Step 13, remove the liner from only one side of the Optical Imaging Film (the side that does not have the yellow dot sticker) and apply the exposed sticky side of the imaging film to the chip, sealing carefully with the Plate Seal Applicator (similar to Figure 10, above). Make sure that the film has adhered completely and evenly on the chip. Remove the side with the yellow dot sticker.
2. Place the sealed chip into an empty ICELL8 Chip Holder that has been prechilled at –80°C (Figure 5). The chip holder should click closed and should close evenly, indicating a proper magnetic seal.
3. Freeze chip and chip holder at –80°C for a minimum of 30 min before proceeding to RT (Section V.E, below).

SAFE STOPPING POINT: The chip can be frozen and stored in the chip holder at –80°C for subsequent processing.

D. Protocol: Analyze Nanowells with ICELL8 cx CellSelect Software

Either accept the automatic cell candidacy suggested by CellSelect Software or, if desired, manually inspect the selected nanowells in CellSelect Software to exclude or include one or more candidate wells. Please refer to the ICELL8 cx CellSelect v2.5 Software User Manual for more information about the manual triage process.

NOTES:

- A filter file **MUST** be used in this workflow. As noted in the table footnote in Section II, only enough reagents are included in the kit to dispense to 2,000 wells per chip. Please triage your nanowells accordingly.
- We recommend using automated threshold detection (auto-tune) to determine candidate wells for the RT dispense. Refer to Appendix F of the ICELL8 cx CellSelect v2.5 Software User Manual for more details.
- If you opt out of generating the filter file in CellSelect software, it must be manually created. See Appendix D "Advanced Filter File Configuration" in the ICELL8 cx Single-Cell System User Manual for detailed instructions about constructing the file.

E. Protocol: First Strand cDNA Synthesis by RT (2nd Dispense)

First-strand cDNA is synthesized by the oligo dT primer (SMART-Seq Pro CDS) and followed by the template switching oligo (SMART-Seq Pro Oligonucleotide) for template switching at the 5′ end of transcripts.

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Reagent Kit
    - Nuclease-Free Water, SMART-Seq Pro Lysis Buffer, SMART-Seq Pro RT Buffer, SMART-Seq Pro CDS, SMART-Seq Pro Oligonucleotide, RNase Inhibitor, SMARTScribe Reverse Transcriptase
  - ICELL8 Loading Kit – B
    - Blotting Paper, RC Film
- From the ICELL8 384-Well Source Plate and Seal: a 384-well source plate and plate seal
- A Plate Seal Applicator and film sealing roller

2. Before You Start

- If needed, perform a once-a-day warmup on the ICELL8 cx instrument.
- Set the centrifuge(s) used for spinning the ICELL8 chip and 384-Well Source Plate to 4°C.
- Preheat the thermal cycler lid temperature to 72°C and the block temperature to 47.2°C.
- Preprogram the ICELL8 cx thermal cycler with the RT cycle (Step 11) before the experiment. Run and hold the program so the thermal cycler is preheated before the reaction (Step 9).
- Thaw all reagents on ice except for the enzymes (RNase Inhibitor and SMARTScribe Reverse Transcriptase). The enzymes should remain in storage at –20°C storage until just prior to use and kept on ice at all times. Gently mix and spin down all thawed reagents and enzymes.

3. Procedure

1. Remove the ICELL8 Chip Holder containing the ICELL8 350v Chip from the –80°C freezer. Thaw the chip in the chip holder until it reaches room temperature (about 10 min) to lyse the sample(s).
2. While the chip is thawing, mix all components in the order listed in a 1.5-ml tube and then vortex it briefly.

   NOTE: Remove the RNase Inhibitor from the freezer for this step and add to the mix.

17.6 µl	Nuclease-Free Water
19.0 µl	SMART-Seq Pro Lysis Buffer
100.0 µl	SMART-Seq Pro RT Buffer
2.4 µl	SMART-Seq Pro CDS
10.0 µl	SMART-Seq Pro Oligonucleotide
11.0 µl	RNase Inhibitor
160.0 µl	Total volume

3. Take the thawed chip out of the chip holder. Use a Kimwipe to dry any liquid on the chip surface, and then centrifuge the chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4°C. Keep the chip on ice until ready for RT dispense.

   NOTE: This is not a stopping point; immediately proceed to Steps 4–7 and the RT Master Mix dispense.

4. Add the SMARTScribe Reverse Transcriptase to the mixture from Step 2 to create the RT Master Mix.

   NOTE: Remove the reverse transcriptase from the freezer, gently tap the tube to mix, and add to the RT reaction mix.

160.0 µl	Step 2 Mixture
40.0 µl	SMARTScribe Reverse Transcriptase
200.0 µl	Total volume (RT Master Mix)

   Mix by gently vortexing for 1–2 sec and spin the tube briefly in a minicentrifuge to collect contents.

5. Pipette 48 µl of RT Master Mix into the 384-plate source wells A3, B3, C3, and D3 highlighted in Figure 13, below.

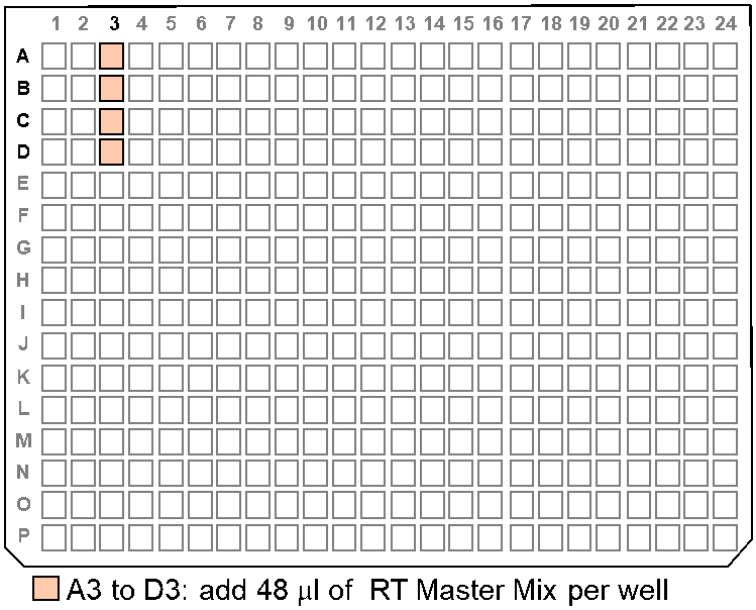


Figure 13. Aliquoting 48 µl of RT Master Mix into each of the orange source wells.

6. Seal the 384-Well Source Plate with a 384-Well Source Plate Seal using the Plate Seal Applicator.
7. Centrifuge the source plate at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.
8. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:
   a. Start in Section X.F, Step 5, with loading the 384-well source plate into the plate nest.
   b. At Step 8, click the [Dispense RT (35 nl filtered)] button (Figure 14).

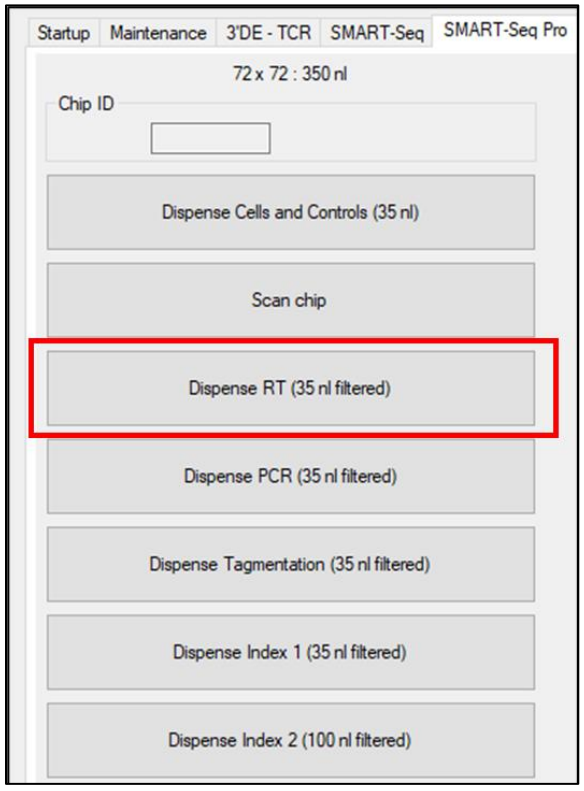


Figure 14. Using CELLSTUDIO software to dispense the RT mix.

c. Follow Steps 9–15 as written in Section X.F.

d. For Step 15, seal with RC Film.

e. For Step 16, centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.

9. During centrifugation, perform the [Tip Clean] procedure 3–4 times on the instrument and prepare the ICELL8 cx thermal cycler. Run and immediately hold the RT program outlined in Step 11, below.

10. Make sure the thermal cycler lid temperature is at 72°C and the block temperature is at 47.2°C. After centrifugation, place the chip into the thermal cycler.

11. Resume the RT program:

47.2°C	5 sec
41.2°C	3 hr
74.1°C	10 sec
70°C	3 min
4°C	forever

SAFE STOPPING POINT: The chip can be left in the thermal cycler at 4°C overnight.

F. Protocol: Full-Length cDNA Amplification by PCR 1 (3rd Dispense)

The first-strand cDNA is used as template for 2nd-strand cDNA synthesis and amplification during PCR 1.

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Reagent Kit
    - Nuclease-Free Water, SMART-Seq Pro PCR 1 Buffer, Terra Direct PCR Polymerase Mix
  - ICELL8 Loading Kit – B
    - Blotting Paper, RC Film
- From the ICELL8 384-Well Source Plate and Seal: a 384-well source plate and plate seal
- A Plate Seal Applicator and film sealing roller

2. Before You Start

- If needed, perform a once-a-day warmup on the ICELL8 cx instrument.
- Set the centrifuges used for spinning the ICELL8 chip and 384-Well Source Plate to 4°C.
- Preheat the thermal cycler lid temperature to 72°C and the block temperature to 47.2°C.
- Preprogram the thermal cycler with PCR 1 (Step 9) before the experiment. Run and hold the program before the reaction (Step 7).
- Thaw all reagents on ice. Gently mix and spin down all thawed reagents.

3. Procedure

1. Remove the chip from the thermal cycler (Section V.E, Step 11). Centrifuge the chip at 3,220*g* for 3 min at 4°C. Keep the chip on ice until ready for PCR 1 dispense.
2. Mix all components in the order listed in a 1.5-ml tube and then vortex gently.

158.0 µl	Nuclease-Free Water
30.0 µl	SMART-Seq Pro PCR 1 Buffer
12.0 µl	Terra Direct PCR Polymerase Mix
200.0 µl	Total volume (PCR 1 Master Mix)

3. Pipette 48 µl of PCR 1 Master Mix into the 384-plate source wells A11, B11, C11, and D11 highlighted in Figure 15, below.

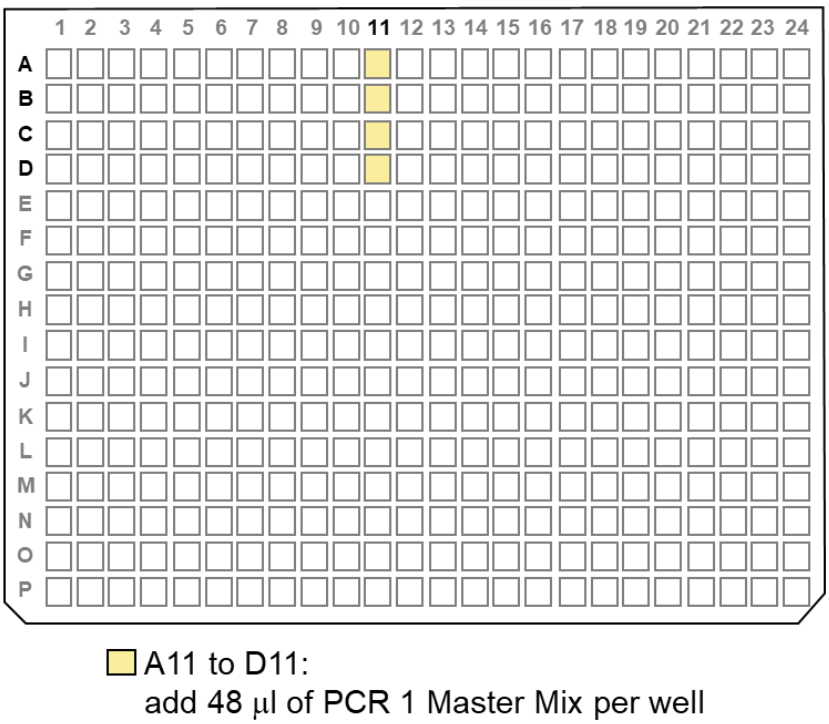


Figure 15. Aliquoting 48 µl of PCR 1 Master Mix into each of the yellow source wells.

4. Seal the 384-Well Source Plate with a 384-Well Source Plate Seal using the Plate Seal Applicator.
5. Centrifuge the source plate at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.

6. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:

   a. Start in Section X.F, Step 5, with loading the 384-well source plate into the plate nest.

   b. At Step 8, click the [Dispense PCR (35 nl filtered)] button (Figure 16).

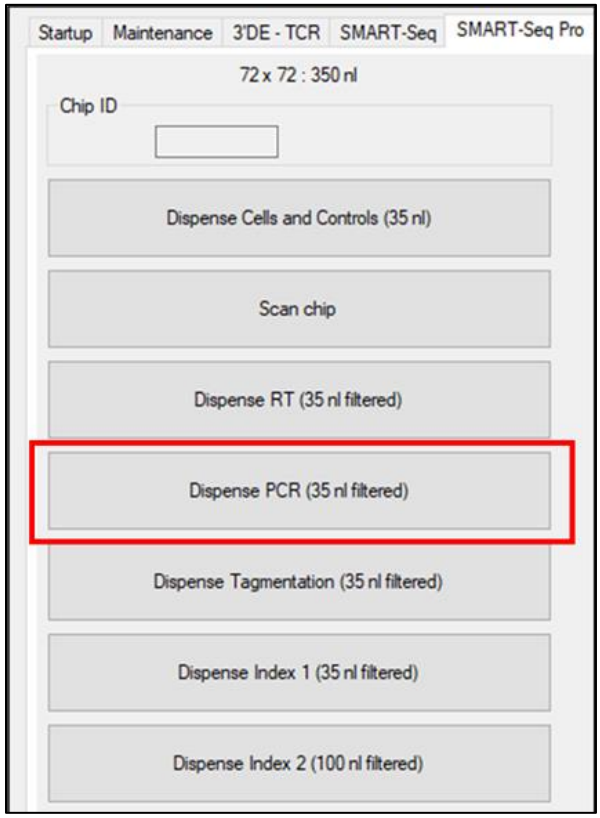


Figure 16. Using CELLSTUDIO software to dispense the PCR mix.

   c. Follow Steps 9–14 as written in Section X.F.

   d. For Step 15, seal with RC Film.

   e. For Step 16, centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.

7. During centrifugation, perform the [Tip Clean] procedure 3–4 times on the instrument and prepare the ICELL8 cx thermal cycler. Run and immediately hold the RT program outlined in Step 9, below.

8. Make sure the thermal cycler lid temperature is at 72°C and the block temperature is at 47.2°C. After centrifugation, place the chip into the thermal cycler.

9. Once the chip is loaded into the thermal cycler, resume the PCR 1 program:

96.5°C	1 min
14 cycles*:	
100°C	10 sec
59.6°C	5 sec
64.8°C	25 sec
72.2°C	9 sec
67.9°C	2min 51 sec
74.1°C	10 sec
70.0°C	9 min 50 sec
4°C	

*While 14 cycles of PCR during the PCR 1 step will provide a good library size and sequencing data for many cell types and nuclei, other sample types may have different optimal cycle numbers during PCR. For example, cells with a high mRNA content (like K-562 cell lines) may require as few as 10 cycles of PCR to reach the proper sequencing library size for Illumina sequencers. Low mRNA content samples may require more than 14 cycles.

Please contact technical support for further assistance if you are seeing issues after library validation and quantification (Section V.O).

SAFE STOPPING POINT: The chip can be left in the thermal cycler at 4°C overnight.

G. Protocol: Preparation of the SMART-Seq Pro Indexing Primer Plate

72 forward indexing primers and 72 reverse indexing primers are resuspended with Index Resuspension Buffer for the 5th and 6th dispenses.

IMPORTANT: Index plate preparation should be performed prior to or directly after the Tagmentation dispense step (Section V.H). It is important to proceed with the forward indexing primer dispense right after the Tagmentation incubation has completed, as that is a safe stopping point.

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Indexing Primer Set - A -OR- SMART-Seq Pro Indexing Primer Set - B
    NOTE: Use the set specific to your run, as selected in Section V.B, Step 11
    - SMART-Seq Pro Indexing Primer Plate, Plate Sealing Film
  - SMART-Seq Pro Reagent Kit
    - SMART-Seq Pro Index Resuspension Buffer
- A Plate Seal Applicator

2. Before You Start

- Thaw the SMART-Seq Pro Index Resuspension Buffer at room temperature

3. Procedure

1. Take the 384-well SMART-Seq Pro Indexing Primer Plate out of the bubble wrap.
   NOTE: Do not open the sealing foil yet.
2. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 3 min at room temperature.
3. Carefully remove sealing foil from the SMART-Seq Pro Indexing Primer Plate.

4. Add resuspension buffer into the primer plate wells using the following scheme:
   - 38.5 µl in to wells A5–P8, A9–D9, and A10–D10 (Figure 17, pink)
   - 80 µl in to wells A13–P16, A17–D17, and A18–D18 (Figure 17, blue)

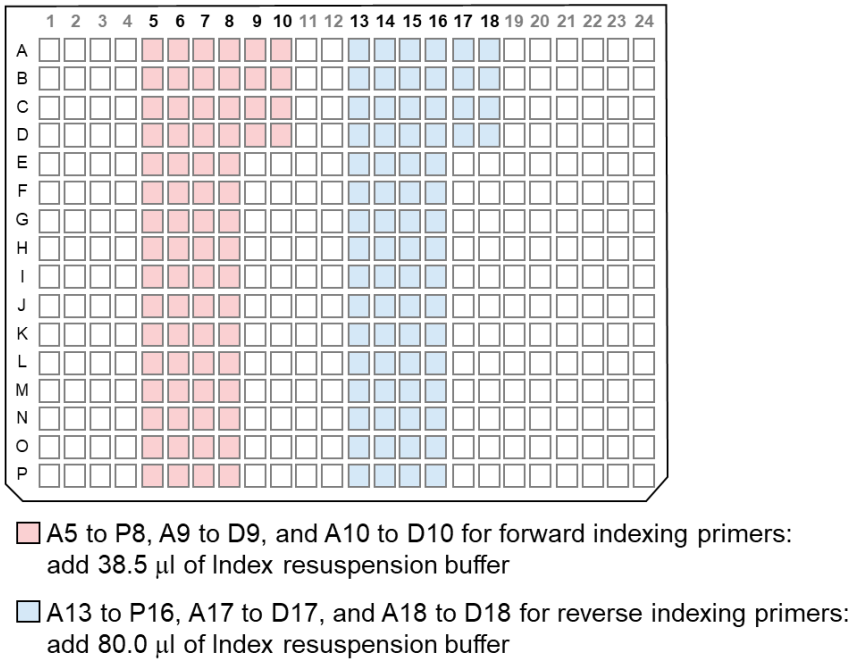


Figure 17. The SMART-Seq Pro Indexing Primer Plate containing 72 forward and 72 reverse indexing primers. Add Index resuspension buffer to wells of forward indexing primers in pink (38.5 µl per well) and reverse indexing primers in blue (80 µl per well). See Appendix A for barcode sequences.

5. Seal the plate with the Plate Sealing Film using a Plate Seal Applicator.
6. Shake the plate with a plate shaker with 3 mm mixing orbit for 5 min at room temperature.
7. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 3 min at room temperature to avoid condensation on the film.
8. Keep the plate at room temperature until the dispense of forward indexing primers (5th dispense, Section V.I).

H. Protocol: Tagmentation (4th Dispense)

Amplified full-length cDNAs are tagmented by Bead-Linked Transposome (BLT).

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Reagent Kit
    - Nuclease-Free Water, SMART-Seq Pro Tagmentation Buffer, $MgCl_2$, Terra Direct PCR Polymerase Mix
  - ICELL8 Loading Kit – B
    - Blotting Paper, RC Film
- From the ICELL8 384-Well Source Plate and Seal: a 384-well source plate and plate seal
- From the ILMN DNA LP (M) Tagmentation: Illumina BLT
- A Plate Seal Applicator and film sealing roller

2. Before You Start

- Preprogram the thermal cycler with the Tagmentation program (Step 8) before the experiment. Run and hold the program before the reaction (Step 6).
- Preheat the lid of the thermal cycler to 40°C and the block temperature to 42.3°C.
- Thaw Nuclease-Free Water, $MgCl_2$, and the Terra Direct PCR Polymerase Mix on ice. Gently mix and spin down the thawed $MgCl_2$ and Terra Direct PCR Polymerase Mix. Keep them on ice during the procedure.

3. Procedure

1. Remove the chip from the thermal cycler (Section V.F, Step 9). Centrifuge the chip at 3,220*g* for 3 min at 4°C. Keep the chip on ice until ready for Tagmentation dispense.
2. Make the Tagmentation Master Mix by mixing all components in the order listed in the table below in a 1.5-ml tube and then vortex gently.

57.75 µl	Nuclease-Free Water
105.6 µl	SMART-Seq Pro Tagmentation Buffer
3.85 µl	$MgCl_2$
30.8 µl	Terra Direct PCR Polymerase Mix
22.0 µl	Illumina BLT
220.0 µl	Total volume (Tagmentation master mix)

3. Pipette 48 µl into the 384-plate source wells M3, N3, O3, and P3 as highlighted in Figure 18, below. Be sure that there are no air bubbles in the wells.

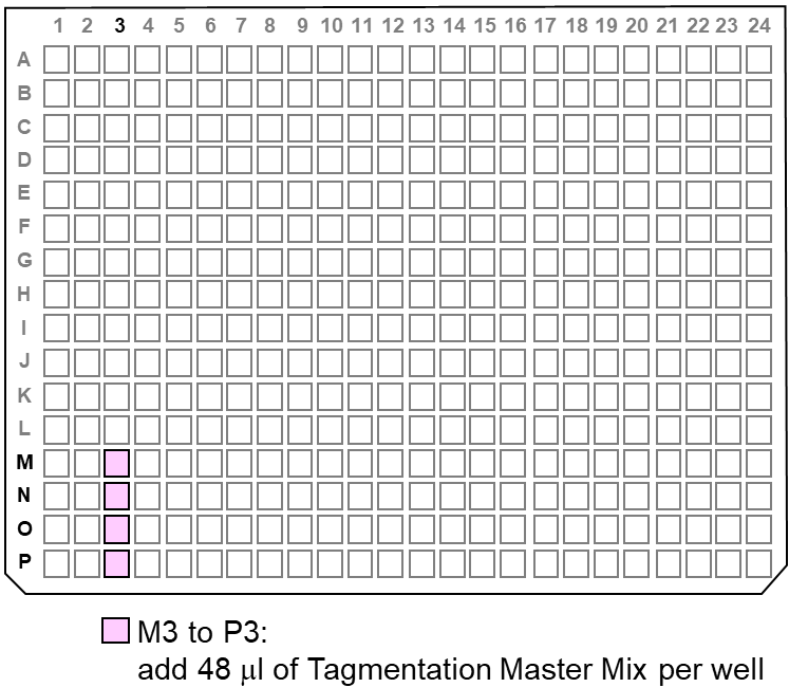


Figure 18. Add Tagmentation Master Mix to the source plate wells M3–P3. Shown in pink.

4. Seal the 384-Well Source Plate with a 384-Well Source Plate Seal using the Plate Seal Applicator. Do not centrifuge the plate.

IMPORTANT: Proceed to the next step quickly to avoid settling of BLT beads. Do not centrifuge the plate. Make sure that there are no air bubbles on the bottom of the wells.

5. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:

   a. Start in Section X.F, Step 5, with loading the 384-well source plate into the plate nest.

   b. At Step 8, click the [Dispense Tagmentation (35 nl filtered)] button (Figure 19).

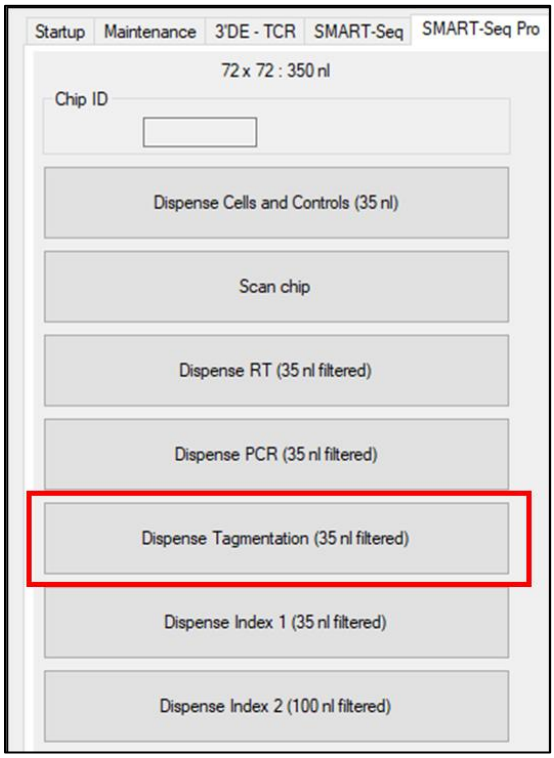


Figure 19. Using CELLSTUDIO software to dispense the Tagmentation mix.

   c. Follow Steps 9–14 as written in Section X.F.

   d. For Step 15, seal with RC Film.

   e. For Step 16, centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.

6. During centrifugation, perform the [Tip Clean] procedure 3–4 times on the instrument and prepare the ICELL8 cx thermal cycler. Run and immediately hold the Tagmentation program outlined in Step 8 below.

7. Make sure the thermal cycler lid temperature is at 40°C and the block temperature is at 42.3°C. After centrifugation, place the chip into the thermal cycler.

8. Once the chip is loaded into the thermal cycler, resume the Tagmentation program:

42.3°C	5 sec
36.6°C	30 min
4°C	forever

IMPORTANT: This is NOT a safe place to stop. After the tagmentation program has reached 4°C, immediately proceed to the next step of the procedure (Section V.I), the dispense of forward indexing primers.

I. Protocol: Forward Indexing Primers (5th Dispense)

72 forward indexing primers are dispensed from a SMART-Seq Pro Indexing Primer Plate (prepared in Section V.G) and used during PCR 2.

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Indexing Primer Plate (prepared in Section V.G)
  - ICELL8 Loading Kit – B
    - Blotting Paper, RC Film
- A razor blade
- Film sealing roller

2. Before You Start

- Set the centrifuge(s) used for spinning the ICELL8 chip and Indexing Primer Plate to 4°C.
- Visually check the prepared Indexing Primer Plate for bubbles in the well. Bubbles should not be present given shaking and centrifuge steps earlier (Section V.G, Steps 6 & 7), but if bubbles are observed:
  - Prior to continuing, centrifuge again for 3,220*g* (minimum 2,600*g*) for 3 min at room temperature -or-
  - Remove the bubbles gently by pipette after removing the seal (Step 3, below)

3. Procedure

1. Remove the chip from the thermal cycler (Section V.H, Step 10). Centrifuge the chip at 3,220*g* for 3 min at 4°C.
2. Using the razor blade, cut the plate seal into two halves between columns 11 and 12.

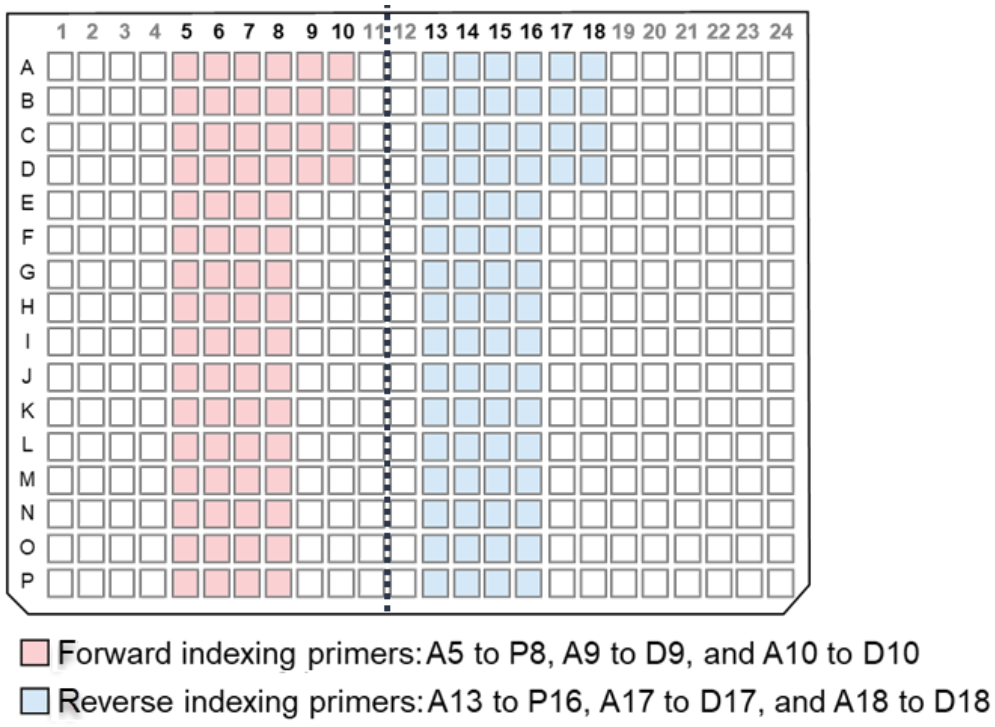


Figure 20. Plate map of the Indexing Primers, indicating the cut line for use in the 5th dispense. The indexes (shown in pink and blue) were reconstituted in Section V.G. See Appendix A for barcode sequences. The razor cut placement is shown by the black dotted line.

3. Remove the plate seal on the left-hand side (columns 1–11) to expose the reconstituted forward indexing primers (Figure 21).

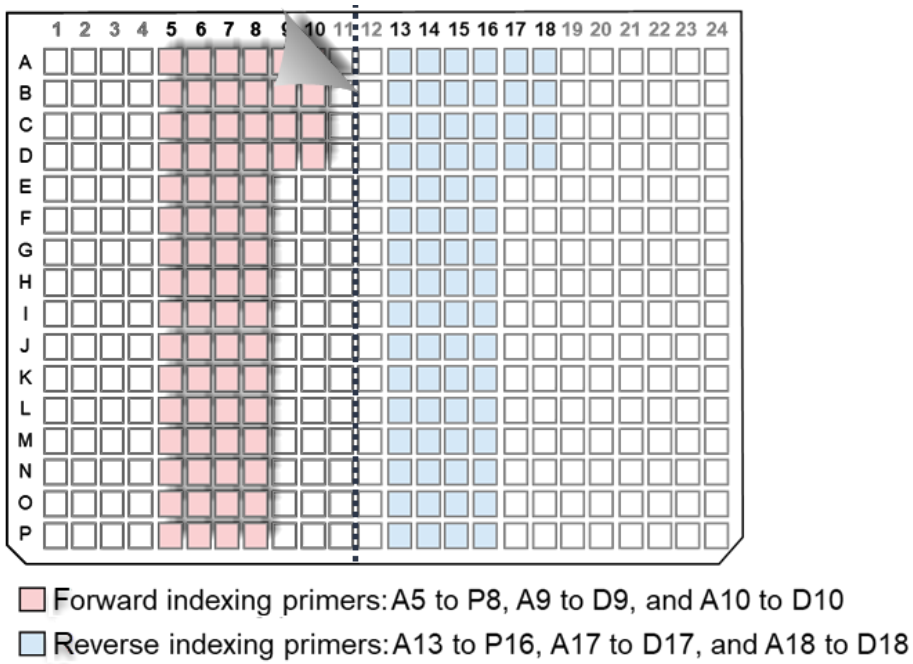


Figure 21. Peeling the plate seal from the forward indexing primers. The forward indexing primers are shown in pink. The plate seal should remain on the right-half of the plate, covering the reverse indexing primers (blue).

4. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:

   a. Start in Section X.F, Step 5, with loading the 384-well source plate into the plate nest.
   b. At Step 8, click the [Dispense Index 1 (35 nl filtered)] button (Figure 22).

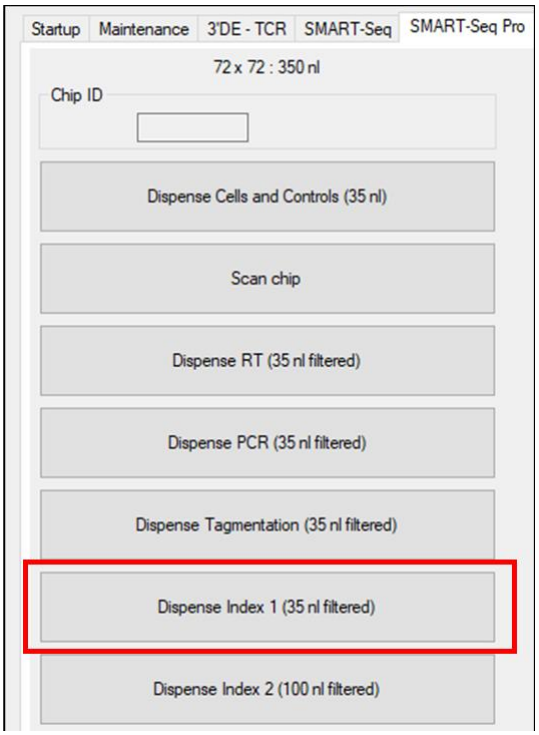


Figure 22. Using CELLSTUDIO software to dispense Index 1.

c. Follow Steps 9–14 as written in Section X.F.

d. For Step 15, seal with RC Film.

e. For Step 16, centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.

5. During centrifugation, perform the [Tip Clean] procedure 3–4 times on the instrument.

IMPORTANT: This is NOT a safe place to stop. Immediately proceed to the next step (Section V.J), the dispense of reverse indexing primers.

J. Protocol: Reverse Indexing Primers (6th Dispense) and PCR 2

72 reverse indexing primers are dispensed from a SMART-Seq Pro Indexing Primer Plate (also used in the previous section) and used during PCR 2.

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Indexing Primer Plate (from Section V.I)
  - ICELL8 Loading Kit – B
    - Blotting Paper, RC Film
- Film sealing roller

2. Before You Start

- Set the centrifuge(s) used for spinning the ICELL8 chip and the Indexing Primer Plate to 4°C.
- Preheat the thermal cycler lid temperature to 72°C and the block temperature to 72.1°C.
- Preprogram the thermal cycler with the PCR 2 program (Step 5) before the experiment. Run and hold the program before the reaction (Step 3).

3. Procedure

1. Remove the rest of the seal from the Indexing Primer Plate to expose the reverse indexing primers (columns 12–24, Figure 23).

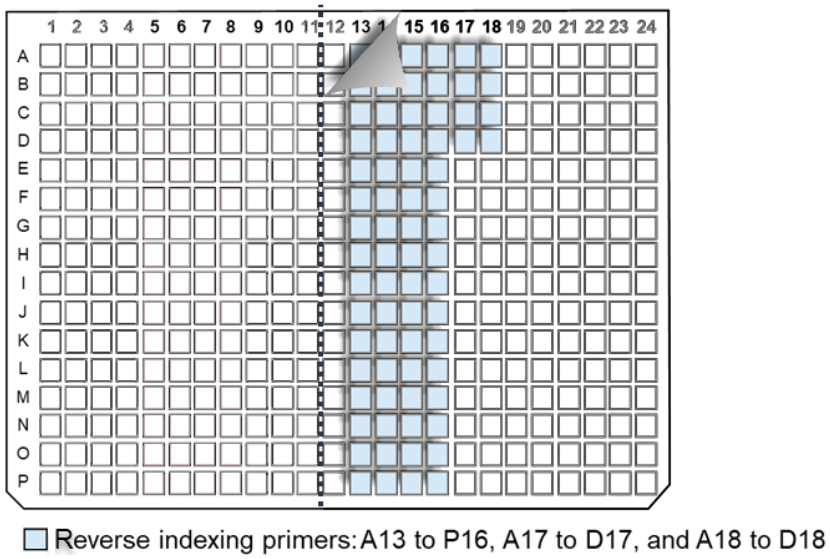


Figure 23. Peeling the plate seal from the reverse indexing primers. Remove the remaining seal from the right-side of the plate.

IMPORTANT: If bubbles were noted in the Indexing Primer Plate wells for the reverse primers during the previous protocol (Section V.I) and the plate was not centrifuged to remove them, remove the bubbles gently by pipette now.

2. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:
   a. Start in Section X.F, Step 5, with loading the 384-well source plate into the plate nest.
   b. At Step 8, click the [Dispense Index 2 (100 nl filtered)] button (Figure 24).

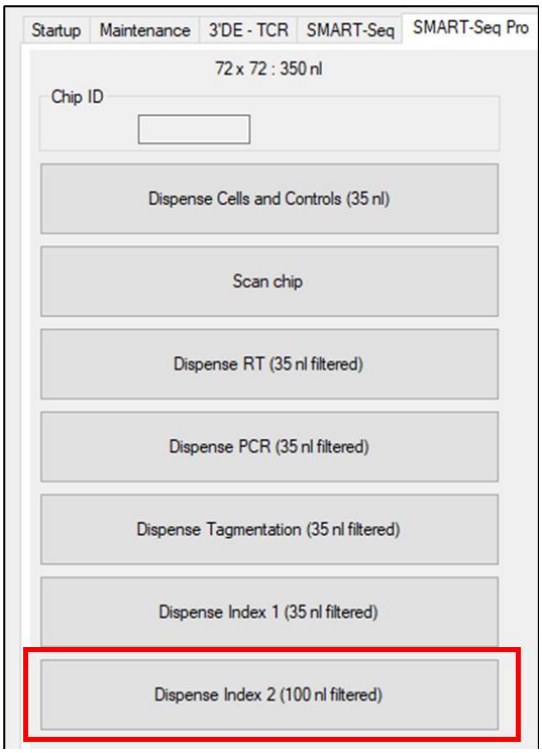


Figure 24. Using CELLSTUDIO software to dispense Index 2.

   c. Follow Steps 9–14 as written in Section X.F.
   d. For Step 15, seal with RC Film.
   e. For Step 16, centrifuge at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.
3. During centrifugation, perform the [Tip Clean] procedure 3–4 times on the instrument and prepare the ICELL8 cx thermal cycler. Run and immediately hold the PCR 2 program outlined in Step 5, below.
4. Make sure the thermal cycler lid temperature is at 72°C and the block temperature is at 72.1°C. After centrifugation, place the chip into the thermal cycler.
5. Once the chip is loaded into the thermal cycler, resume the PCR 2 program:

72.1°C	3 min
98.2°C	18 sec
96.5°C	42 sec
5 cycles:	
100°C	10 sec
54.4°C	5 sec
59.6°C	10 sec
72.2°C	9 sec
67.9°C	1 min 51 sec
4°C	forever

SAFE STOPPING POINT: The chip can be left in the thermal cycler at 4°C overnight.

K. Protocol: Library Extraction from the Chip

This protocol extracts the library amplified by PCR 2 from the chip.

Refer to the ICELL8 cx Single-Cell System User Manual, Section XI "Protocol: Extract library from the chip" for the procedure. The collected volume should be no less than 70% of the theoretical maximum potential volume.

SAFE STOPPING POINT: The eluate can be frozen at –20°C.

L. Protocol: Purification of the Extracted Library

In this protocol, the extracted library is purified twice using a 1:1 proportion of magnetic beads.

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Reagent Kit: Elution Buffer (10 mM)
- Prealiquoted tube of NucleoMag NGS Clean-up and Size Select (see note in Section III)
- Fresh 80% (v/v) ethanol from an anhydrous ethanol stock

2. Before You Start

- Equilibrate a prealiquoted 1.5-ml tube of NucleoMag NGS Clean-up and Size Select beads to room temperature for ~30 min prior to use.
- Immediately prior to 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.
- Prepare fresh 80% (v/v) ethanol before each cleanup (2 ml each). Use an anhydrous ethanol stock.

3. Procedure

1. Transfer half of the volume of the extracted library (from Section V.K) to a 1.5-ml tube and store in a freezer.

   NOTE: This half is reserved in case further analysis is needed.

2. Add 1:1 volume of well-vortexed NucleoMag NGS Clean-up and Size Select beads to the collection tube and the remaining half of the extracted library.

   Example: add 200 µl of magnetic beads to 200 µl of the extracted library.

3. Vortex the tube to mix well.
4. Incubate the tube at room temperature for 8 min to let the DNA bind to the beads.
5. Place the tube in the 1.5-ml tube magnetic separator for ~5 min or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.

   NOTE: During the incubation, if there are beads not against the magnet, use the supernatant to resuspend them and pipette them onto the magnet with the rest of the beads.

6. When the liquid appears clear, keep the tube on the magnetic separator and pipette out the supernatant.

7. Keep the tube on the magnetic separator and add 1 ml of freshly made 80% ethanol to the tube 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.
8. Repeat Step 7 one time.
9. Spin down the tube briefly to collect the liquid at the bottom of the well.
10. Place the tube on the magnetic separator for 30 sec and then remove all remaining ethanol with a pipette.

    NOTE: It is important to make sure all ethanol is removed so the beads elute well and recovery is efficient.

11. Leave the tube at room temperature for ~5 min or longer until the pellet appears dry.

    NOTE: Check the pellet frequently during this time and continue to Step 12 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you'd like additional assistance with this determination.

    https://www.takarabio.com/learning-centers/next-generation-sequencing/faqs-and-tips/rna-seq-tips

    If the beads are overdried, there will be cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be reduced.

12. Once the beads are dried, remove the samples from the magnetic separation device and add 50 µl of Elution Buffer to cover the beads.
13. Incubate the tube with the beads and Elution Buffer at room temperature for 2 min to rehydrate.
14. Mix the pellet by pipetting up and down 10 times to elute DNA from the beads, then put the tube back on the magnetic separator for 1 minute or longer until the solution is completely clear.

    NOTE: During the incubation on the magnet, there may be a small portion of beads not pelleting against the magnet. Use the supernatant to resuspend them by pipetting up and down, and pipette them onto the magnet with the rest of the beads. Continue incubation until there are no beads left in the supernatant.

15. Transfer clear supernatant containing purified library to a fresh 0.2-ml PCR tube.
16. Add 50 µl of NucleoMag NGS Clean-up and Size Select beads to the purified library.
17. Repeat Steps 3–15 with the SMARTer-Seq Magnetic Separator - PCR Strip using 200 µl fresh 80% ethanol washes at Step 7 and 14 µl of Elution Buffer at Step 12. The 0.2-ml PCR tube after Step 15 will be used in library amplification protocol (Section V.M).

M. Protocol: Library Amplification (PCR 3)

This PCR step amplifies and yields the final sequencing-ready library.

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Reagent Kit
    - SeqAmp CB PCR Buffer, 5X Primer Mix, Terra Direct PCR Polymerase Mix

2. Procedure

1. Preprogram the PCR thermal cycler with the PCR 3 program (Step 6). Run and hold the program to preheat the lid and block.
2. Take all required reagents out of the freezer and thaw the 5X Primer Mix.
3. Gently mix each reagent tube and spin down briefly. Store on ice.
4. Add all reagents in the order below to the 0.2 ml PCR tube with the purified library that results from Section V.L:

14 µl	Purified library
25 µl	SeqAmp CB PCR Buffer
10 µl	5X Primer Mix
1 µl	Terra Direct PCR Polymerase Mix
50 µl	Total volume

5. Make sure the PCR thermal cycler lid temperature is at 105°C and the block temperature is at 95°C.
6. Load the tube with the purified library and reagents into the thermal cycler and run the following PCR 3 program:

	95°C	1 min
5 cycles:		
	98°C	10 sec
	60°C	15 sec
	68°C	2 min
	4°C	forever

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

N. Protocol: Purification of the Sequencing-Ready Library

In this protocol, the sequencing-ready library is purified using a 1:1 proportion of magnetic beads.

1. Required Components

- From the SMART-Seq Pro Application Kit - 2 Chip:
  - SMART-Seq Pro Reagent Kit: Elution Buffer (10 mM)
- Prealiquoted tube of NucleoMag NGS Clean-up and Size Select (see note in Section III)
- Fresh 80% (v/v) ethanol from an anhydrous ethanol stock

2. Before You Start

- Equilibrate a prealiquoted 1.5-ml tube of NucleoMag NGS Clean-up and Size Select beads to room temperature for ~30 min prior to use.
- Immediately prior to 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.
- Prepare at least 400 µl fresh 80% (v/v) ethanol before each cleanup. Use an anhydrous ethanol stock.

3. Procedure

1. Remove the PCR tube from the PCR thermal cycler (Section V.M). Spin it down briefly.
2. Add 50 µl (1:1 v/v) of well-vortexed magnetic beads to the tube.
3. Vortex the tube to mix well.
4. Incubate the tube at room temperature for 8 min to let the DNA bind to the beads.
5. Place the tube on Magnetic Stand for ~5 min or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.

   NOTE: During the incubation, if there are beads not against the magnet, use the supernatant to resuspend them and pipette them onto the magnet with the rest of the beads.
6. When the liquid appears clear, keep the tube on the magnetic stand and pipette out the supernatant.
7. Keep the tube on the magnetic stand and add 200 µl of freshly made 80% ethanol to the tube 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.
8. Repeat Step 7 one time.
9. Spin down the tube briefly to collect the liquid at the bottom of the well.
10. Place the tube on the magnetic stand for 30 sec, and then remove all remaining ethanol using a pipette.

    NOTE: It is important to make sure all ethanol is removed so the beads elute well and recovery is efficient.
11. Leave the tube at room temperature for ~5 min or longer until the pellet appears dry.

    NOTE: Check the pellet frequently during this time and continue to Step 12 when it is dry enough. Do not over dry. See note in Section V.L, Step 11 for more details, if needed.
12. Once the beads are dried, add 17 µl Elution Buffer to cover the beads and incubate it at room temperature for 2 min to rehydrate.
13. Mix the pellet by pipetting up and down 10 times to elute DNA from the beads, then put the tube back on the magnetic stand for 1 min or longer until the solution is completely clear.

    NOTE: During the incubation on the magnet, there may be a small portion of beads not pelleting against the magnet. Use the supernatant to resuspend them by pipetting up and down, and pipette them onto the magnet with the rest of the beads. Continue incubation until there are no beads left in the supernatant.
14. Transfer the clear supernatant containing the purified sequencing-ready library to a clean 1.5-ml tube.

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

O. Protocol: Library Validation and Quantification

To determine whether library production and purification were successful, we recommend analyzing and quantifying the final libraries by a bioanalyzer using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626) and by qPCR using the Library Quantification Kit (Takara Bio, Cat. No. 638324). Please refer to the corresponding user manuals for detailed instructions.

Procedure

1. Measure the concentration of the purified sequencing-ready library using 1 µl of the library, a Qubit fluorometer, and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851). Refer to the Qubit dsDNA HS assay kit user manual for sample prep instructions.
2. Based on the Qubit measurement, dilute the amplicon to 0.2 to 2.0 ng/µl of library.

   NOTE: We recommend using several concentrations for the Bioanalyzer (e.g., 0.2, 0.5, 1, and 2 ng/µl).
3. Use 1 µl of each concentration to load the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Chip from Agilent's High Sensitivity DNA Kit for validation. Be careful not to transfer beads with your sample.

   See the user manual for the Agilent High Sensitivity DNA Kit for instructions.
4. Use the bioanalyzer results to determine library quality and average size for qPCR. An example of a typical bioanalyzer profile for an NGS library that has been successfully purified and size selected is shown in Figure 25, below.

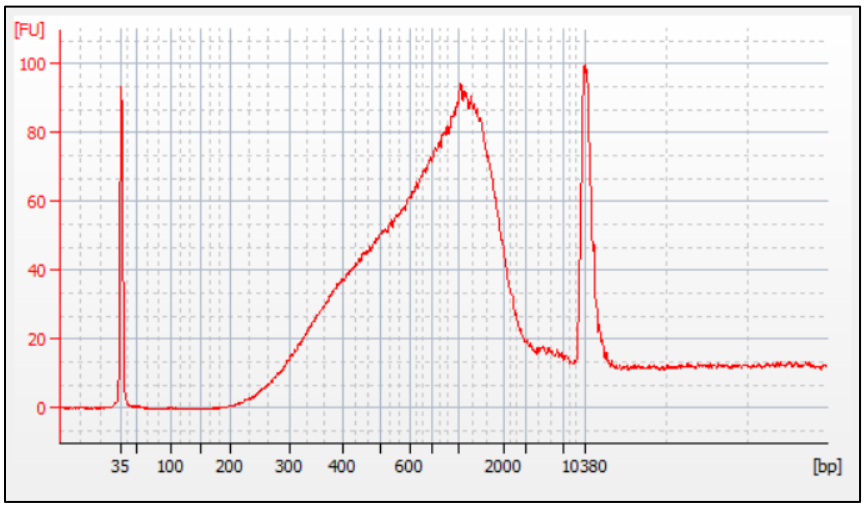


Figure 25. An electropherogram example from a library prepared from PBMC and quantified using the Agilent 2100 Bioanalyzer.

5. Run qPCR using the Library Quantification Kit. Refer to the Library Quantification Kit User Manual for instructions. Use the average size as determined by the bioanalyzer to calculate the molar library concentration.
6. Use the qPCR result to determine the final library quantity for sequencing.
7. Store the sequencing library at –20°C until ready to sequence. See Appendix B for sequencing guidelines.
8. Refer to Appendix C for data analysis guidelines.

Appendix A: SMART-Seq Pro Indexing Primer Set Barcodes

A shortened name is used in the table below for simplicity, instead of using the full name of each index. For example, "Forward Index 1" below stands for "SMART-Seq Pro Indexing Primer Set - A, Forward Index 1".

Sample sheets will be required if Illumina's bcl2fastq Conversion Software is used for demultiplexing the sequencing reads. Please refer to Appendix C for details.

Table 3. SMART-Seq Pro Indexing Primer Set - A and Set B, forward indexing primers 1–72 barcodes. Set A and Set B have identical forward primers.

Forward indexing (i5) primers			Forward indexes (i5) on sample sheet	
Index number	Well position	Index sequence in primers	MiSeq®, HiSeq® 2000/2500, NovaSeq™	MiniSeq™, NextSeq®, HiSeq 3000/4000, HiSeq X
Forward Index 1	A5	AACCAACG	AACCAACG	CGTTGGTT
Forward Index 2	B5	AACGATAG	AACGATAG	CTATCGTT
Forward Index 3	C5	AAGAAGAC	AAGAAGAC	GTCTTCTT
Forward Index 4	D5	AGAGCCTA	AGAGCCTA	TAGGCTCT
Forward Index 5	E5	ATAGTCAA	ATAGTCAA	TTGACTAT
Forward Index 6	F5	CAACTGCA	CAACTGCA	TGCAGTTG
Forward Index 7	G5	CAGCATGA	CAGCATGA	TCATGCTG
Forward Index 8	H5	CCGCCTAA	CCGCCTAA	TTAGGCGG
Forward Index 9	I5	CCTAGCGA	CCTAGCGA	TCGCTAGG
Forward Index 10	J5	CGCAACGG	CGCAACGG	CCGTTGCG
Forward Index 11	K5	CTTGGCCT	CTTGGCCT	AGGCCAAG
Forward Index 12	L5	GCGGTTCT	GCGGTTCT	AGAACCGC
Forward Index 13	M5	GCTTGATG	GCTTGATG	CATCAAGC
Forward Index 14	N5	GGCTCTCT	GGCTCTCT	AGAGAGCC
Forward Index 15	O5	TCAATGCT	TCAATGCT	AGCATTGA
Forward Index 16	P5	TGGTAATT	TGGTAATT	AATTACCA
Forward Index 17	A6	TTCTGAAC	TTCTGAAC	GTTCAGAA
Forward Index 18	B6	AACCAGAA	AACCAGAA	TTCTGGTT
Forward Index 19	C6	AACCGAAC	AACCGAAC	GTTCGGTT
Forward Index 20	D6	AACCGCCA	AACCGCCA	TGGCGGTT
Forward Index 21	E6	AATAAGGA	AATAAGGA	TCCTTATT
Forward Index 22	F6	ACCTTATT	ACCTTATT	AATAAGGT
Forward Index 23	G6	TGGTCCTG	TGGTCCTG	CAGGACCA
Forward Index 24	H6	CAACGAGG	CAACGAGG	CCTCGTTG
Forward Index 25	I6	CCAATGGA	CCAATGGA	TCCATTGG
Forward Index 26	J6	CGCCTATG	CGCCTATG	CATAGGCG
Forward Index 27	K6	CTCTCCAA	CTCTCCAA	TTGGAGAG
Forward Index 28	L6	GGCTTGAA	GGCTTGAA	TTCAAGCC
Forward Index 29	M6	GTTAAGTT	GTTAAGTT	AACTTAAC
Forward Index 30	N6	TCAAGTAT	TCAAGTAT	ATACTTGA
Forward Index 31	O6	TCGCGGAT	TCGCGGAT	ATCCGCGA
Forward Index 32	P6	TGAGTCCT	TGAGTCCT	AGGACTCA
Forward Index 33	A7	AGTTGAAC	AGTTGAAC	GTTCAACT
Forward Index 34	B7	AACCTCAG	AACCTCAG	CTGAGGTT

Forward indexing (i5) primers			Forward indexes (i5) on sample sheet	
Index number	**Well position**	**Index sequence in primers**	**MiSeq®, HiSeq® 2000/2500, NovaSeq™**	**MiniSeq™, NextSeq®, HiSeq 3000/4000, HiSeq X**
Forward Index 35	C7	AACGGTCT	AACGGTCT	AGACCGTT
Forward Index 36	D7	AACTCAAG	AACTCAAG	CTTGAGTT
Forward Index 37	E7	AACTCCGA	AACTCCGA	TCGGAGTT
Forward Index 38	F7	AAGGTTCA	AAGGTTCA	TGAACCTT
Forward Index 39	G7	AATTCGGT	AATTCGGT	ACCGAATT
Forward Index 40	H7	ACCAGACC	ACCAGACC	GGTCTGGT
Forward Index 41	I7	ACTTAGTA	ACTTAGTA	TACTAAGT
Forward Index 42	J7	AGCGGCAA	AGCGGCAA	TTGCCGCT
Forward Index 43	K7	AGGTCGAA	AGGTCGAA	TTCGACCT
Forward Index 44	L7	AGTCTGGA	AGTCTGGA	TCCAGACT
Forward Index 45	M7	ATAATGGT	ATAATGGT	ACCATTAT
Forward Index 46	N7	ATCCATTG	ATCCATTG	CAATGGAT
Forward Index 47	O7	ATGAATCT	ATGAATCT	AGATTCAT
Forward Index 48	P7	CAAGATTG	CAAGATTG	CAATCTTG
Forward Index 49	A8	CCGAATTG	CCGAATTG	CAATTCGG
Forward Index 50	B8	CCGGAGTT	CCGGAGTT	AACTCCGG
Forward Index 51	C8	CCTTCAGG	CCTTCAGG	CCTGAAGG
Forward Index 52	D8	CGAATATT	CGAATATT	AATATTCG
Forward Index 53	E8	CGGAGACT	CGGAGACT	AGTCTCCG
Forward Index 54	F8	CGTAGGCA	CGTAGGCA	TGCCTACG
Forward Index 55	G8	GAACTAAG	GAACTAAG	CTTAGTTC
Forward Index 56	H8	GAAGCTCG	GAAGCTCG	CGAGCTTC
Forward Index 57	I8	GACTATTG	GACTATTG	CAATAGTC
Forward Index 58	J8	GAGTAACG	GAGTAACG	CGTTACTC
Forward Index 59	K8	GCAGTCCA	GCAGTCCA	TGGACTGC
Forward Index 60	L8	GCTCAAGG	GCTCAAGG	CCTTGAGC
Forward Index 61	M8	GGATATCG	GGATATCG	CGATATCC
Forward Index 62	N8	GGTCAGAT	GGTCAGAT	ATCTGACC
Forward Index 63	O8	GTAAGAAG	GTAAGAAG	CTTCTTAC
Forward Index 64	P8	GTAGAAGT	GTAGAAGT	ACTTCTAC
Forward Index 65	A9	GTATCTGA	GTATCTGA	TCAGATAC
Forward Index 66	B9	GTCATCTA	GTCATCTA	TAGATGAC
Forward Index 67	C9	GTCCGCAA	GTCCGCAA	TTGCGGAC
Forward Index 68	D9	GTTCAATA	GTTCAATA	TATTGAAC
Forward Index 69	A10	TAACGTCG	TAACGTCG	CGACGTTA
Forward Index 70	B10	TCGGAACG	TCGGAACG	CGTTCCGA
Forward Index 71	C10	CATTCTAC	CATTCTAC	GTAGAATG
Forward Index 72	D10	TTACTTCT	TTACTTCT	AGAAGTAA

Table 4. SMART-Seq Pro Indexing Primer Set - A, reverse indexing primers 1–72 barcodes.

Reverse indexing (i7) primers			Reverse indexes (i7) on sample sheet
Index number	**Well position**	**Index sequence in primers**	**MiSeq, MiniSeq, NextSeq, HiSeq 2000/2500, HiSeq 3000/4000, HiSeq X, NovaSeq**
Reverse Index 1	A13	AACCGGTT	AACCGGTT
Reverse Index 2	B13	AACCTAGA	TCTAGGTT
Reverse Index 3	C13	AAGACCAG	CTGGTCTT
Reverse Index 4	D13	AGAACGAC	GTCGTTCT
Reverse Index 5	E13	AGAACTCT	AGAGTTCT
Reverse Index 6	F13	ATTCAGCT	AGCTGAAT
Reverse Index 7	G13	CATACGTC	GACGTATG
Reverse Index 8	H13	CCATTATG	CATAATGG
Reverse Index 9	I13	CCTTGAAT	ATTCAAGG
Reverse Index 10	J13	CGGAATCA	TGATTCCG
Reverse Index 11	K13	CTAGTTGC	GCAACTAG
Reverse Index 12	L13	CTCGCGTA	TACGCGAG
Reverse Index 13	M13	CTTGAGTC	GACTCAAG
Reverse Index 14	N13	GAACGTAT	ATACGTTC
Reverse Index 15	O13	GACTGCGG	CCGCAGTC
Reverse Index 16	P13	GCGTACGG	CCGTACGC
Reverse Index 17	A14	GCTTCTCC	GGAGAAGC
Reverse Index 18	B14	GGAGGCTC	GAGCCTCC
Reverse Index 19	C14	GGAGTATG	CATACTCC
Reverse Index 20	D14	GTCGCTAG	CTAGCGAC
Reverse Index 21	E14	TCGTTCGA	TCGAACGA
Reverse Index 22	F14	TCTCTACC	GGTAGAGA
Reverse Index 23	G14	TGGCGACG	CGTCGCCA
Reverse Index 24	H14	TTCGATGA	TCATCGAA
Reverse Index 25	I14	TTGATCCA	TGGATCAA
Reverse Index 26	J14	AACCTGCC	GGCAGGTT
Reverse Index 27	K14	AACGCATC	GATGCGTT
Reverse Index 28	L14	AACGCCAT	ATGGCGTT
Reverse Index 29	M14	AACGCGCA	TGCGCGTT
Reverse Index 30	N14	AAGAATGG	CCATTCTT
Reverse Index 31	O14	AAGACGCT	AGCGTCTT
Reverse Index 32	P14	ACCAACCG	CGGTTGGT
Reverse Index 33	A15	ACCGAATG	CATTCGGT
Reverse Index 34	B15	ACTCGCTA	TAGCGAGT
Reverse Index 35	C15	AGAAGAGC	GCTCTTCT
Reverse Index 36	D15	AGAATCTC	GAGATTCT
Reverse Index 37	E15	ATGCTTAG	CTAAGCAT
Reverse Index 38	F15	CAGACCTT	AAGGTCTG
Reverse Index 39	G15	CCGCTAGG	CCTAGCGG
Reverse Index 40	H15	CCGGTTAG	CTAACCGG
Reverse Index 41	I15	CCTCGACG	CGTCGAGG
Reverse Index 42	J15	CGAAGCTG	CAGCTTCG

Reverse indexing (i7) primers			Reverse indexes (i7) on sample sheet
Index number	Well position	Index sequence in primers	MiSeq, MiniSeq, NextSeq, HiSeq 2000/2500, HiSeq 3000/4000, HiSeq X, NovaSeq
Reverse Index 43	K15	CGACCGCG	CGCGGTCG
Reverse Index 44	L15	CGTCATAA	TTATGACG
Reverse Index 45	M15	CTAGGAGA	TCTCCTAG
Reverse Index 46	N15	CTATTCAT	ATGAATAG
Reverse Index 47	O15	CTCTACTT	AAGTAGAG
Reverse Index 48	P15	CTGATTGA	TCAATCAG
Reverse Index 49	A16	CTTCGTTA	TAACGAAG
Reverse Index 50	B16	GAAGCAGC	GCTGCTTC
Reverse Index 51	C16	GAATAGGC	GCCTATTC
Reverse Index 52	D16	GCTCTGCT	AGCAGAGC
Reverse Index 53	E16	GGAGCGCA	TGCGCTCC
Reverse Index 54	F16	GGCGGTAT	ATACCGCC
Reverse Index 55	G16	GGTAACGC	GCGTTACC
Reverse Index 56	H16	GGTACGCC	GGCGTACC
Reverse Index 57	I16	GGTAGAAT	ATTCTACC
Reverse Index 58	J16	GGTTAGTC	GACTAACC
Reverse Index 59	K16	GTCTCGCG	CGCGAGAC
Reverse Index 60	L16	GTTCTACG	CGTAGAAC
Reverse Index 61	M16	TAGTATCT	AGATACTA
Reverse Index 62	N16	TAGTTAGG	CCTAACTA
Reverse Index 63	O16	TATTGCGC	GCGCAATA
Reverse Index 64	P16	TCAGTTAA	TTAACTGA
Reverse Index 65	A17	TCCGTATA	TATACGGA
Reverse Index 66	B17	TCCTGAGA	TCTCAGGA
Reverse Index 67	C17	TCGTCGCC	GGCGACGA
Reverse Index 68	D17	TGGCGTTA	TAACGCCA
Reverse Index 69	A18	TGGTATGA	TCATACCA
Reverse Index 70	B18	TTAAGCGT	ACGCTTAA
Reverse Index 71	C18	TTCGCGAC	GTCGCGAA
Reverse Index 72	D18	TTGCATAT	ATATGCAA

Table 5. SMART-Seq Pro Indexing Primer Set - B, reverse indexing primers 73–144 barcodes.

Reverse indexing (i7) primers			Reverse indexes (i7) on sample sheet
Index number	Well position	Index sequence in primers	MiSeq, MiniSeq, NextSeq, HiSeq 2000/2500, HiSeq 3000/4000, HiSeq X, NovaSeq
Reverse Index 73	A13	AACTCTCC	GGAGAGTT
Reverse Index 74	B13	AACTGATA	TATCAGTT
Reverse Index 75	C13	AAGAGAAT	ATTCTCTT
Reverse Index 76	D13	AAGTTGGA	TCCAACTT
Reverse Index 77	E13	ACGAACTT	AAGTTCGT
Reverse Index 78	F13	ACGCAACC	GGTTGCGT

Reverse indexing (i7) primers			Reverse indexes (i7) on sample sheet
Index number	**Well position**	**Index sequence in primers**	**MiSeq, MiniSeq, NextSeq, HiSeq 2000/2500, HiSeq 3000/4000, HiSeq X, NovaSeq**
Reverse Index 79	G13	ACGGAGGA	TCCTCCGT
Reverse Index 80	H13	ACTTACGT	ACGTAAGT
Reverse Index 81	I13	ACTTCTAA	TTAGAAGT
Reverse Index 82	J13	AGACGGAA	TTCCGTCT
Reverse Index 83	K13	AGAGGTCC	GGACCTCT
Reverse Index 84	L13	AGATGCGA	TCGCATCT
Reverse Index 85	M13	AGCAAGGC	GCCTTGCT
Reverse Index 86	N13	AGGCCTTG	CAAGGCCT
Reverse Index 87	O13	AGGTTATG	CATAACCT
Reverse Index 88	P13	AGTATAGT	ACTATACT
Reverse Index 89	A14	ATGGTACT	AGTACCAT
Reverse Index 90	B14	ATTACGAA	TTCGTAAT
Reverse Index 91	C14	CATAACGT	ACGTTATG
Reverse Index 92	D14	CATTAGAA	TTCTAATG
Reverse Index 93	E14	CCAGGCAT	ATGCCTGG
Reverse Index 94	F14	CCGTACTA	TAGTACGG
Reverse Index 95	G14	CGCGCTCA	TGAGCGCG
Reverse Index 96	H14	CGCGGTTG	CAACCGCG
Reverse Index 97	I14	CGCTCTGG	CCAGAGCG
Reverse Index 98	J14	CGGCTAAC	GTTAGCCG
Reverse Index 99	K14	CGTCCTCC	GGAGGACG
Reverse Index 100	L14	CGTTGCGG	CCGCAACG
Reverse Index 101	M14	CTACGTCC	GGACGTAG
Reverse Index 102	N14	CTATCAAG	CTTGATAG
Reverse Index 103	O14	CTCGAGGT	ACCTCGAG
Reverse Index 104	P14	CTCGTCCA	TGGACGAG
Reverse Index 105	A15	CTCTGGCC	GGCCAGAG
Reverse Index 106	B15	CTGCAATG	CATTGCAG
Reverse Index 107	C15	CTGCCTCG	CGAGGCAG
Reverse Index 108	D15	CTTCATGG	CCATGAAG
Reverse Index 109	E15	GAAGTCGT	ACGACTTC
Reverse Index 110	F15	GAATCATG	CATGATTC
Reverse Index 111	G15	GACGGATT	AATCCGTC
Reverse Index 112	H15	GACGTACG	CGTACGTC
Reverse Index 113	I15	GAGGCCAA	TTGGCCTC
Reverse Index 114	J15	GATATATT	AATATATC
Reverse Index 115	K15	GCATTGGT	ACCAATGC
Reverse Index 116	L15	GCGAAGCA	TGCTTCGC
Reverse Index 117	M15	GCGCCTTC	GAAGGCGC
Reverse Index 118	N15	GCGCTCTT	AAGAGCGC
Reverse Index 119	O15	GCTAAGAC	GTCTTAGC
Reverse Index 120	P15	GGAATTGG	CCAATTCC
Reverse Index 121	A16	GGCAGGAC	GTCCTGCC

Reverse indexing (i7) primers			Reverse indexes (i7) on sample sheet
Index number	Well position	Index sequence in primers	MiSeq, MiniSeq, NextSeq, HiSeq 2000/2500, HiSeq 3000/4000, HiSeq X, NovaSeq
Reverse Index 122	B16	GGTACCAA	TTGGTACC
Reverse Index 123	C16	GGTCCTAG	CTAGGACC
Reverse Index 124	D16	GTAATCCG	CGGATTAC
Reverse Index 125	E16	GTCCTAAC	GTTAGGAC
Reverse Index 126	F16	GTTCAGGC	GCCTGAAC
Reverse Index 127	G16	TAATACGT	ACGTATTA
Reverse Index 128	H16	TACGAGTT	AACTCGTA
Reverse Index 129	I16	TACGGTAC	GTACCGTA
Reverse Index 130	J16	TATATGCC	GGCATATA
Reverse Index 131	K16	TATATTGA	TCAATATA
Reverse Index 132	L16	TCAGGCGA	TCGCCTGA
Reverse Index 133	M16	TCATGAAG	CTTCATGA
Reverse Index 134	N16	TCCGACCT	AGGTCGGA
Reverse Index 135	O16	TCGAATAA	TTATTCGA
Reverse Index 136	P16	TCGGTCAT	ATGACCGA
Reverse Index 137	A17	TCTAGAGG	CCTCTAGA
Reverse Index 138	B17	TCTCCGTC	GACGGAGA
Reverse Index 139	C17	TGCGGACT	AGTCCGCA
Reverse Index 140	D17	TTAACCAA	TTGGTTAA
Reverse Index 141	A18	TTACCATT	AATGGTAA
Reverse Index 142	B18	TTATCGTC	GACGATAA
Reverse Index 143	C18	TTCATACG	CGTATGAA
Reverse Index 144	D18	TTCCGGTC	GACCGGAA

Appendix B: Guidelines for Library Sequencing

A. Final Sequencing Library Structure

Libraries generated using the SMART-Seq Pro application have standard Illumina adapters and indexes. The unique combinations of indexes (i5 and i7) are required to discriminate between cells from different wells. Therefore, dual indexes (2 x 8 nt) must be sequenced. This unique combination of i5 and i7 indexes per well in the nanochip is generated using 72 i5 (forward) and 72 i7 (reverse) indexes. The structure of a final sequencing library is shown in Figure 26, below.

For the complete list of sequences of forward and reverse indexing primers, please refer to Appendix A.

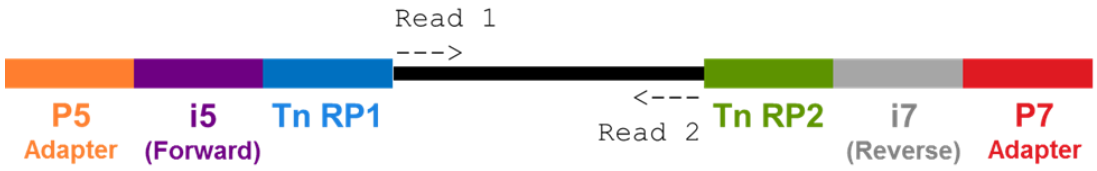


Figure 26. Structure of a final sequencing library. The final library is composed of Tn RP1 and Tn RP2 (Illumina Nextera® read primer 1 and 2 sequences added by the BLT), i5 and i7 indexes, and P5 and P7 adapters.

B. Compatible Illumina Platforms

Full-length libraries resulting from the SMART-Seq Pro application can be run on the following Illumina platforms with either single-end or paired-end sequencing:

- MiSeq
- MiniSeq
- NextSeq series
- HiSeq 2000/2500, 3000/4000
- HiSeq X series (Rapid Run and High Output)
- NovaSeq 6000

Takara Bio has validated libraries using the MiSeq, MiniSeq, NextSeq and NovaSeq.

C. Recommendations for Library Sequencing

Refer to Illumina documentation for instructions on denaturing and diluting libraries.

1. Loading Concentration and PhiX Recommendations

For NextSeq instruments, we recommend a loading concentration of 1.4 pM as a good starting point. Refer to information about individual Illumina instruments on Illumina's website for loading guidelines.

Libraries generated with this protocol do not require the inclusion of PhiX. However, some sample types may not display the well-balanced nucleotide diversity required for base calling. If you are not sure about the performance of your sample, then please include PhiX. Follow Illumina guidelines on how to denature, dilute, and combine a PhiX control library with your own pool of libraries. Make sure to use a fresh and reliable stock of the PhiX control library.

2. Sequencing Depth Recommendations

The sequencing depth is dependent on the purpose of the study. Identification of cell heterogeneity can be achieved with shallow sequencing of about 50,000 paired reads/cell. However, identification of rare genes, alternative splicing transcript isoforms, fusion genes, and immune profiling may need higher sequencing depth. We usually recommend more than 300,000 paired reads per cell.

The following table provides some guidelines for an example in which 1,000 single cells are selected in the experiment. Please refer to the "Illumina sequencing platforms" page for more details: https://www.illumina.com/systems/sequencing-platforms.html.

Table 6. Sequencing depth recommendations.

Platform	Maximum reads per run (millions)	Flow cell type	# of flow cells per run	# of lanes per flow cell	Maximum reads per lane (millions)	Reads per cells* (thousands)†	Platform recommendation‡
MiSeq	25	–	1	1	-	25	No
MiniSeq	25	–	1	1	-	25	No
NextSeq Series (High output)	400	–	1	4	-	400	Yes
HiSeq 4000	5,000	–	1 or 2	8	312.5	312.5	Yes
HiSeq X Series	6,000	–	1 or 2	8	375	375	Yes
NovaSeq 6000	3,200	S1	1 or 2	2	800	800	Yes
NovaSeq 6000	8,200	S2	1 or 2	2	2,050	2,050	Yes
NovaSeq 6000	20,000	S4	1 or 2	4	2,500	2,500	Yes

*We recommend >300,000 reads per cell. However, this number depends on the goal of the experiment.

†Formula used: (# of reads per cell) = (maximum reads per run or lane) / (# of cells).

‡The platform recommendation is based on our recommendation of 300,000 reads per cell. Therefore, it varies based on the number of single cells sequenced as well as on the purpose of the study.

3. Sequencing Run Parameters

SMART-Seq Pro libraries use standard Illumina sequencing primers and do not need custom primers. Dual indexes (2 x 8 cycles) ***must*** be sequenced, and we recommend the number of cycles in Table 6, below. However, cycles of Read 1 and Read 2 can be flexible (e.g., 2 x 100, 2 x 150, and 2 x 250) depending on your experiments.

Single-end sequencing can also be performed with SMART-Seq Pro full-length libraries (e.g., 1 x 100, 1 x 150, and 1 x 250).

Table 7. Sequencing run parameters.

Sequencing read	Recommended # of cycles
Read 1	75
i7 Index	8
i5 Index	8
Read 2	75

Appendix C: Demultiplexing and Data Analysis

There are two options to demultiplex your sequencing data and generate result files.

1. **Use Cogent™ NGS Analysis Pipeline, Takara Bio's single-cell RNA-seq analysis pipeline**

   Cogent NGS Analysis Pipeline (CogentAP) is an end-to-end analysis software that takes sequencing data as input and can perform demultiplexing, alignment, counting, and report generation (in HTML format) with the most commonly used statistics and plots in single-cell analysis. However, if desired, it can be used for de-multiplexing alone. This choice can be made while launching the pipeline.

   Significant advantages of using CogentAP software:

   - Perform end-to-end analysis or just de-multiplexing with ease
   - Provide the well list generated during the experiment (using CellSelect software on the ICELL8 cx instrument) as the input without the need to manually handle barcodes and sample information
   - CogentAP software automatically performs a reverse-complement check and correction of barcodes
   - There is no limit on the number of barcodes that can be processed
   - Output files generated by CogentAP can be loaded into Cogent NGS Discovery Software (CogentDS), our software for extended analysis, to create UMAP analysis popular in scRNA-seq

   Please make sure to keep your version of CogentAP and CogentDS up to date to ensure full compatibility with CELLSTUDIO software. Installation and upgrade information can be found in the user manuals for both CogentAP and CogentDS, available at takarabio.com/icell8-software.

2. **Use Illumina's bcl2fastq Conversion Software**

   After installing bcl2fastq Conversion Software for demultiplexing, a custom sample sheet needs to be generated to use the software. Sample sheets can be created by Illumina Experiment Manager https://support.illumina.com/sequencing/sequencing_software/experiment_manager.html.

   Our indexes are unique sequences that are different from Illumina indexes. Before demultiplexing, the sample sheets created in the Illumina Experiment Manager (above) must be customized with our forward and reverse indexes (see Appendix A).

   Please note that forward indexes (i5) in the sample sheet are dependent on Illumina sequencers, and on some sequencers, the indexes must be entered in the sample sheet in the reverse-complement. The indexes of the wells selected during an experiment can be obtained from the well-list file generated by CellSelect software. Additionally, some versions of bcl2fastq software handle only ~400 barcodes at a time, requiring it to be run multiple times with multiple sample sheets when more than 400 wells are selected. Please see Illumina's User Guide for more details (https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html).

   The resulting demultiplexed FASTQ files can then be analyzed by other compatible custom pipelines.

Appendix D: Reduced Cell Dispense Volumes

In the event you are starting with a smaller sample size (e.g., a low number of cells), it is possible to reduce the volume of the cell dispense mixture.

Reduce the volume of the diluted stained cell suspension to 500 µl (from 1 ml). 60 µl of the suspension is then distributed to each of the eight source wells. See Table 7 for the formulas to follow.

Table 8. Modified sample preparation guidelines for reduced cell-dispense volumes.

Components	Negative control	Positive control	Diluted stained cell suspension	Volume per source well (for each sample)*
Second Diluent (100X)	1.0 µl	1.0 µl	5 µl	0.8 µl
BSA (1%)	1.0 µl	1.0 µl	5 µl	0.8 µl
RNase Inhibitor (40 U/µl)	1.0 µl	1.0 µl	5 µl	0.8 µl
Control K-562 RNA (1 ng/µl)	–	5.7 µl	–	–
Stained cell suspension	–	–	Dilute to 1.4 cells/35 nl*	Dilute to 1.4 cells/35 nl*
1X PBS (Ca^{2+} and Mg^{2+} free)	97 µl	91.3 µl	Up to 500.0 µl	Up to 80 µl
Total	100 µl	100 µl	500 µl†	80 µl‡

*Sufficient stained-cell suspension should be included such that the final concentration in the 500-µl volume of diluted stained cell suspension is 1.4 cells/35 nl (i.e., 40,000 cells/ml).

†The 500-µl total volume of diluted stained cell suspension is sufficient for distributing 60 µl of cell suspension in each of eight source wells.

‡The 80-µl total volume of diluted stained cell suspension is sufficient for distributing 60 µl of cell suspension into a single source well. Multiply appropriately for the number of source wells used for each sample type.

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

This document has been reviewed and approved by the Quality Department.