Takara Bio USA, Inc.

SMART-Seq® ICELL8® Application Kit User Manual

Cat. Nos. 640000, 640219, 640220 & 640221
(021225)

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I. Introduction

High-throughput full-length analysis of single cells

The **SMART-Seq ICELL8 Application Kit** (Cat. Nos. 640219, 640220 & 640221) protocol enables Illumina® sequencing and full-length transcriptome analysis of single cells isolated on the **ICELL8 Single-Cell System** (Cat. No. 640000).

The kit workflow (Figure 1, below) begins with staining and dilution of cell samples and the preparation of positive and negative controls, followed by the dispensing of the cells and controls into the 5,184 nanowells of the ICELL8 250v Chip using the ICELL8 MultiSample NanoDispenser (ICELL8 MSND). As many as eight different samples can be analyzed in a single run, and the dispensing process is completed in approximately 15 min. During this time, cells are maintained in the humidity- and temperature-controlled environment provided by the ICELL8 MSND.

Following the dispensing of the samples and controls, the ICELL8 Imaging System and Micro-Manager software are used to acquire images of the 5,184 nanowells with both Hoechst and Texas Red filters, enabling visualization of Hoechst 33342 and propidium iodide staining, respectively. (Hoechst staining is used to visualize and identify candidate cells based on size/morphology criteria, while propidium iodide staining is used to identify dead cells). Following image acquisition, images are analyzed either automatically or manually using CellSelect® Software, which identifies nanowells containing single, viable cells that meet user-defined criteria. Upon identification and selection of candidate nanowells, CellSelect Software generates a file that will be used to direct the dispensing of RT-PCR reagents to selected nanowells for downstream cDNA synthesis and amplification steps.

After freezing the ICELL8 chip, the chip is returned to the ICELL8 MSND and RT-PCR reagents are distributed to nanowells selected by the software. The ICELL8 chip is then transferred to the ICELL8 Chip Cycler, which is programmed to perform first-strand synthesis and amplification of cDNA in a single run. First-strand cDNA is synthesized by the oligo dT primer (SMART-Seq ICELL8 CDS) and is followed by the template-switching oligo (SMART-Seq ICELL8 Oligonucleotide) and addition of an adapter to the 3′ ends of full-length cDNAs.

The first-strand cDNA is used as template for 2nd-strand cDNA synthesis, in which the added adapter serves as a priming site, allowing for unbiased amplification of full-length cDNA. The full-length cDNA is tagmented by TDE1 (Illumina Tagment DNA Enzyme), and then the tagmented cDNA is amplified using forward and reverse indexing primers. The resulting library is extracted, purified, and amplified to yield the final sequencing-ready library. Following subsequent purification, and validation steps, libraries are ready for sequencing on Illumina platforms.

By leveraging the capabilities of the ICELL8 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.

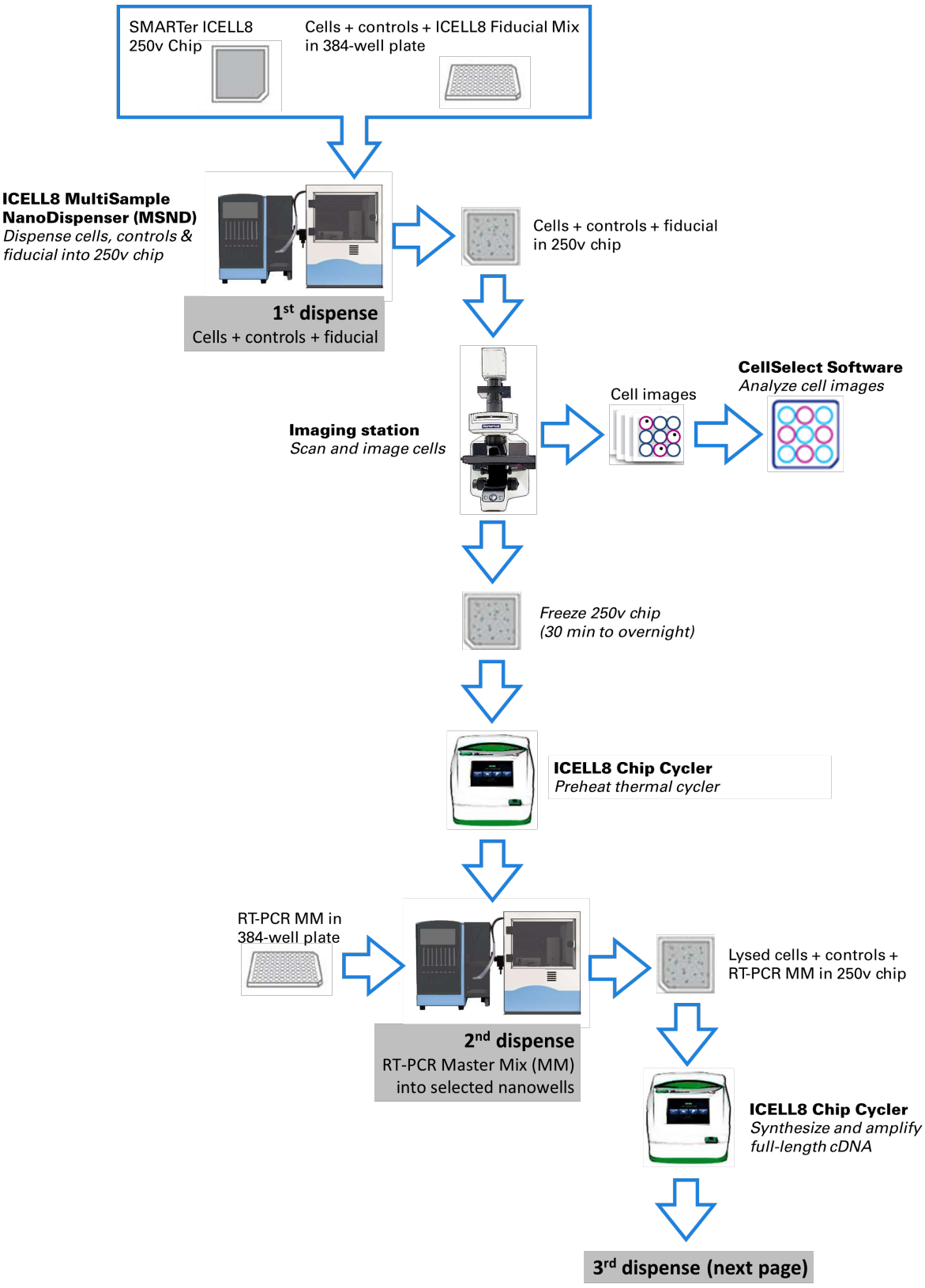


Figure 1. Complete SMART-Seq ICELL8 application kit workflow (continues on next page).

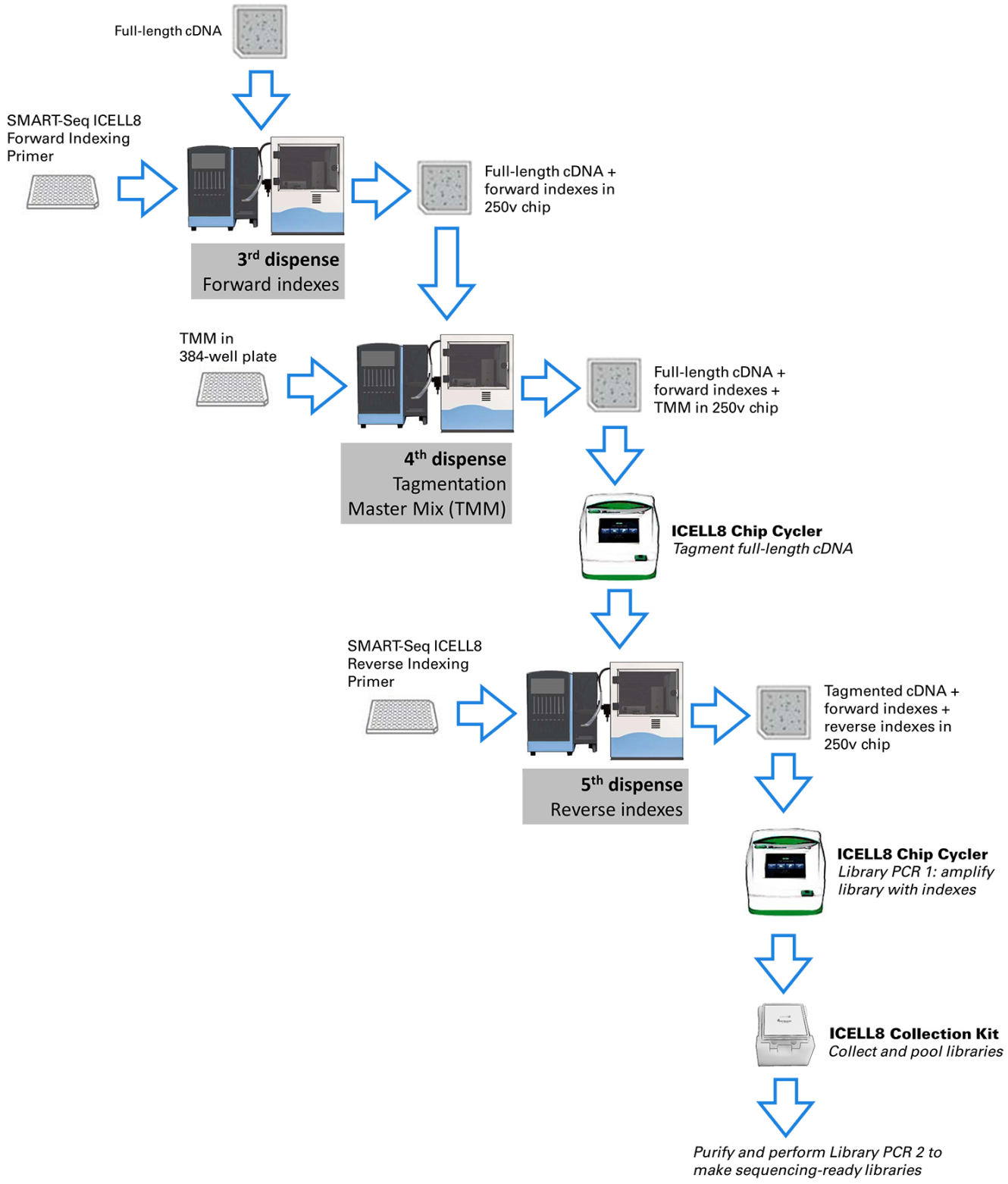


Figure 1. Complete SMART-Seq ICELL8 Application Kit workflow (continues from previous page).

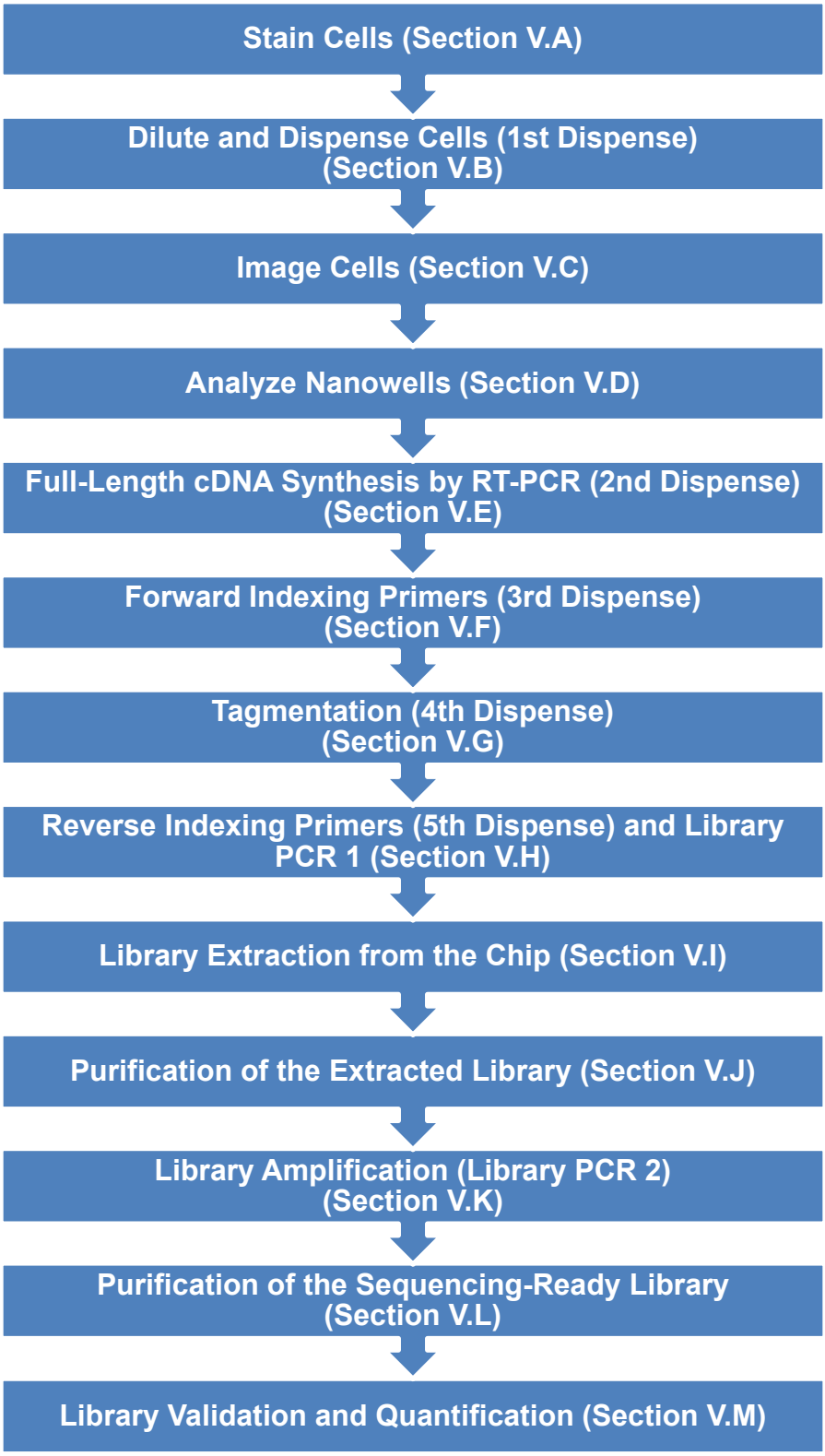


Figure 2. Protocols in the SMART-Seq ICELL8 Application Kit workflow.

II. List of Components

Required products

The following SMART-Seq ICELL8 Application Kits can be used for the workflow:

- SMART-Seq ICELL8 Application Kit – 1 Chip (Cat. No. 640219)
- SMART-Seq ICELL8 Application Kit – 3 Chip (Cat. No. 640220)
- SMART-Seq ICELL8 Application Kit – 5 Chip (Cat. No. 640221)

Each kit contains the necessary chip(s), reagents, and consumables required to run a total of 1, 3, or 5 experiments (respectively) of single-cell, full-length transcriptome analysis.

Table 1. Available SMART-Seq ICELL8 Application Kit contents.

SMART-Seq ICELL8 application kits	640219 (1 Chip)	640220 (3 Chip)	640221 (5 Chip)
Box 1 (ships at room temperature)			
ICELL8 250v Chip* (Cat. No. 640183)	1	3	5
ICELL8 Collection Kit – L*† (Cat. No. 640212)	1	3	5
ICELL8 Loading Kit – B* (Cat. No. 640206)	1	3	5
Box 2 (ships at –70°C)			
SMART-Seq ICELL8 Reagent Kit* (Cat. No. 640202)	1	--	--
SMART-Seq ICELL8 Reagent Kit* (Cat. No. 640203)	--	1	--
SMART-Seq ICELL8 Reagent Kit* (Cat. No. 640204)	--	--	1
SMART-Seq ICELL8 Indexing Primer Set – A* (Cat. No. 640205)	1	2	3
SMART-Seq ICELL8 Indexing Primer Set – B* (Cat. No. 640218)	--	1	2

*Component can also be purchased separately.

†Using the ICELL8 Collection Kit – L requires the ICELL8 Extraction Fixture – L (Cat. No. 640217), purchased separately. Please contact Customer Service (takarabio.com/service) at 800.662.2566, Option 1 to obtain one, if needed.

III. Additional Materials Required

ICELL8 Single-Cell System

This protocol requires use of an ICELL8 Single-Cell System (Takara Bio, Cat. No. 640000). The ICELL8 system consists of the following components:

Table 2. ICELL8 Single-Cell System components.

ICELL8 Single-Cell System (Cat. No. 640000)	
Component	**Quantity per system**
ICELL8 Single-Cell Instrument	1
ICELL8 150v Chip*	3
ICELL8 3′ DE Chip*	3
ICELL8 3′ DE for UMI Reagent Kit*	6
ICELL8 Collection Kit	6
ICELL8 Loading Kit	6
MSND 384-Well Source Plate and Seals	1 (pack of 20)
ICELL8 Imaging Station Installation Kit	1
ICELL8 Imaging Film	1
ICELL8 Chip Holder	2

*These chips are not compatible with the SMART-Seq ICELL8 application kit workflow. An ICELL8 250v Chip must be purchased to perform the protocols detailed in this user manual.

General lab supplies

- Personal protective equipment (PPE): powder-free gloves, safety glasses, lab coat, sleeve protectors, etc.
- Film Sealing Roller for PCR Plates (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
- Vortex mixer
- Centrifuges and rotors for conical tubes and plates. Recommended:
  - Eppendorf 5810R with swinging plate buckets
  - Kubota 3740 with rotor SF-240 for cell preparation (Kubota)
- MSND 384-Well Source Plates and Seals (20 Pack) is included with the instrument; additional plates/seals in various sizes can be ordered separately (Takara Bio, Cat. Nos. 640192, 640018, or 640037)
- Nuclease-free 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: 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
- Two pairs of tweezers for handling chips during imaging
- Nuclease-decontamination solution
- Exhaust hood system with UV

For ICELL8 MSND general operation

- Deionized water (for pressure reservoir and humidifier)
- Freshly mixed 0.2% sodium hypochlorite solution
- Helium (>99.9% purity)

NOTE: The ICELL8 MSND has been qualified to work with either helium (preferred) or argon gas if helium is unavailable. References to 'noble gas' in this document should be interpreted to mean whichever of these two are being used in your system.

IMPORTANT: Other noble gases (neon, krypton, etc.) should NOT BE USED.

For staining and dispensing cells

- ICELL8 Chip Holder (Takara Bio, Cat. No. 640008); two chip holders are included with the instrument, and additional chip holders can be ordered separately.
- 1X PBS (no Ca^{2+}, Mg^{2+}, phenol red, or serum, pH 7.4; Thermo Fisher Scientific, Cat. No. 10010-023)
- ReadyProbes Cell Viability Imaging Kit, Blue/Red (contains Hoechst 33342 and propidium iodide; Thermo Fisher Scientific, Cat. No. R37610)
- Appropriate cell culture medium*
- TrypLE Express (Thermo Fisher Scientific, Cat. No. 12604-021)*

*Required only if performing dissociation of adherent cells.

For tagmentation

- Illumina Tagment DNA TDE1 Enzyme and Buffer Small Kit (Illumina, Cat. No. 20034197) or Illumina Tagment DNA TDE1 Enzyme and Buffer Large Kit (Illumina, Cat. No. 20034198).

NOTES:
- Illumina Tagment DNA TDE1 Enzyme and Buffer Kits include Tagment DNA Enzyme (TDE1) and Tagment DNA Buffer (TD).
  - The Small Kit includes 0.17 mL (1 tube) of TDE1 and 1.24 mL (1 tube) of TD.
  - The Large Kit includes 0.65 mL (1 tube) of TDE1 and 1.24 mL (2 tubes) of TD.
- SMART-Seq ICELL8 Application Kit requires only TDE1. Do not use TD.

For library purification and validation

- SMARTer-Seq® Magnetic Separator - PCR Strip (Takara Bio, Cat. No. 635011)
- Agencourt AMPure XP PCR purification system (5 ml; Beckman Coulter, Cat. No. A63880)

NOTES:
- Agencourt AMPure XP beads need to come to room temperature before the container is opened. Therefore, we strongly recommend aliquoting the beads into 1.5 ml tubes upon receipt, and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min). This aliquoting process is also essential for minimizing 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) or similar
- Agilent High Sensitivity DNA Kit (110 samples; Agilent, Cat. No. 5067-4626)

NOTE: Alternatively, the libraries can be quantified by qPCR using the NGS Library Quantification Kit (Takara Bio, Cat No. 638324).

- Qubit Fluorometer or similar (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 (ORFLO, Cat. No. MXZ001) with MOXI Z Cell Count Cassettes, Type M (25 pack; ORFLO, Cat. No. MXC001) or MOXI Z Cell Count Cassettes, Type S (25 pack; ORFLO, Cat. No. MXC002)

NOTES:
- Refer to a Moxi Z user manual 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. SMART-Seq ICELL8 Indexing Primer Set

IMPORTANT: Prepare the 384-well plates containing indexing primers before starting the experiment.

– If processing one chip, do the following steps for either SMART-Seq ICELL8 Indexing Primer Set – A or SMART-Seq ICELL8 Indexing Primer Set – B.

– If processing two chips for sequencing together, do the following steps for each of SMART-Seq ICELL8 Indexing Primer Set – A and SMART-Seq ICELL8 Indexing Primer Set – B.

1. Take the 384-well plate out of the –80°C freezer and seal it using a film applicator immediately before thawing the plate. (Do not open the sealing foil.)

   IMPORTANT: The sealing foil is securely sealed when manufactured. However, the foil might get partly opened during shipment. Therefore, it is important to seal it completely to avoid index contamination among wells.

2. Without opening the sealing foil, thaw the frozen indexes at room temperature and seal again using a film applicator.
3. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 3 min between 4°C and room temperature.
4. Shake the plate with a 384-well plate shaker for 1 min at room temperature.
5. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 3 min between 4°C and room temperature. If centrifuging at 4°C, bring the plate back to room temperature prior to the dispense.

   IMPORTANT: If you observe that the sealing foil is not securely sealed, please contact TBUSA Technical Support at technical_support@takarabio.com or 800.662.2566.

B. Sample Recommendations

This protocol is typically performed with several milliliters of healthy cell culture suspension as starting material. We recommend maintaining a cell density between 1 x 10^5 and 7.5 x 10^6 cells/ml prior to starting the protocol.

C. 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, etc. 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 protocol.
- Because of the large volume or viscosity of mixtures subject to purification using AMPure 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.
- Use deionized (e.g., Milli-Q) water for the ICELL8 MSND pressure bottle and humidifier; clean these reservoirs routinely. Use nuclease-free molecular biology-grade water for the ICELL8 MSND wash bottle.
- UV treat reagent reservoirs, seals, pipettes, filter tips, and compatible reagents prior to use.

D. 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 ICELL8 single-cell systems 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.

E. ICELL8 MSND Application Notes

Refer to the "ICELL8 MultiSample Nanodispenser User Manual" for full details. Included below are general reminders.

- Before operating the instrument, check the water level in the pressure reservoir. Refill the pressure reservoir if the weighing scale below the pressure reservoir displays a value ≤4 kg (≤8 lbs).
- Add deionized water (e.g., Milli-Q water) to the reservoir as indicated in the following steps, and in Figure 3 (below):
  a. Close the helium (or argon) in-line stopcock and open the vent stopcock.
  b. Fill the pressure reservoir with deionized water to the fill line (top of the protective cover rim). The weighing scale below the pressure reservoir should display a value of ~4 kg.

c. Reattach the cap and let sit for ~20 min to degas. Make sure that the cap is properly threaded and securely fastened to prevent the noble gas from leaking out.

d. Close the system by opening the noble gas in-line stopcock and closing the vent stopcock.

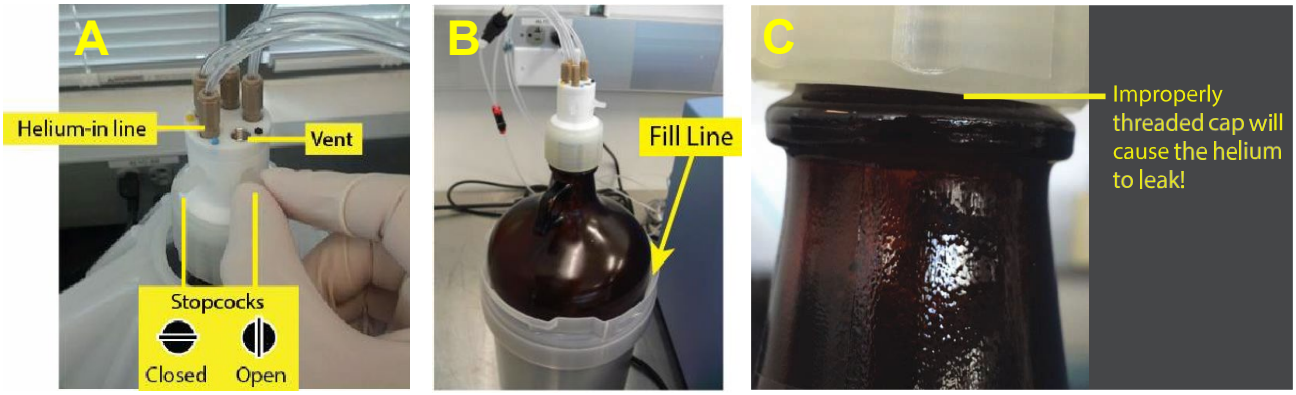


Figure 3. Filling the pressure reservoir. **Panel A.** Close the noble gas in-line stopcock and open the vent stopcock. **Panel B.** Add Milli-Q water to the fill line of the pressure reservoir. **Panel C.** Make sure that the cap on the pressure reservoir is properly attached.

- Check the water level in the humidifier and fill if needed. Clean the humidifier regularly.
- Check the pressure on the noble gas tank. The regulator should be set to a supply input of >500 psi (3.5 MPa) and an output of 30–40 psi. If the tank pressure drops to <500 psi, replace it with a full tank.
- Check the sodium hypochlorite level in the wash bottle. If it is <25% full or 3–4 days old, replace it with 500 ml of freshly made 0.2% sodium hypochlorite solution prepared from reagent-grade sodium hypochlorite in deionized, filtered water (e.g., Milli-Q, ELGA system, or equivalent with 0.2-µm filtration).
- Check the waste container. If full, dispose the liquid waste appropriately and replace with an empty container.
- All dispensing steps in the ICELL8 MSND Stage Module should be performed with the MSND 384-Well Source Plate oriented with the A1 well positioned at the top-right corner of the 384-well Plate Nest, and the chip oriented with the chamfered (notched) corner positioned towards the lower-right corner of the Dispensing Platform (Figures 4–6, below).

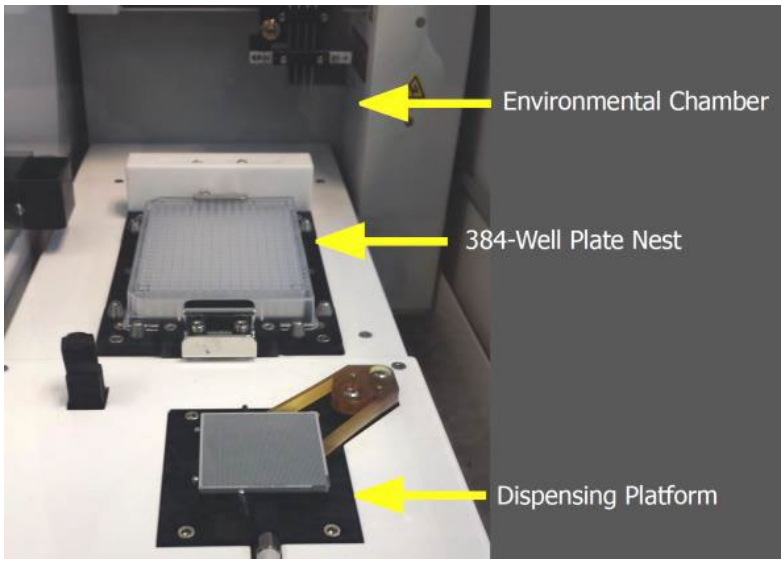


Figure 4. ICELL8 MSND Stage Module.

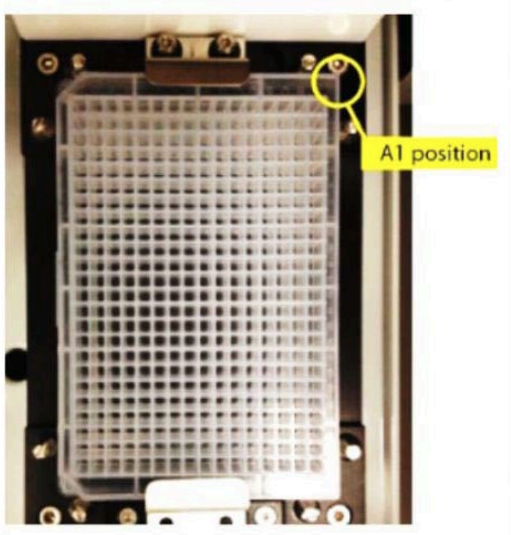


Figure 5. MSND 384-Well Source Plate in the Plate Nest.

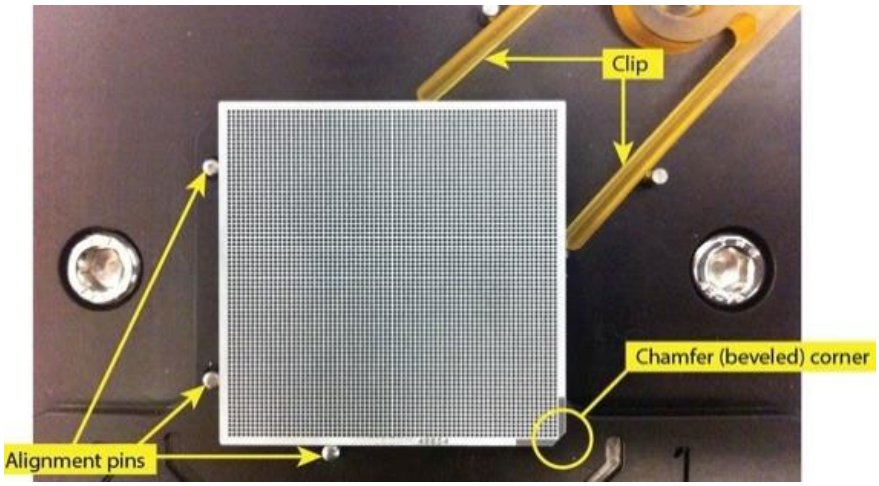


Figure 6. Chip seated in the Dispensing Platform. Stretch the arms of the clip apart and angle the chip onto the Dispensing Platform with the chamfered (notched) corner of the chip oriented towards the lower-right corner of the Dispensing Platform. The edges of the chip should be pressed against the three alignment pins.

IMPORTANT: Make sure that the entire chip lies perfectly flat and sits squarely on the Dispensing Platform. Make sure that the alignment pins do not improperly catch on the side walls of the chip.

F. ICELL8 Imaging System and CellSelect Software

Included below are instructions for using the ICELL8 Imaging System and CellSelect Software to perform the protocol described in this user manual. Please refer to the ICELL8 Imaging System User Manual and the CellSelect Software User Manual for more detailed information.

G. ICELL8 250v Chip

- Each ICELL8 250v Chip is engraved with a unique number (Figure 7). You can use this number to link your chip images and other experimental record files.

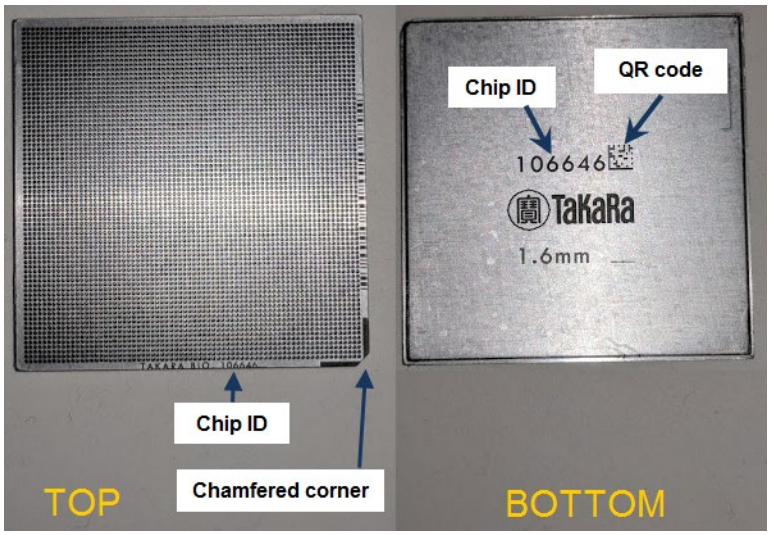


Figure 7. ICELL8 250v Chip features. **TOP.** In addition to the 5,184 nanowells, note the chamfered (notched) corner at the bottom right. The "TaKaRa" logo and the chip ID (unique to each chip) are engraved on the chip border, near the chamfered corner. **BOTTOM.** The chip ID is also engraved on the 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.

V. Protocols

A. Protocol: Stain Cells

In this protocol, sample cells are stained with Hoechst 33342 and propidium iodide dyes that enable imaging, analysis, and selection of candidates suitable for downstream analysis following dispensation into the ICELL8 250v Chip. If starting from a nonadherent suspension culture, like human K-562 cells, use Procedure 1 (below); if starting from an adherent culture, use Procedure 2 (below), then stain the trypsinized cell suspension using Procedure 1.

Cell handling notes

- This protocol requires several milliliters of healthy cell culture suspension with a cell density between 1 x 10^5 and 7.5 x 10^6 cells/ml. Some cell lines may require trypsinization to achieve a single-cell suspension.
- 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.

Before you start

- Perform a once-a-day warmup: initialize the ICELL8 MSND; from the *Startup* tab, complete each step in the Daily Checklist, and then click the [Daily Warmup] button. After this process is complete, go to the *Advanced* tab and execute the **Wash Prime** function followed by **Tip Clean**. Refer to the "ICELL8 MultiSample Nanodispenser User Manual" for more details.
- Prefreeze ICELL8 Chip Holder(s) at –80°C.
- Set the chip centrifuge to 22°C.
- Start the ICELL8 Imaging System by turning on system components in the following order: the fluorescence light source, the camera, the controllers for the microscope imaging filters and stage, and then the computer. Please note that the fluorescence light source requires a warmup period of ~5 min. Refer to the ICELL8 Imaging System User Manual for detailed setup instructions before first use.
- Thaw Second Diluent (100X; light blue cap) and ICELL8 Fiducial Mix (1X) on ice. Once thawed, keep on ice for the remainder of the protocol.
- Prewarm 1X PBS (Ca^{2+} and Mg^{2+} free, pH 7.4), TrypLE Express*, and cell culture medium* at 37°C.
  *Required only if performing dissociation of adherent cells.
- Dilute Control K-562 RNA (1 µg/µl; yellow cap) to 10 ng/µl for use in the next protocol (Section V.B, Table 3) as indicated in the following steps and keep the dilution on ice:
  1. Dilute Control K-562 RNA to 50 ng/µl by mixing 38 µl of Nuclease-Free Water (white cap) with 2 µl of Control K-562 RNA (1 µg/µl) in a sterile nuclease-free microcentrifuge tube.
  2. Dilute Control K-562 RNA to 10 ng/µl by mixing 8 µl of Nuclease-Free Water with 2 µl of Control K-562 RNA (50 ng/µl) in a sterile nuclease-free microcentrifuge tube.

NOTES:
- Return Control K-562 RNA (50 ng/µl) stock solution to storage at –70°C.
- Diluted Control K-562 RNA should be kept on ice at all times.

1. Staining cells in suspension

1. Prepare a 1:1 mixture of Hoechst 33342 and propidium iodide. Combine 80 µl of each dye per ml of cells to be stained. 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 cell concentration using a Moxi automated cell counter and Moxi Z cassette or your preferred method (e.g., use Moxi Z cassette MF-M for K-562 cells; refer to a Moxi Z user manual for guidance in selecting an appropriate cassette size for the cells being analyzed). Use a pipette to transfer 75 µl of cell suspension to one end of the loading platform. Take a reading and record the cell concentration.
4. Add 320 µl of the premixed Hoechst 33342 and propidium iodide dye mix to the 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 1X PBS (prewarmed to 37°C) to stained cells. For the example described here, 2 ml of prewarmed 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 100*g* for 3 min at room temperature. 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: 100g for 3 min.
   - PBMCs or similarly sized cells: 500g for 3 min.
9. Gently remove 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 tube top using a fresh Kimwipe such that the last remaining drop on the tilted tube top is gently removed.
12. Gently add 1 ml of 1X PBS (prewarmed to 37°C) to the tube side wall.
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 a Moxi automated cell counter and an appropriate cassette (or your preferred method). Take two readings for each stained cell sample and average the results. The recommended concentration range of stained cell suspension is 1.2–5.0 x 10^5 cells/ml. If you end up with a concentration lower than 1.2 x 10^5 cells/ml, repellet the stained cell suspension and resuspend in a lower volume of 1X PBS to achieve a concentration in the recommended range.
15. Proceed to the next protocol (Section V.B).

2. Preparing adherent cell types from a 75-cm culture flask*

*Adjust volumes accordingly for different-sized flasks.

1. To a 75-cm flask containing adherent cells, exchange cell media with 10 ml of 1X PBS prewarmed to 37°C by dispensing the PBS on the side walls of flask.

   NOTE: Do not pour PBS directly on cells.
2. Wash the cells by tilting the flask gently. Do not mix by pipetting.
3. Remove the PBS from the cells using a standard large-bore tissue culture pipette.
4. Add 3 ml of TrypLE Express prewarmed to 37°C to the flask to dissociate the cells.
5. The efficiency of cell dissociation from the flask surface may vary with cell type. Monitor the process visually using a microscope.
6. Once cell dissociation has occurred, neutralize the trypsinization reaction by gently adding 7 ml of complete media (containing 10% serum; prewarmed to 37°C). Do not vortex or overagitate cells.
7. Follow the procedure "Staining cells in suspension" (Section V.A), 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 MSND 384-Well Source Plate and dispensed into the ICELL8 250v Chip using the ICELL8 MSND. Refer to the "ICELL8 MultiSample Nanodispenser User Manual" for detailed information about instrument setup and operation.

Before you start

- Confirm that initialization and setup procedures for the ICELL8 MSND and ICELL8 Imaging System have been completed (see "Before you start" in Section V.A, above).
- Prefreeze the empty ICELL8 Chip Holder (Figure 8, below) 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 control.
- This protocol requires an MSND 384-Well Source Plate (with seal) and the following components: Control K-562 RNA (diluted to 10 ng/µl in the previous protocol), Second Diluent (100X; light blue cap), BSA (1%; red cap), ICELL8 Fiducial Mix, RNase Inhibitor (40 U/µl; white cap), SMART-Seq ICELL8 CDS (blue cap)—all from the SMART-Seq ICELL8 Reagent Kit—plus Blotting Paper and Optical Imaging Film (from the ICELL8 Loading Kit – B).
- Thaw Second Diluent (100X), BSA (1%), ICELL8 Fiducial Mix (1X), and SMART-Seq ICELL8 CDS 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.1) and the information in Table 3 (below) to calculate the volumes of stained cell suspension and 1X PBS that should be combined for a final concentration of 1 cell/35 nl in a total volume of 1 ml.

Figure 8. ICELL8 Chip Holder.

Procedure

Prepare diluted stained cell suspension

1. Vortex Second Diluent, RNase Inhibitor, BSA (1%), and SMART-Seq ICELL8 CDS and spin the tubes briefly to collect contents at the bottom.
2. In a 1.5 ml microcentrifuge tube, combine the volumes of prewarmed 1X PBS, RNase Inhibitor, Second Diluent, BSA (1%), and SMART-Seq ICELL8 CDS indicated in the corresponding column of

Table 3 (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.

3. To the 1.5 ml microcentrifuge tube from the previous step (containing 1X PBS, RNase Inhibitor, Second Diluent, BSA (1%), and SMART-Seq ICELL8 CDS), add the calculated volume of stained cell suspension that will yield a final concentration of 1 cell/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 or 200 µl 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

4. Prepare negative and positive controls in separate 1.5 ml microcentrifuge tubes using the volumes indicated in Table 3 (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 RNA should be 10 pg/35 nl (equivalent to the total RNA content from 1 cell).

Table 3. Sample preparation guidelines.

Components	Cap color	Negative Control	Positive Control	Diluted Stained Cell Suspension	For 8 samples (volume per sample)
Second Diluent (100X)	Light blue	1.00 µl	1.00 µl	10.00 µl	1.00 µl
BSA (1%)	Red	1.00 µl	1.00 µl	10.00 µl	1.00 µl
RNase Inhibitor (40 U/µl)	White	1.00 µl	1.00 µl	10.00 µl	1.00 µl
SMART-Seq ICELL8 CDS	Blue	1.92 µl	1.92 µl	19.20 µl	1.92 µl
Control K-562 RNA (10 ng/µl)	–	–	2.86 µl	–	–
Stained cell suspension	–	–	–	Dilute to 1 cell/35 nl*	Dilute to 1 cell/35 nl*
1X PBS (Ca^{2+} and Mg^{2+} free)	–	95.08 µl	92.22 µl	Up to 1,000.00 µl	Up to 100 µl
Total		100.00 µl	100.00 µl	1,000.00 µl†	100.00 µl‡

*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 cell/35 nl (i.e., 28,600 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 9, 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 cell dispense source plate

Refer to the "ICELL8 MultiSample Nanodispenser User Manual" for detailed information about instrument setup and operation.

5. Using a wide-bore 1 ml pipette tip, gently mix the diluted stained cell suspension prepared in Step 3 (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.

6. Using a wide-bore 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 an MSND 384-Well Source Plate as indicated in Figure 9 (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 MSND 384-Well Source Plate.
   - Do not vortex or spin down the MSND 384-Well Source Plate.
   - Do not tap plate. If any bubbles are present, remove using a pipette tip.

7. Add positive control, negative control, and ICELL8 Fiducial Mix (1X) to the MSND 384-Well Source Plate as indicated in the following steps and in Figure 9 (below):
   - Add 25 µl of prepared positive control to well P24.
   - Add 25 µl of prepared negative control to well A24.
   - Add 25 µl of ICELL8 Fiducial Mix (1X) to well P1.

   NOTES:
   - Be careful not to splash liquid into neighboring wells.
   - Do not introduce bubbles when adding reagents to the MSND 384-Well Source Plate.
   - Do not vortex or spin down the MSND 384-Well Source Plate.
   - Do not tap plate. If any bubbles are present, remove using a pipette tip.

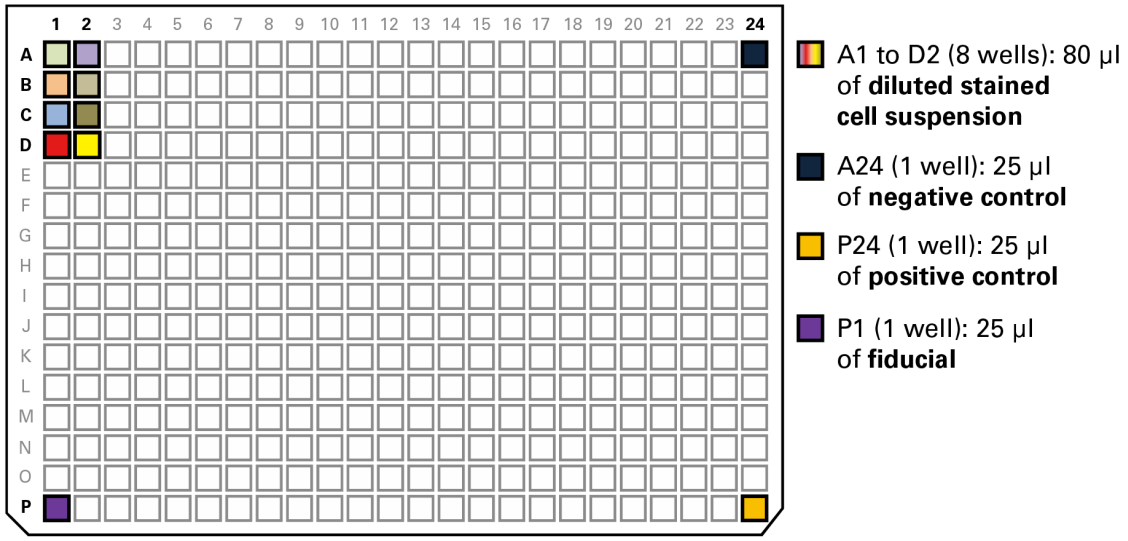


Figure 9. Setting up the MSND 384-Well Source Plate for dispensing cell samples and controls.

8. Seal the MSND 384-Well Source Plate with the provided Plate Seal. Do not centrifuge the plate.
9. Open the packet containing the ICELL8 250v Chip.
10. On the ICELL8 MSND software screen, click the *FLA* tab and then click the box under the "ChipID" field. Scan the barcode on the back of the chip or enter the chip ID into the text field.

CAUTION: Be sure to avert your eyes from the scanner light emitted by the Barcode Reader.

11. Place the chip on the chuck of the Dispensing Platform in the ICELL8 MSND with the chamfered (notched) corner of the chip at the lower right corner of the Dispensing Platform, aligned with the chamfered corner of the chuck (see Figure 6, above).

IMPORTANT: The edges of the chip should be pressed against the three alignment pins; **make sure that the chip is flat on the chuck from all sides and is in the correct orientation** (see Figure 6, above).

12. Place the MSND 384-Well Source Plate in the ICELL8 MSND with the A1 corner positioned at the top-right corner of the Plate Nest (see Figures 4 and 5, above). The beveled corners of the MSND 384-Well Source Plate should be on the left side.
13. Gently remove the seal from the MSND 384-Well Source Plate and lock the plate into position then close the ICELL8 MSND environmental chamber doors.
14. On the ICELL8 MSND Software screen, under the *FLA* tab, click [Dispense cells] (Figure 10, below) then click [OK] after carefully going through all questions prompted. The ICELL8 MSND will start dispensing 35 nl of cell suspension, positive/negative control, or fiducial to the appropriate nanowells.

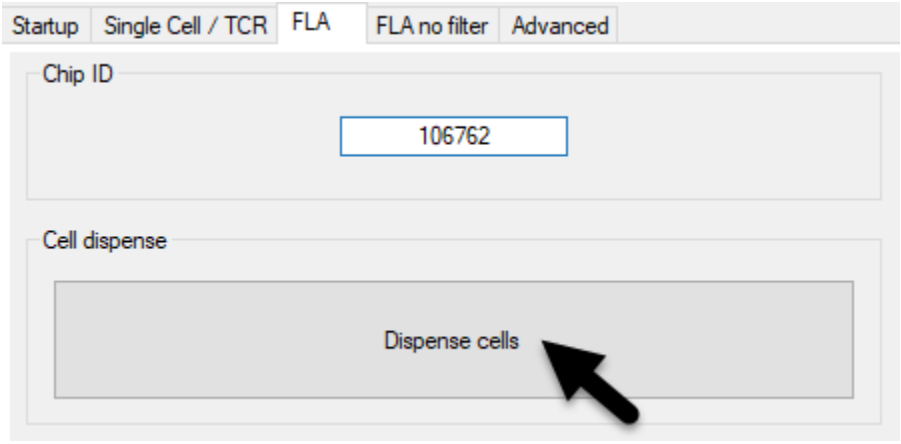


Figure 10. Using the ICELL8 MSND software to dispense cells into the ICELL8 250v Chip.

15. After dispensing is completed, remove the chip from the Dispensing Platform and gently blot for two seconds with a fresh piece Blotting Paper (provided in the ICELL8 Loading Kit – B) and Blotter.

Figure 11. Blot the chip with Blotting Paper and Blotter. A Blotter is provided with the ICELL8 Single-Cell System.

16. Remove the liner from **only one side** of an Optical Imaging Film and apply the exposed sticky side of the imaging film to the blotted chip (Figure 12, below).
17. Use a film applicator (left panel of Figure 12) to make sure chip is securely sealed to avoid well-to-well contamination. **Note the orientations of the chamfered corner and the imaging film tab** (right panel of Figure 12).

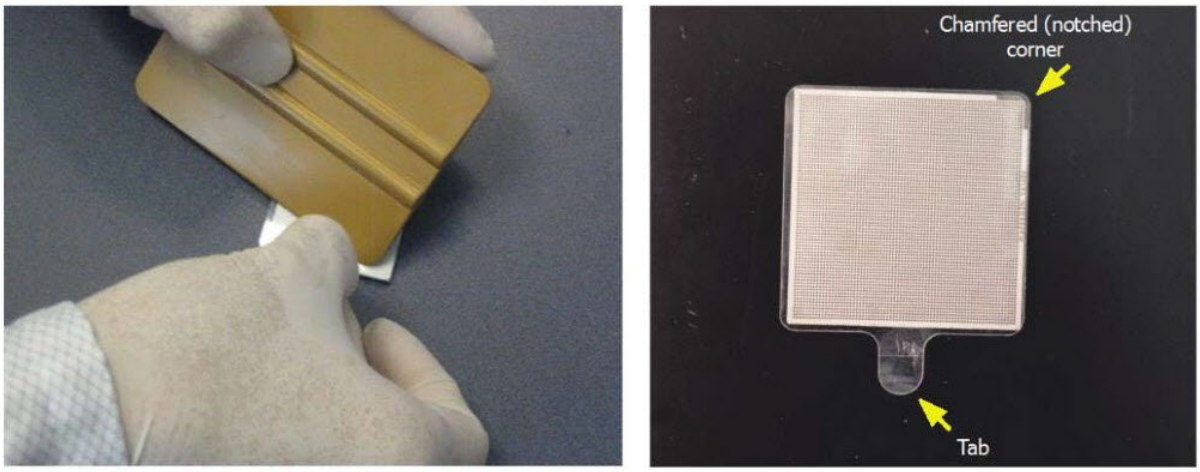


Figure 12. Sealing the blotted chip. Seal the blotted chip using a film applicator (left). The tab of the film should be oriented downward relative to the chamfered corner of the chip (right).

18. Place the sealed chip on a Chip Spinner and centrifuge the sealed chip at 300*g* for 5 min at room temperature with full acceleration and full brake (Figure 13, 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.

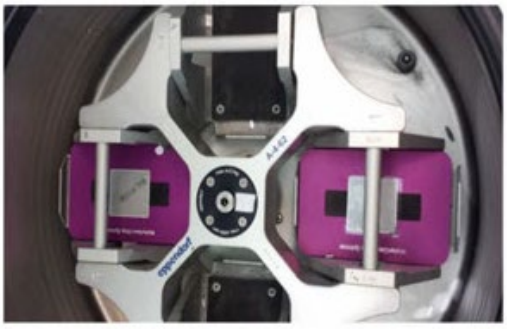


Figure 13. Centrifuge Chip Spinner.

C. Protocol: Image Cells

In this protocol, images of all 5,184 nanowells of the ICELL8 250v Chip are acquired. Refer to the ICELL8 Imaging System User Manual for detailed information about instrument setup and operation.

ICELL8 Imaging System notes

- The light source requires a warm-up period of ~5 min. A blue light should be visible indicating that the burner is ready.
- The bulb has a life span of ~2,000 hours. Please contact Takara Bio Field Support for assistance with changing the bulb. After a new bulb is installed, let it run for 1 hour before normal operation.
- After the burner is turned on, please allow at least 30 min before turning it off.
- Wait for at least 30 min before turning on a previously turned-off burner.
- Do not turn the burner on and off more than 4 times a day; it should be left on if you plan to use it frequently throughout the day.

- Allow the imaging system's camera to warm up for fifteen seconds (green light is on and has stopped blinking) before starting the Micro-Manager software.

> **NOTE:** Failure to allow the camera to warm up before starting the software could result in a hardware configuration error. If the error occurs, close the software and restart the camera**.**

- Micro-Manager software application should remain closed until all other pieces of hardware are turned on.

Procedure

Start software and input chip information

1. Start the Micro-Manager software. Ensure that the manual shutter is in the Open position.
2. Click **Live** in the *Main* window to open a window showing a live image from the camera.
3. Click **Multi-D Acq.** to open the *Multi-Dimensional Acquisition* window.
4. Make sure that the **Multiple positions (XY)** box is checked.
5. Click **Load Chip type** and load the configuration file `72.72.pos` (if it is not preloaded).
6. Position the cursor so that it resides in the "Chip Number" field. Input the chip ID or use the barcode scanner to scan the two-dimensional barcode on the back of the chip WITHOUT inverting the chip.

>  **CAUTION:** Be sure to avert your eyes from the scanner light emitted by the Barcode Reader.

7. Enter a short experimental name and the chip number in the file name prefix (`%PATH%\WafergenData\<experiment name_chip number>`). The image files will be saved under this named folder.

Position chip on microscope

> **NOTE:** The chip should be centrifuged prior to imaging.

8. Place the chip on the microscope Holding Platform with the chamfered (notched) corner facing the upper right corner (Figure 14, below).

> **NOTE:** Ensure that the chip is sitting flat on the chuck from all sides and is in the correct orientation (Figure 6, above).

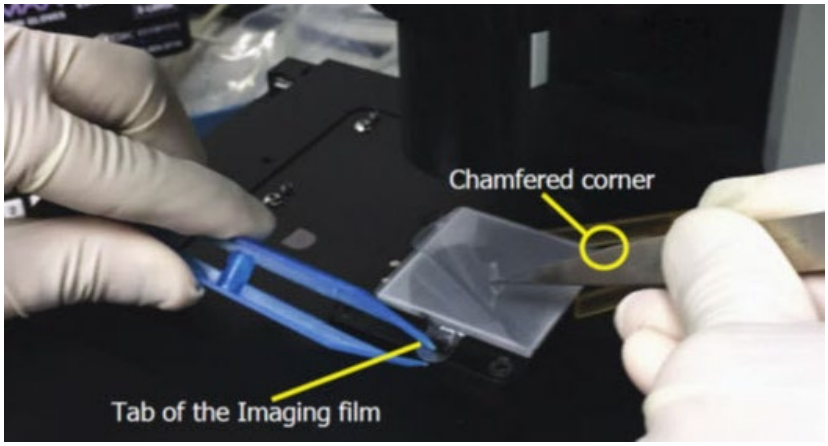


Figure 14. Seating the chip on the microscope holding platform.

9. Ensure the chip is perfectly flat and seated square relative to the pins before removing the second release liner. Peel off the top layer release liner of the Optical Imaging Film using tweezers (Figure 14, above). The chip will remain sealed by the double-sided intermediate film layer.

IMPORTANT: Save the release liner for reapplication in Step 20.

10. Move the objective over the chip. Ensure that the objective is vertical and centered (you should hear/feel a slight click).
11. Select the row "Pos0" in the *Stage Position List* window (Figure 15), and then click the [Go to] button.

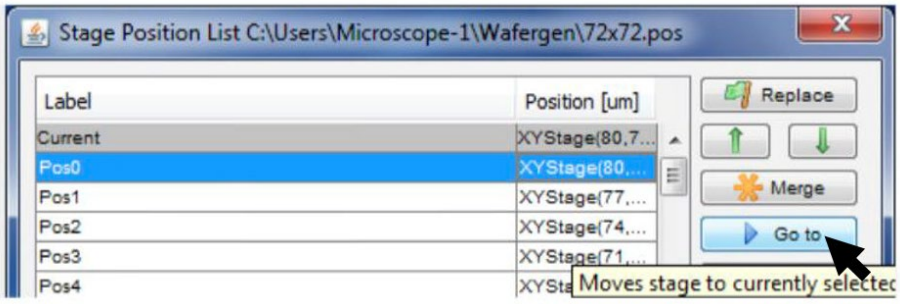


Figure 15. Moving the motorized stage to Position 0.

12. In the *Main* window, under "Configuration settings", select **Texas Red** as the illumination channel for examination of fiducial-containing nanowells (Figure 16).

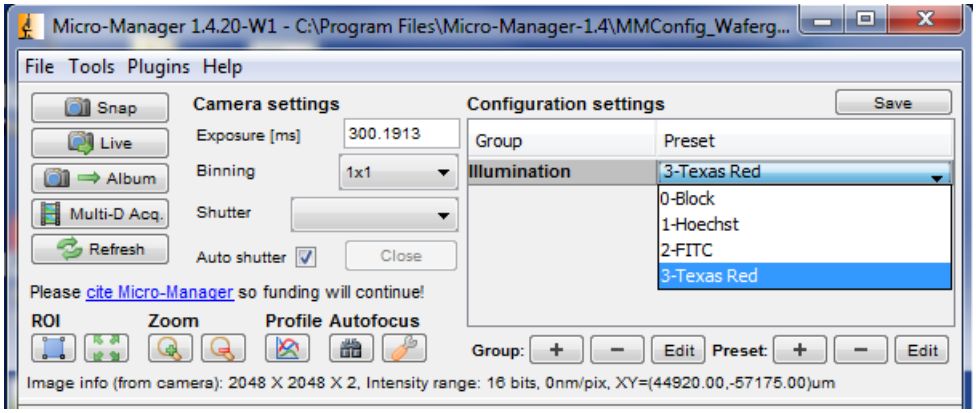


Figure 16. Setting the illumination channel to Texas Red.

13. Confirm that five fiducial-containing wells are present, forming an "X" shape at Pos0 (Figure 17). There are four fields of view across an entire chip that include fiducial-containing nanowells (Pos0, Pos11, Pos132, and Pos143). Each field of view includes 36 nanowells (a 6 x 6 grid).

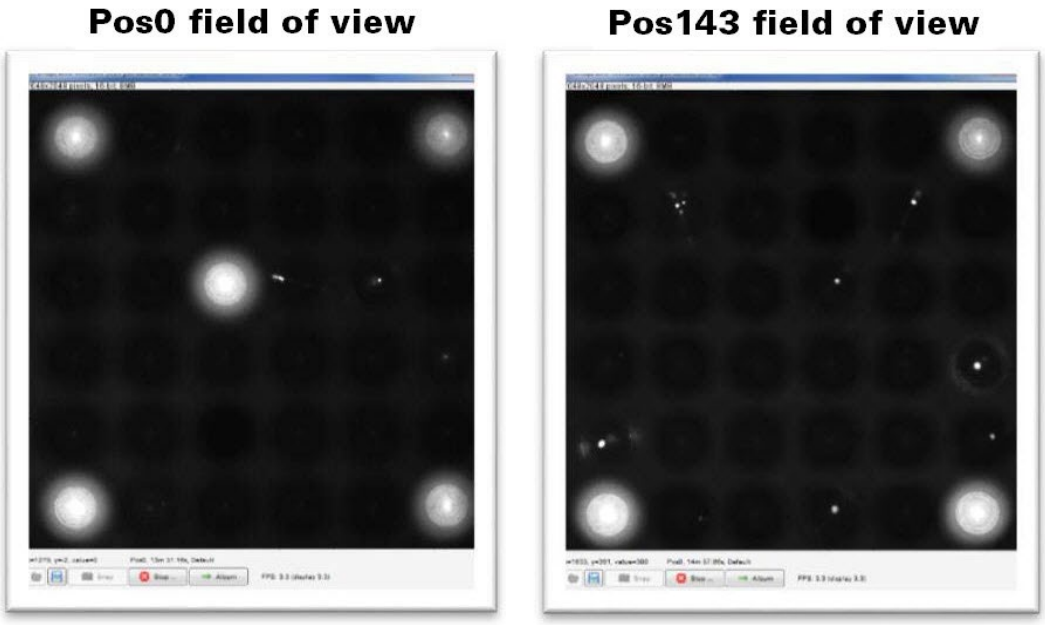


Figure 17. Multiwell images including fiducial-containing nanowells.

14. Select "Pos143" in the *Stage Position List* window and confirm that all 36 nanowells are properly captured in the image. There will be four fiducial-containing nanowells in the shape of a square, as shown in Figure 17 (above). Pos143 is located at the lower right corner of the stage fixture.

Optimize image focus

15. Adjust image contrast and focus settings for optimal visualization of single cells (refer to the "ICELL8 Imaging System User Manual" available from the takarabio.com website for more details):
    a. In the *Main* window, under "Configuration settings," select **Hoechst**—the name of the illumination channel for examination of Hoechst-stained cells.
    b. Use the microscope's manual focus to achieve the optimal Z-plane focal depth to visualize a field of single cells.
    c. Use the zoom function to zoom in on a well containing one or a few cells that are off center (i.e., not in the center of a well).
    d. Adjust the fine contrast knob back and forth to find the optimal focal point for a cell (smallest, sharpest view) or the optimal focal point between two cells on the Z-plane (blue box in Figure 18, below).
    e. Zoom out to view all 36 nanowells at Pos143 and confirm that the focus is optimal.

> **NOTE:** If the cells are still out of focus after these steps, ensure that the chip is perfectly flat on the Dispense Platform.

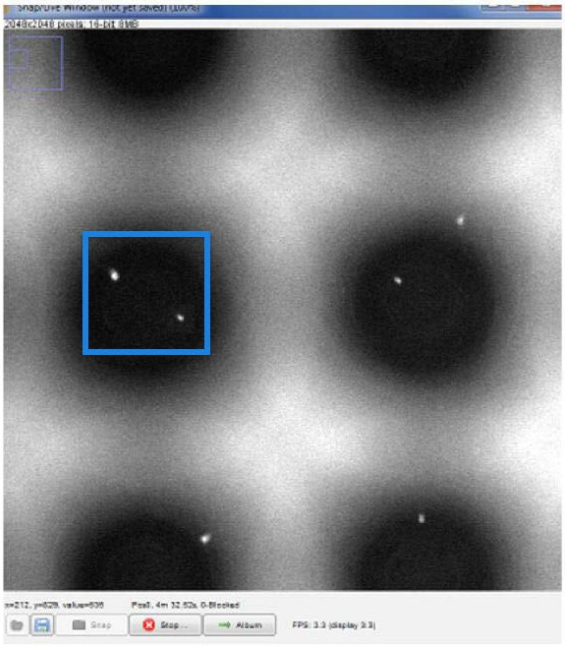


Figure 18. Adjusting the focus on cells.

16. Close the *Stage Position List* window.

Acquire images

17. In the "Channels" table of the *Multi-Dimensional Acquisition* window, the following two preconfigured channels should be preselected: **Hoechst** (exposure: 200) and **Texas Red** (exposure: 300). The channel named "Hoechst" enables visualization of Hoechst staining, while the channel named "Texas Red" is used for visualization of propidium iodide staining.
18. Click [Acquire!] and accept the prompt to create a new folder based on the current date. The camera will acquire TIFF images from 144 fields of view (Pos0–Pos143) using Hoechst and Texas Red channels, respectively, with each field of view including 36 nanowells in a 6 x 6 grid. Following acquisition, the entire image folder should contain a total of 288 TIFF files comprising the 144 fields of view in both the Hoechst and Texas Red channels.
    a. In the *Main* window, select the *Contrast* tab and ensure that "Camera Depth" menu is set to **12 bit (0-4095)** for Hoechst and **10 bit (0-1023)** bit for Texas Red (in the purple box in Figure 19, below).
    b. Next, confirm that the [Autostretch] and [Ignore] boxes are selected, and that the percentage value is in the range of **1–3%** for **Ignore** (blue box in Figure 19. If this does not provide optimal contrast, drag the ends of the diagonal line (see arrows in Figure 19) closer to the range of the histogram peaks and zoom out when satisfied with the focus in the Z-plane.

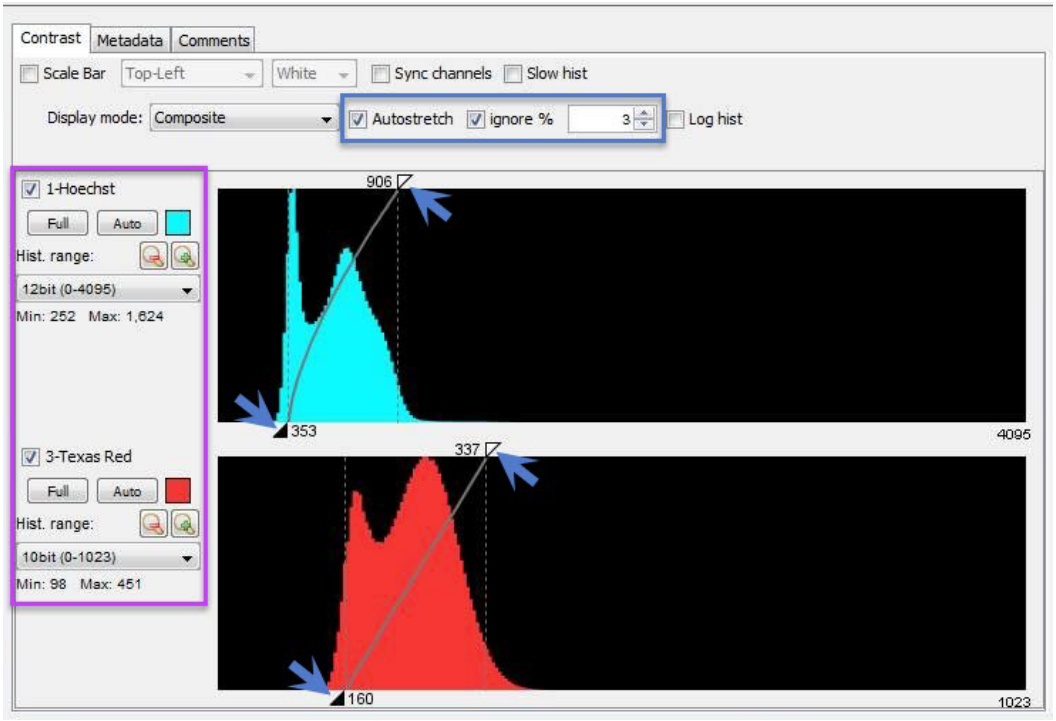


Figure 19. Adjusting camera depth and image contrast.

19. After imaging is complete, the software will provide the prompt "Run CellSelect with images from: `C:\Wafergen\WafergenData` For Chip: <Chip ID>?". If proceeding to the next protocol (Section V.D, below), click **Yes**. This will launch the CellSelect Software. If you plan to analyze the images later, click **No** and close the Micro-Manager software.

> **NOTE:** The directory will be different if users select to save the data elsewhere.

20. Reapply the peeled release liner (from Step 9) onto the top side of the double-sided intermediate film layer on the chip.
21. Place the imaged chip into an empty ICELL8 Chip Holder prechilled at –80°C (Figure 8, above). Make sure that the Optical Imaging Film is well sealed over the chip. The chip holder should click closed and should close evenly, indicating a proper magnetic seal.
22. Freeze cells at –80°C for a minimum of 30 min before proceeding to Preheat and RT-PCR (Section V.E, below).
23. Turn off the controllers for the microscope imaging filters and stage, the camera, and the fluorescence light source when the imaging is completed.

> **SAFE STOPPING POINT:** The chip can be frozen in the chip holder at –80°C overnight.

D. Protocol: Analyze Nanowells with CellSelect Software

In this protocol, CellSelect Software is used to analyze the images of the ICELL8 250v Chip in order to identify nanowells containing viable single cells that are suitable for further processing and analysis. Refer to the CellSelect Software User Manual for detailed information about using the software.

Procedure

Load images

1. If continuing from the previous protocol (Section V.C, above) skip to Step 2. Otherwise, start the CellSelect Software by clicking the **CellSelect** icon below and import the chip folder by clicking the **File > Open chip folder** menu option.

2. Click [Yes] when prompted to convert the detected Micro-Manager image stack.

Figure 20. Converting image stack.

NOTE: This prompt will only display the first time that chip images are loaded into the CellSelect Software.

3. Check the chip number when prompted for Chip ID.

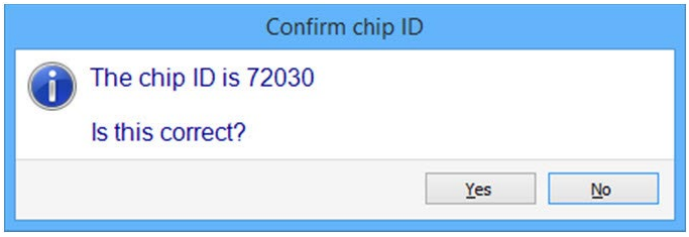


Figure 21. Confirming the chip ID.

4. Add a description, if helpful, when prompted.

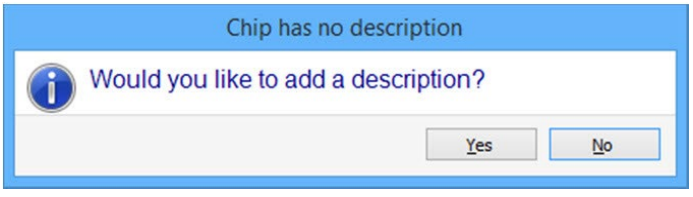


Figure 22. Adding a description for the chip.

5. Load the settings file, `StandardCellSettings-V5.1.xml`, shown below.

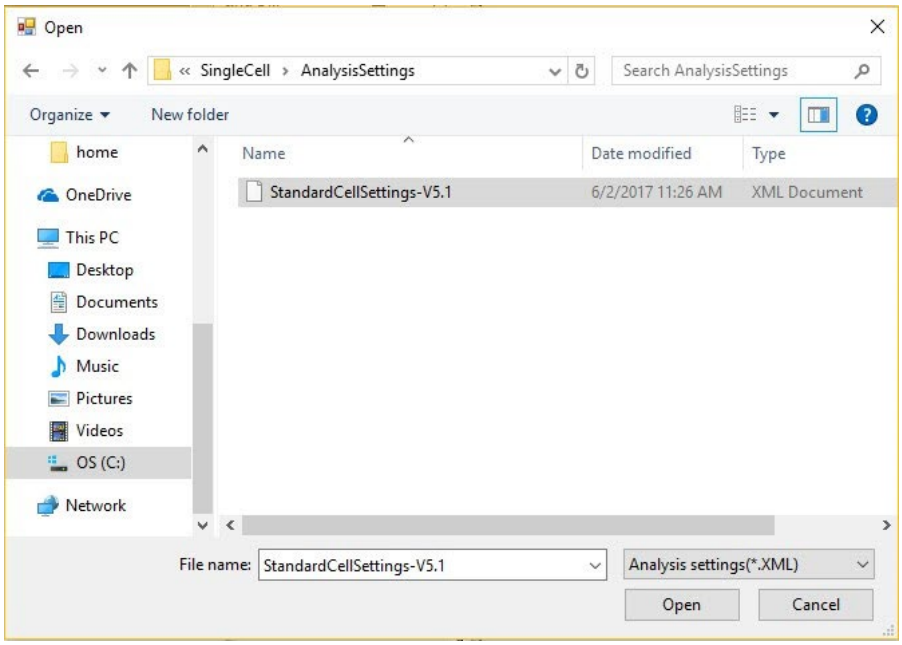


Figure 23. Loading the settings file.

Define fiducials

6. The software will automatically detect fiducials and prompts you with four consecutive fiducial images. The [Define fiducials] box in the top-left portion in the *Main* window will be highlighted as this process occurs (see Figure 24). The first fiducial image (Pos0) shows five green circles, while the other three fiducial images (Pos11, Pos132, Pos143) show four green circles. The green circles highlight the centers of the nanowells (Figure 24).

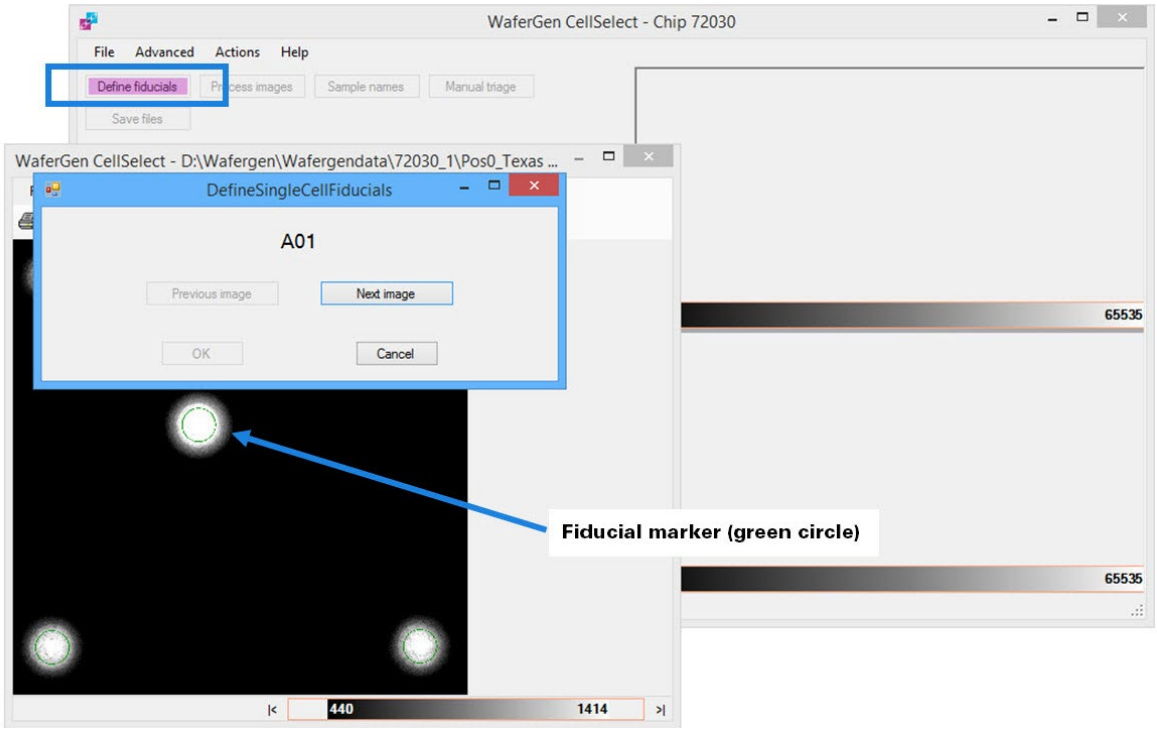


Figure 24. Defining fiducials.

7. Inspect the fiducials in each fiducial image for proper positioning.

   a. Click and drag the *DefineSingleCellFiducials* prompt window to the side to view the fiducials image (see Figure 25, below).

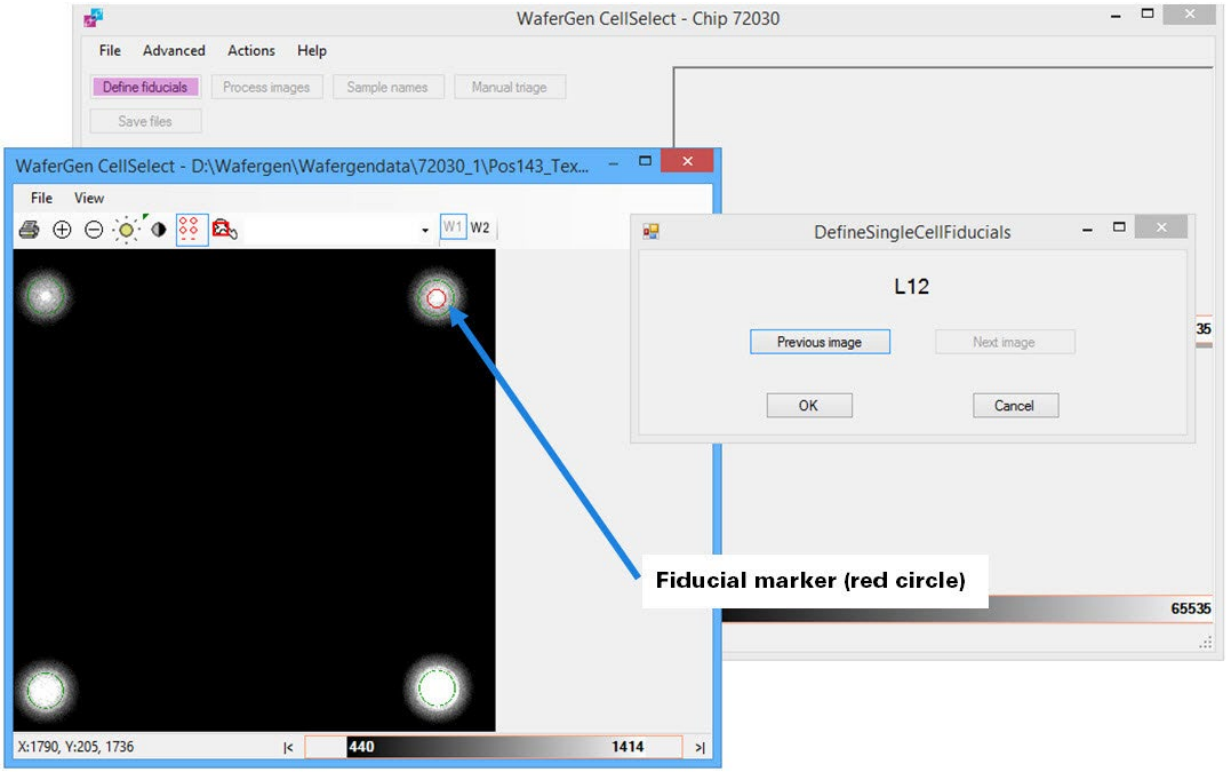


Figure 25. Inspecting and adjusting fiducials.

   b. If the green circles are not centered properly, click on the nanowell centers with the mouse pointer (changes to red circle; see Figure 25, above).

   c. If necessary, resize the fiducial image to view a 6 x 6 grid (36 nanowells). Use the following tools in the *Image Viewer* toolbar to check and correct fiducials: **Zoom in/Zoom out**, **Image contrast**, **Auto contrast**, **Well overlay**, **Wavelength 1 or 2** (W1, W2). Figure 26 provides a legend for the *Image Viewer* toolbar icons.

Figure 26. *Image Viewer* toolbar icons.

d. Click through all four fiducial images and confirm that the chip was oriented correctly for dispensing samples. The image for the Pos0 field of view should show five fiducial-containing nanowells, while the images for the Pos11, Pos132, and Pos143 fields of view should each show four fiducial-containing nanowells. If the layout of fiducial-containing nanowells is correct, click [OK] and close the last fiducial image. If the layout of fiducial-containing nanowells is incorrect, contact Technical Support.

Load barcode file

8. In the *Main* window, select the **File > Load barcode file** menu option.

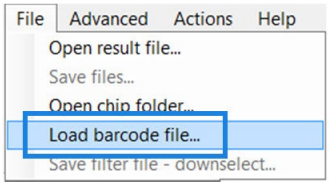


Figure 27. File menu.

9. From the pop-up window of the barcode file, select the preloaded file: `SMART-seq_SetA.xml` or `SMART_seq_SetB.xml`.

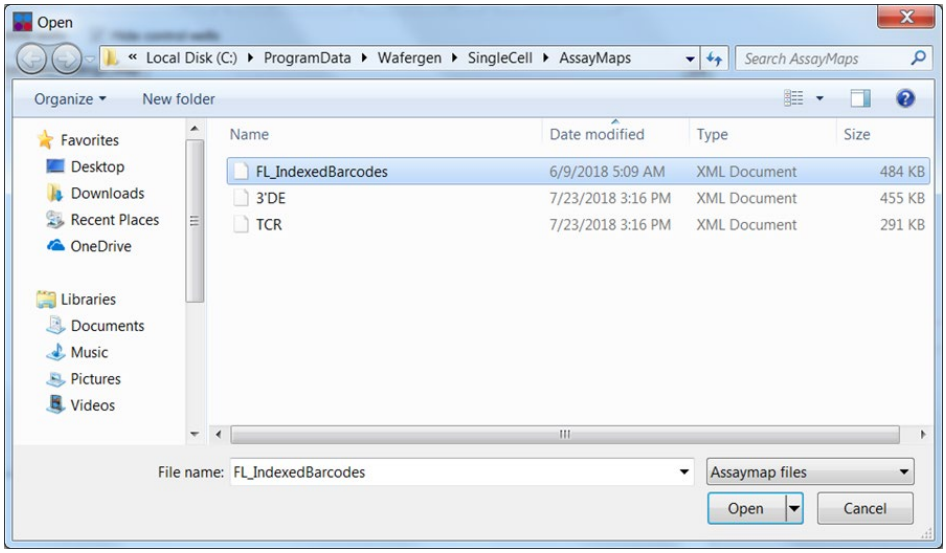


Figure 28. Loading the barcode file for the chip.

Process images

10. Click [Process Images]. The software will analyze two sets of 144 multi-well images taken using the Hoechst and Texas Red channels, respectively, and automatically identify and select all nanowells that contain viable, single cells (i.e., "candidates") and controls based on the parameters defined in *Settings*.

NOTE: For each image, the following criteria must be satisfied for the software to identify a cell as being a candidate for downstream analysis:

- The cell must appear in the Hoechst channel and NOT in the Texas Red channel.
- No other cells are visible in the well.
- The cell must satisfy the size/shape parameters specified in Settings.

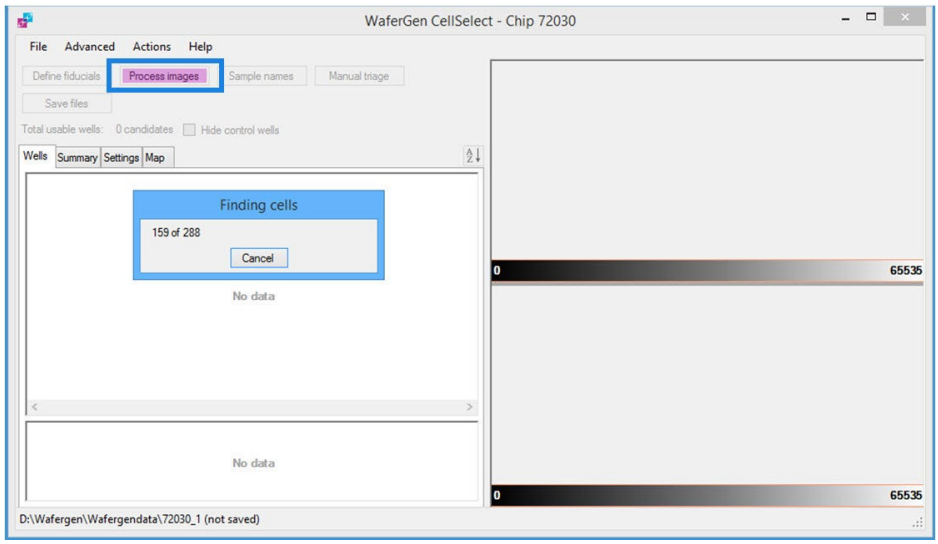
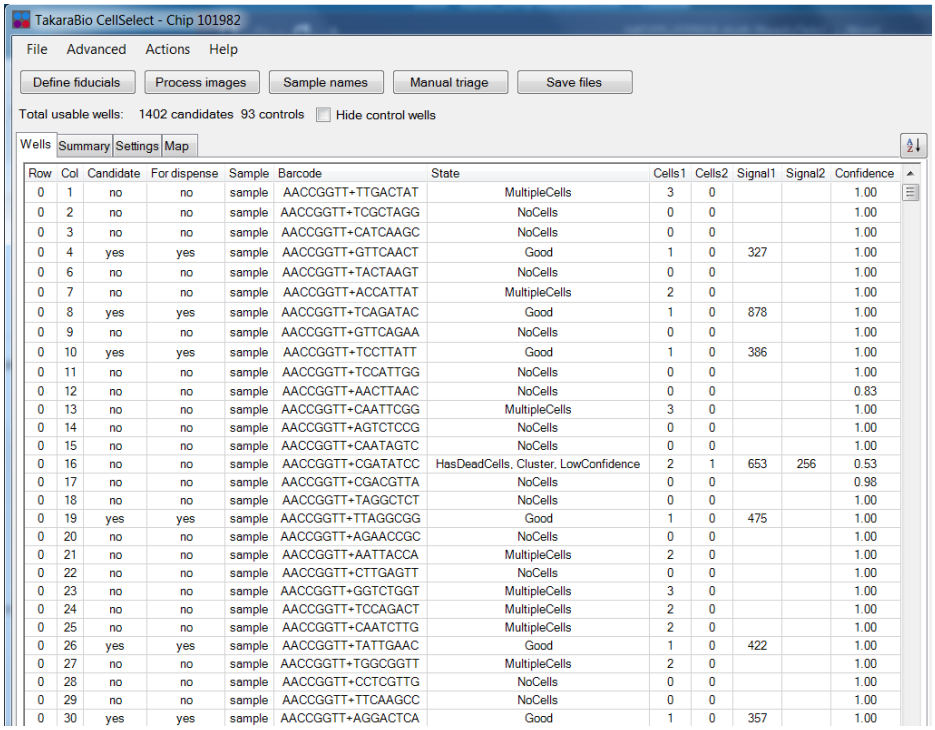


Figure 29. Having the software find cells based on parameters defined in *Settings*.

11. After processing is complete, the software will provide a prompt for saving results. Click [Yes] and input a file name prefixed with the chip number (`<Chip ID>_<Date>.wcd`). The software will then do the following:
    - Select wells among all those that are checked under Candidate according to the best parameters defined in the *Settings* tab.
    - Save the result file under the specified name (`<Chip ID>_<Date>.wcd`). The result file will contain the selected nanowells and setting parameters.
    - Automatically generate the filter file for dispensing RT reagents (`<Chip ID>_FilterFile.csv`). The selected wells in the filter file will be displayed in the *For Dispense* tab.
12. Load the results along with the settings from the saved WCD file (Figure 30, below). During the 3rd and 5th dispenses, a unique combination of barcodes will be dispensed into each selected well in the chip, as shown in the Barcode column. The software uses the following legend when examining nanowells:
    - **Teal circle around nanowell** = candidate well
    - **Pink circle around nanowell** = non-candidate well
    - **Green circle around nanowell** = control well
    - **Green outline around object** = a live cell
    - **Yellow outline around object** = artifact, ignored
    - **Blue circle around object** = a reflection artifact
    - **Green dot in center of nanowell** = marks the center of the well; not a cell

    Refer to the CellSelect Software User Manual for more information.



Row	Col	Candidate	For dispense	Sample	Barcode	State	Cells1	Cells2	Signal1	Signal2	Confidence
0	1	no	no	sample	AACCGGTT+TTGACTAT	MultipleCells	3	0			1.00
0	2	no	no	sample	AACCGGTT+TCGCTAGG	NoCells	0	0			1.00
0	3	no	no	sample	AACCGGTT+CATCAAGC	NoCells	0	0			1.00
0	4	yes	yes	sample	AACCGGTT+GTTCAACT	Good	1	0	327		1.00
0	6	no	no	sample	AACCGGTT+TACTAAGT	NoCells	0	0			1.00
0	7	no	no	sample	AACCGGTT+ACCATTAT	MultipleCells	2	0			1.00
0	8	yes	yes	sample	AACCGGTT+TCAGATAC	Good	1	0	878		1.00
0	9	no	no	sample	AACCGGTT+GTTCAGAA	NoCells	0	0			1.00
0	10	yes	yes	sample	AACCGGTT+TCCTTATT	Good	1	0	386		1.00
0	11	no	no	sample	AACCGGTT+TCCATTGG	NoCells	0	0			1.00
0	12	no	no	sample	AACCGGTT+AACTTAAC	NoCells	0	0			0.83
0	13	no	no	sample	AACCGGTT+CAATTCGG	MultipleCells	3	0			1.00
0	14	no	no	sample	AACCGGTT+AGTCTCCG	NoCells	0	0			1.00
0	15	no	no	sample	AACCGGTT+CAATAGTC	NoCells	0	0			1.00
0	16	no	no	sample	AACCGGTT+CGATATCC	HasDeadCells, Cluster, LowConfidence	2	1	653	256	0.53
0	17	no	no	sample	AACCGGTT+CGACGTTA	NoCells	0	0			0.98
0	18	no	no	sample	AACCGGTT+TAGGCTCT	NoCells	0	0			1.00
0	19	yes	yes	sample	AACCGGTT+TTAGGCGG	Good	1	0	475		1.00
0	20	no	no	sample	AACCGGTT+AGAACCGC	NoCells	0	0			1.00
0	21	no	no	sample	AACCGGTT+AATTACCA	MultipleCells	2	0			1.00
0	22	no	no	sample	AACCGGTT+CTTGAGTT	NoCells	0	0			1.00
0	23	no	no	sample	AACCGGTT+GGTCTGGT	MultipleCells	3	0			1.00
0	24	no	no	sample	AACCGGTT+TCCAGACT	MultipleCells	2	0			1.00
0	25	no	no	sample	AACCGGTT+CAATCTTG	MultipleCells	2	0			1.00
0	26	yes	yes	sample	AACCGGTT+TATTGAAC	Good	1	0	422		1.00
0	27	no	no	sample	AACCGGTT+TGGCGGTT	MultipleCells	2	0			1.00
0	28	no	no	sample	AACCGGTT+CCTCGTTG	NoCells	0	0			1.00
0	29	no	no	sample	AACCGGTT+TTCAAGCC	NoCells	0	0			1.00
0	30	yes	yes	sample	AACCGGTT+AGGACTCA	Good	1	0	357		1.00

Figure 30. Viewing the results list after reading the image stack.

Specify sample names

13. Click [Sample names] and enter the sample description; enter one sample name for all samples (Figure 31, below), or multiple sample names based on the source plate configuration layout in Figure 9, Section V.B (above).

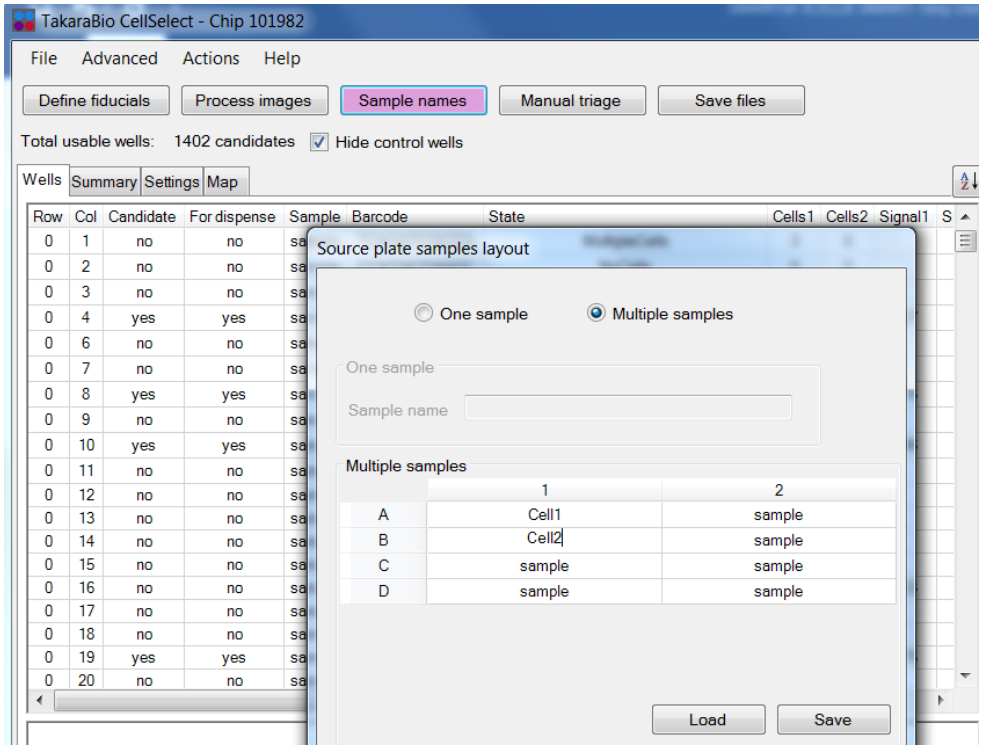


Figure 31. Specifying sample names. For the One sample option, "Test" is repeated down the "Sample" column. Control and fiducial wells are identified automatically.

Review images

14. Click the *Wells* tab and sort the nanowell statistics in the "Candidate" column by clicking the **Candidate** heading (see Figure 32, below). The software will sort and consolidate all selected nanowells (i.e., "candidates") to the top of the table.

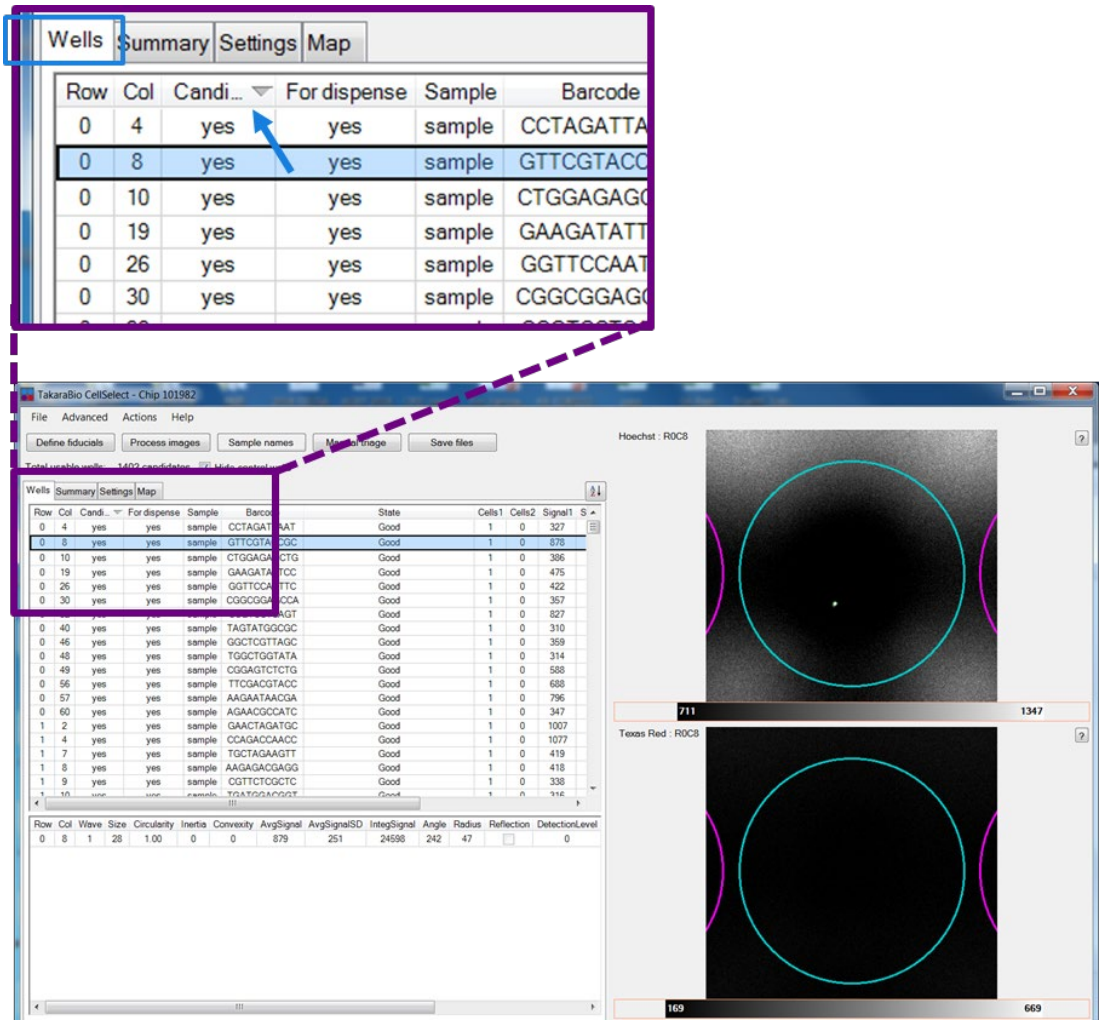


Figure 32. Sorting the candidate column to consolidate all selected nanowells to the top of the table.

15. If desired, manually inspect the selected nanowells. Click any row for closeup views of the selected nanowell in both the Hoechst and Texas Red channels (top and bottom single-well images, respectively, in Figure 32, above).
16. Selected nanowells can also be viewed in the context of the corresponding multi-well image via the *Image Viewer* window: click the **Advanced > Image viewer** menu item (Figure 33, below). A multi-well image highlighting the nanowell selected in the results table (on the *Wells* tab) will appear.
17. Arrange the windows as demonstrated in Figure 33 (below). When you click on any of the Candidate rows in the results table (on the *Wells* tab), the software will present corresponding single-well images, and display the corresponding multi-well image in the *Image Viewer* window.
18. To manually exclude one or more candidate wells, right-click the highlighted rows on the *Wells* tab and select **Exclude selected wells**. To exclude several candidate wells, consider using the [Manual

triage] function. Refer to the corresponding section of the CellSelect Software User Manual for more information.

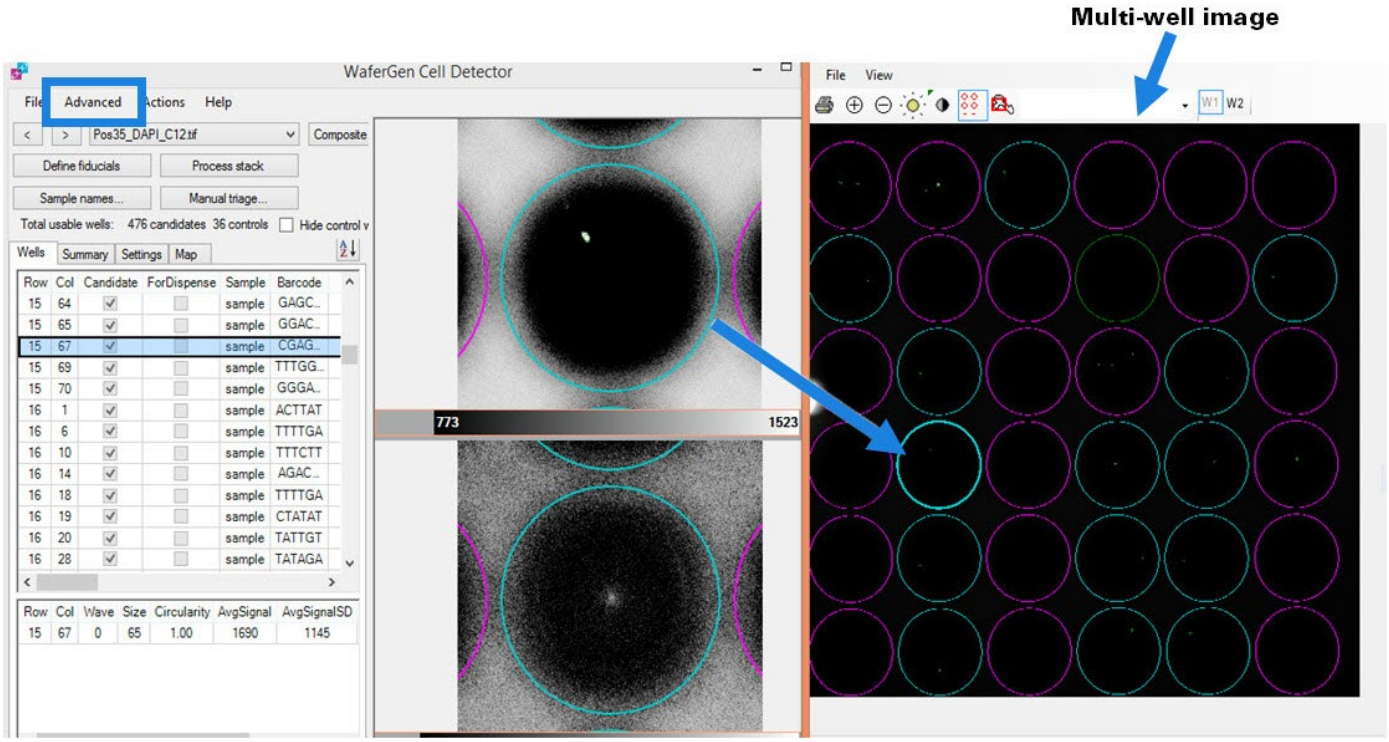


Figure 33. Arrangement of CellSelect Software windows. The image on the far right is the multi-well image. The horizontally split images in the center show a single cell in a nanowell stained by Hoechst 33342 (top) and the absence of propidium iodide stain in the corresponding position (dead-cell stain; bottom). In the bottom image, the green dot in the center (if seen) marks the bottom of the nanowell and is probably not from a dead cell because it does not match the location of the Hoechst-stained cell.

Save files

19. Click the **File > Save Files** to save any changes you have made to the results file. We strongly recommend using a new file name to avoid overwriting the original results file. The file is saved with the `.wcd` file extension and stored in the `C:\Users\ICELL8\WafergenData` folder.

    NOTE: If the image files were saved in a folder or directory other than the default (`C:\Users\ICELL8\WafergenData`), the CellSelect Software will save to the same directory used where the images were saved.

20. Select the *Summary* tab to view the number of wells selected for dispensing of RT-PCR Master Mix in the next protocol.

21. Depending on your experimental aims, you may wish to "downselect," or reduce the number of wells that are processed further. If you would like to downselect nanowells, click the **Actions > Downselect**.

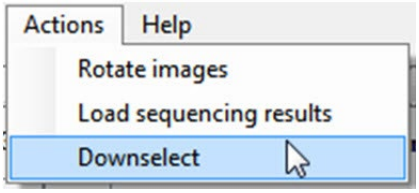


Figure 34. Choosing Downselect from the Actions menu.

22. Enter the desired number of control and sample wells to be included (Figure 35, below).

> **NOTE:** If you enter numbers much greater than the number of wells checked under the "Candidate" column, the software will automatically select the maximum possible number of candidate wells (this is a quick and easy way to select all candidate wells, especially when working with multiple different samples).

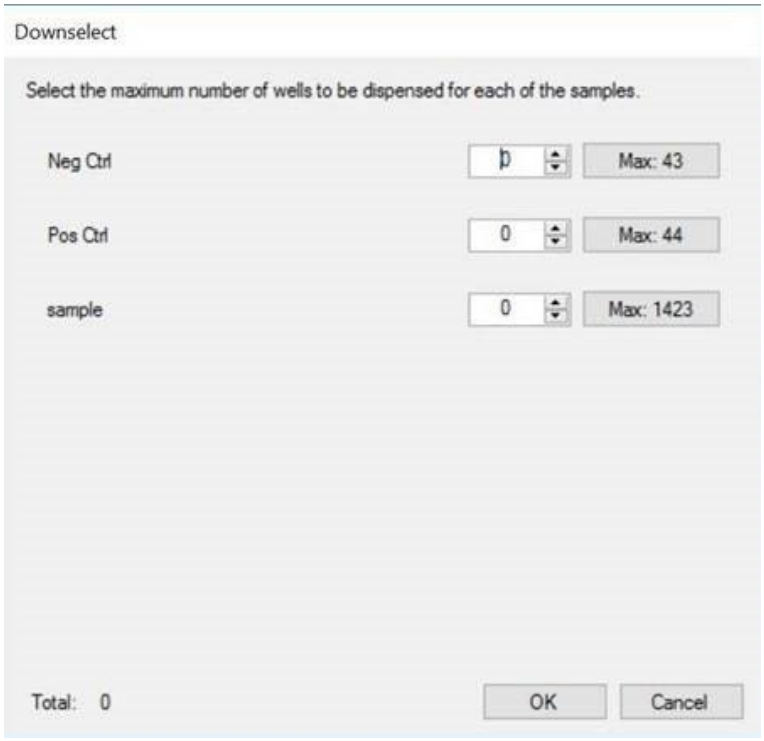


Figure 35. Downselecting nanowells for the filter file.

23. Save the filter file after downselecting the number of wells. A 72 x 72 grid of the entire chip will be saved as a `.csv` file automatically named as `<Chip ID>.csv`, in which nanowells to be included in subsequent dispensations are marked with a 1, and nanowells to be excluded are marked with a 0.
24. Copy the filter file (`.csv`) and bring it with you to the ICELL8 MSND. It will be used for dispensing the RT-PCR reaction mix in the next protocol (Section V.E, below).

E. Protocol: Full-Length cDNA Synthesis by RT-PCR (2nd Dispense)

First-strand cDNA is synthesized by the oligo dT primer (SMART-Seq ICELL8 CDS) and followed by the template switching oligo (SMART-Seq ICELL8 Oligonucleotide) for template switching at the 5′ end of transcripts. The first-strand cDNA is used as template for 2nd strand-cDNA synthesis and the amplification during PCR.

Before you start

- If needed, perform a once-a-day warmup: initialize the ICELL8 MSND; from the *Startup* tab, complete each step in the Daily Checklist, and then click the [Daily Warmup] button. After this process is complete, go to the *Advanced* tab and execute the **Wash Prime** function followed by **Tip Clean**, and then repeat the **Tip Clean** function 2–3 more times.

- Prefreeze the chip Cold Block at –80°C.
- Set the centrifuge(s) used for spinning the chip and MSND 384-Well Source Plate to 4°C.
- Preprogram the thermal cycler with Preheat (Step 1) and RT-PCR (Step 6) before the experiments.
- Run and hold the programs before the reactions.
- This protocol requires a 384-Well Source Plate (with seal) and the following components: Nuclease-Free Water (white cap), SMART-Seq ICELL8 RT-PCR Buffer (red cap), SMART-Seq ICELL8 Oligonucleotide (orange cap), RNase Inhibitor (white cap), Terra PCR Direct Polymerase Mix (pink cap), and SMARTScribe Reverse Transcriptase (purple cap)—all from the SMART-Seq ICELL8 Reagent Kit—plus Blotting Paper and RC Film (from the ICELL8 Loading Kit – B).
- Thaw all reagents on ice except for the enzymes. Remove the enzymes from –20°C storage just prior to use and keep them on ice at all times. Gently vortex and spin down all thawed reagents and enzymes.
- Remove the ICELL8 Chip Holder containing the ICELL8 250v Chip from the –80°C freezer. Thaw the chip in the chip holder until it reaches room temperature (about 10 min) to lyse cells. Take the 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 Preheat.

Procedure

1. Run and hold the Preheat program below. Place the ICELL8 250v Chip into the ICELL8 Chip Cycler that has been preheated to 76°C and a heated-lid temperature of 72°C. Resume the following Preheat program:

76°C	5 sec
72°C	3 min

2. After the 3 min incubation at 72°C, immediately place the chip on a chip Cold Block on ice at least for 2 min.
3. Centrifuge the chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4°C and keep the chip on chip Cold Block on ice until ready for RT-PCR dispense.
4. Run and hold the program for RT-PCR with a heated lid.
5. Mix all components except for SMARTScribe Reverse Transcriptase in the order listed in Step 6, and then vortex it briefly.
6. Add the SMARTScribe Reverse Transcriptase to the Master Mix immediately prior to use. Mix it by vortexing gently.

   IMPORTANT: Remove the reverse transcriptase and PCR polymerase from the freezer, gently tap the tubes to mix, and add to the RT-PCR reaction mix. Mix by gently vortexing for 1–2 seconds and spin the tube briefly in a minicentrifuge to collect contents.

83.6 µl	Nuclease-Free Water (white)
105.6 µl	SMART-Seq ICELL8 RT-PCR Buffer (red)
8.8 µl	SMART-Seq ICELL8 Oligonucleotide (orange)
4.4 µl	RNase Inhibitor (white)
8.8 µl	Terra PCR Direct Polymerase Mix (pink)
8.8 µl	SMARTScribe Reverse Transcriptase (200 U/µl; purple)
220.0 µl	Total volume

7. Bring the RT-PCR Master Mix to room temperature and pipette 50 µl of RT-PCR Master Mix into the 384-plate source wells (A3, B3, C3, and D3) highlighted in the figure below.

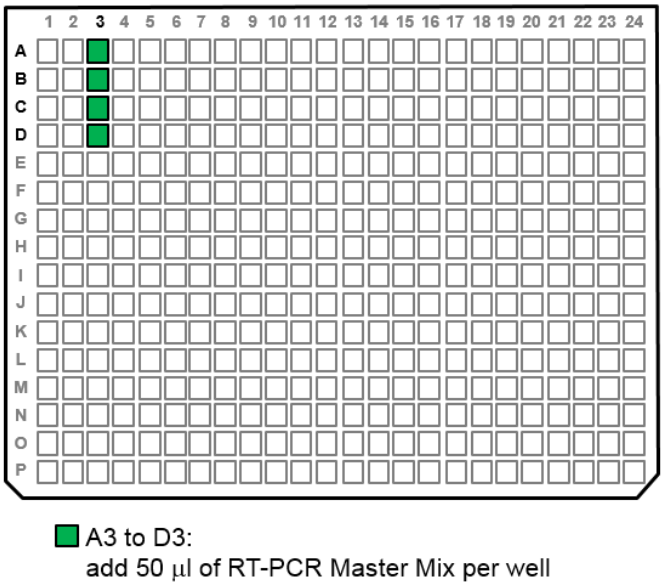


Figure 36. Aliquot 50 µl of RT-PCR Master Mix to the colored source wells.

8. Seal the MSND 384-Well Source Plate with a MSND 384-well plate seal.
9. Centrifuge the chip in the Chip Spinner and the source plate separately at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.
10. Remove the seal and place the MSND 384-Well Source Plate in the ICELL8 MSND.
11. Peel off the imaging film from the chip and place it on the Dispensing Platform. Make sure that the chamfered corner is facing the bottom right.

    IMPORTANT: The edges of the chip should be pressed against the three alignment pins; **make sure that the chip is flat on the chuck from all sides and is in the correct orientation** (see Section IV.E, Figure 6, above).

12. On the ICELL8 MSND software screen, under the *FLA* tab, type or scan the chip number in the "Chip ID" field.
13. Under the *FLA* tab, click the browse button next to the Filter file field to load the `<Chip Number>.CSV` filter file (Figure 37).

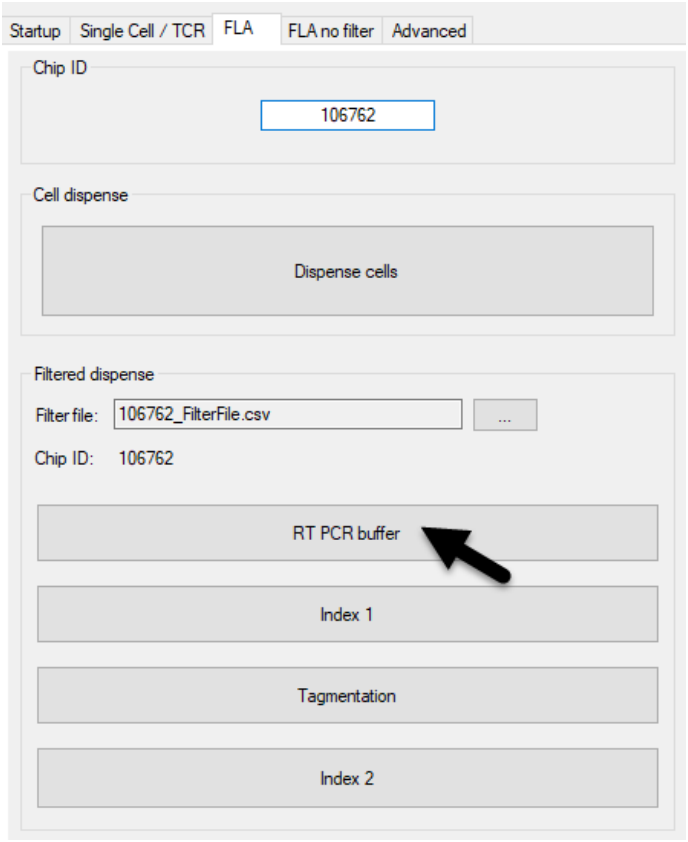


Figure 37. Load the PredispensedFilter.csv file into the ICELL8 MSND software and choose [RT PCR buffer].

14. Click [RT PCR buffer] and carefully go through all questions prompted and then click **OK**. The ICELL8 MSND will dispense 35 nl of RT-PCR Master Mix into each nanowell.
15. After dispensation is completed, remove the chip from the Dispensing Platform and gently blot for two seconds with a fresh Blotting Paper and a Blotter (Section V.B, Figure 11).
16. Seal the blotted chip with a RC Film using a Film Sealing Roller (Figure 38, below).

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

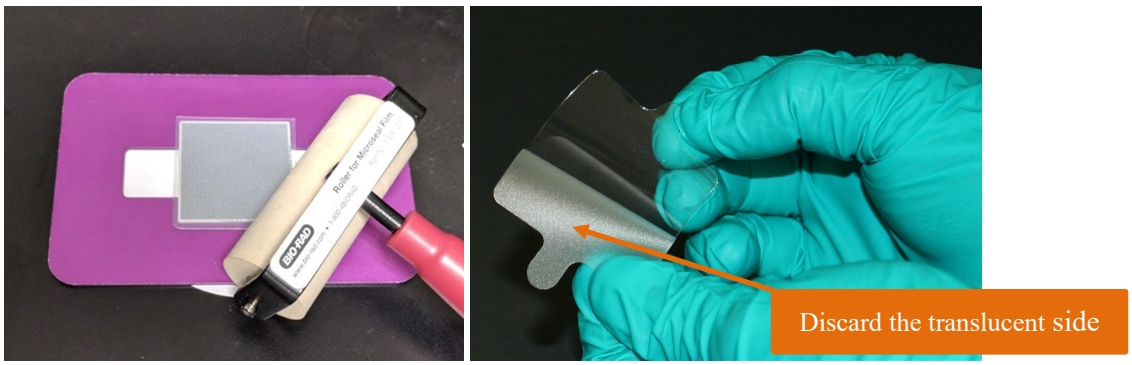


Figure 38. Preparing and adhering the RC film. RC film is composed of two layers. One layer is clear, and the other layer is translucent. **Left panel.** When sealing a chip, remove the translucent layer and discard it, and then put the remaining layer on the chip. **Right Panel.** Tightly adhere the film using the film sealing roller. Make sure that the chip is securely sealed to avoid well-to-well contamination and evaporation.

17. On the ICELL8 MSND, perform the **Tip Clean** procedure 3–4 times.

18. Centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.
19. Place the chip into the ICELL8 Chip Cycler with a heated-lid temperature of 72°C, and hold the RT-PCR program at the first step (Section V.E, Step 4).
20. Resume the RT-PCR program:

42.3°C	5 sec
36.6°C	3 hours
99°C	9 sec
95.5°C	1 min
14 cycles*:	
100°C	5 sec
99°C	7 sec
59°C	5 sec
64°C	30 sec
69.5°C	5 sec
67.5°C	6 min
4°C	forever

*See Appendix B for cycling guidelines.

SAFE STOPPING POINT: The chip can be left in the ICELL8 Chip Cycler at 4°C overnight.

F. Protocol: Forward Indexing Primers (3rd Dispense)

72 forward indexing primers are dispensed from the prealiquoted 384-well plate (stored at –70ºC), which is used for Library PCR 1.

Before you start

- If needed, perform a once-a-day warmup: initialize the ICELL8 MSND; from the *Startup* tab, complete each step in the Daily Checklist, and then click the [Daily Warmup] button. After this process is complete, go to the *Advanced* tab and execute the **Wash Prime** function followed by **Tip Clean**, and then repeat the **Tip Clean** function 2–3 more times.
- Set the centrifuge(s) used for spinning the chip and MSND 384-Well Source Plate to 4°C.
- This protocol requires the 384-well plate containing 72 forward indexing primers (stored at –70°C), a Plate Seal Applicator, a film sealing roller, and the following components: Blotting Paper and RC Film (from the ICELL8 Loading Kit – B).
- Prepare the 384-well plate containing 72 forward indexing primers:

  IMPORTANT: Steps a)–e) should be performed before you begin the entire experiment.

  a. Take the 384-well plate out of the –80°C freezer and seal it using a film applicator immediately before thawing the plate. (Do not open the sealing foil.)

  IMPORTANT: The sealing foil is securely sealed when manufactured. However, the foil might get partly opened during shipment. Therefore, it is important to seal it again to avoid index contamination among wells.

b. Without opening the sealing foil, thaw the frozen indexes at room temperature and seal again using a film applicator.

c. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 3 min between 4°C and room temperature.

d. Shake the plate with a 384-well plate shaker for 1 min at room temperature.

e. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 3 min between 4°C and room temperature. If centrifuging at 4°C, bring the plate back to room temperature prior to dispensing.

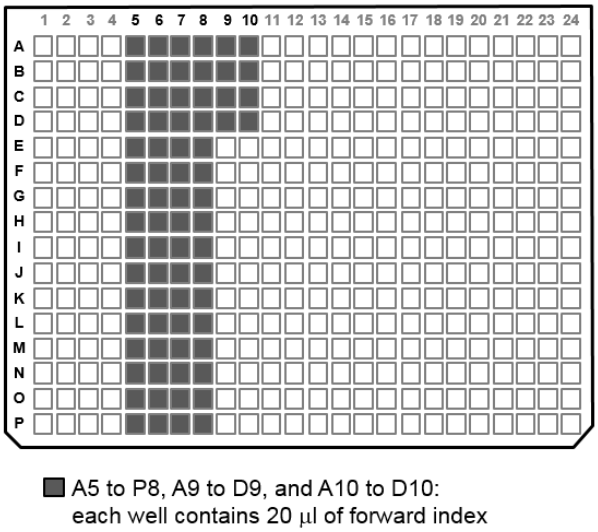


Figure 39. SMART-Seq ICELL8 forward indexing primers. The 72 forward indexes shown in black are prealiquoted into a 384-well plate (20 µl per well). See Appendix A for barcode sequences.

Procedure

1. Remove the chip from the ICELL8 Chip Cycler (Section V.E, Step 20). Centrifuge the chip at 3,220*g* for 3 min at 4°C.
2. Peel off the RC Film from the chip. Place the chip on the Dispensing Platform and make sure that the chamfered corner is facing the bottom right. Make sure that the **Tip Clean** procedure was done 3–4 times.
3. Remove the sealing foil from 384-well plate containing forward indexing primers and place it in the ICELL8 MSND.
4. On the ICELL8 MSND software screen, under the *FLA* tab, type or scan the chip number in the "Chip ID" field.
5. Under the *FLA* tab, click the browse button next to the Filter file field to load the `<Chip Number>.CSV` filter file (Figure 40).

IMPORTANT: Make sure that the Chip ID matches the filter file selected.

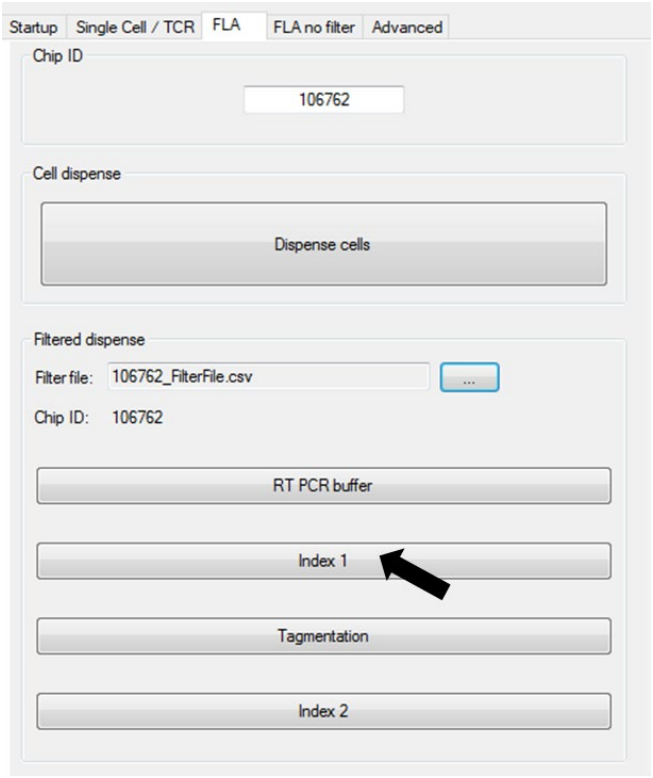


Figure 40. Load the PredispensedFilter.csv file into the ICELL8 MSND software and choose [Index 1].

6. Click [Index 1] and carefully go through all questions prompted and then click **OK**. The ICELL8 MSND will dispense 35 nl of forward indexing primers into each nanowell.

> **IMPORTANT:** Make sure that the chip is flat on the chuck and the 384-well index place is fully seated in the correct position.

7. After dispensation is completed, remove the chip from the Dispensing Platform and gently blot for two seconds with a fresh Blotting Paper and a Blotter (Figure 11).
8. Seal the blotted chip with a RC Film using a film sealing roller (Section V.B, Figure 12). Make sure that the chip is securely sealed to avoid well-to-well contamination.

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

9. On the ICELL8 MSND, perform the **Tip Clean** procedure 3–4 times.
10. Centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.

G. Protocol: Tagmentation (4th Dispense)

Synthesized full-length cDNAs are tagmented by Tagment DNA Enzyme (TDE1).

Before you start

- Preprogram the thermal cycler with the Tagmentation program (Step 14) before the experiment.
- Run and hold the program before the reaction.

- This protocol requires an MSND 384-Well Source Plate (with seal), TDE1, and the following components: Nuclease-Free Water (white cap), Terra PCR Direct Polymerase Mix (pink cap), TRH (white cap), $MgCl_2$ (white cap)—all from the SMART-Seq ICELL8 Reagent Kit—Blotting Paper, and RC Film (from the ICELL8 Loading Kit – B).
- Thaw Nuclease-Free Water and $MgCl_2$ and remove the enzymes from –20°C storage just prior to use and keep them on ice at all times. Gently vortex and spin down the thawed $MgCl_2$ and enzymes.

Procedure

1. Make Tagmentation Master Mix by mixing all components in the order listed in table below. Vortex briefly.

52.80 µl	Nuclease-Free Water (white)
139.04 µl	$MgCl_2$ (white)
17.60 µl	TDE1
8.80 µl	Terra PCR Direct Polymerase Mix (pink)
1.76 µl	TRH (white)
220.0 µl	Total volume

2. Bring the Master Mix to room temperature and pipette 50 µl into the 384-plate source wells (A11, B11, C11, and D11) as highlighted in Figure 41, below.

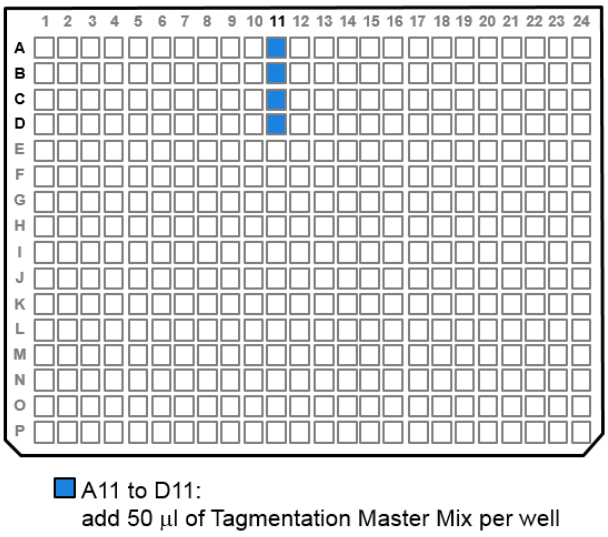


Figure 41. Add Tagmentation Master Mix to the source plate wells shown in blue.

3. Seal the MSND 384-Well Source Plate with the plate seal.
4. Centrifuge the source plate at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC. Remove the seal and place the MSND 384-Well Source Plate in the ICELL8 MSND.
5. Peel off the RC Film from the chip after 3rd dispense (Section V.F, Step 10). Place the chip on the Dispensing Platform and make sure that the chamfered corner is facing the bottom right. Make sure that the **Tip Clean** procedure was done 3–4 times after the 3rd dispense.
6. On the ICELL8 MSND software screen, under the *FLA* tab, type or scan the chip number in the "Chip ID" field.
7. Under the *FLA* tab, click the browse button next to the Filter file field to load the `<Chip Number>.CSV` filter file (Figure 42, below).

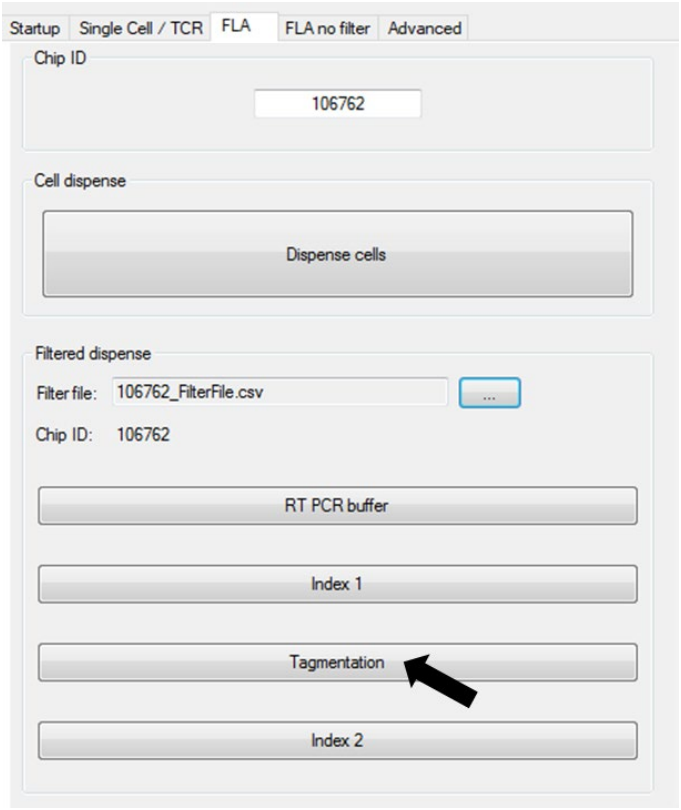


Figure 42. Load the `PredispensedFilter.csv` file into the ICELL8 MSND software and choose [Tagmentation].

8. Click [Tagmentation] and carefully go through all questions prompted and then click **OK**. The ICELL8 MSND will dispense 35 nl of tagmentation Master Mix into each nanowell.
9. After dispensation is completed, remove the chip from the Dispensing Platform and gently blot for two seconds with a fresh Blotting Paper and a Blotter (Figure 11).
10. Seal the blotted chip with a RC Film using a film sealing roller (Section V.B, Figure 12). Make sure that the chip is securely sealed to avoid well-to-well contamination.

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

11. On the ICELL8 MSND, perform the **Tip Clean** procedure 3–4 times.
12. Centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.
13. Place the chip into the ICELL8 Chip Cycler with a heated-lid temperature of 40°C to perform tagmentation.
14. Run the Tagmentation program:

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

H. Protocol: Reverse Indexing Primers (5th Dispense) and Library PCR 1

Reverse indexing primers are dispensed from the prealiquoted 384-well plate (stored at –70ºC), which is used for Library PCR 1.

Before you start

- If needed, perform a once-a-day warmup: initialize the ICELL8 MSND; from the *Startup* tab, complete each step in the Daily Checklist, and then click the [Daily Warmup] button. After this process is complete, go to the *Advanced* tab and execute the **Wash Prime** function followed by **Tip Clean**, and then repeat the **Tip Clean** function 2–3 more times.
- Set the centrifuge(s) used for spinning the chip and MSND 384-Well Source Plate to 4°C.
- Preprogram the thermal cycler with the Library PCR 1 program (Step 12) before the experiment.
- Run and hold the program before the reaction.
- This protocol requires the 384-well plate containing 72 reverse indexing primers (stored at –70°C) and the following components from the ICELL8 Loading Kit – B: Blotting Paper and RC Film.
- Prepare the 384-well plate containing 72 reverse indexing primers.

IMPORTANT: Steps 1–5 should be performed before you begin the entire experiment.

1. Take the 384-well plate out of the –80°C freezer and seal it using a film applicator immediately before thawing the plate. (Do not open the sealing foil.)

   IMPORTANT: The sealing foil is securely sealed when manufactured. However, the foil might get partly opened during shipment. Therefore, it is important to seal it again to avoid index contamination among wells.

2. Without opening the sealing foil, thaw the frozen indexes at room temperature and seal again using a film applicator.
3. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 3 min between 4°C and room temperature.
4. Shake the plate with a 384-well plate shaker for 1 min at room temperature.
5. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 3 min between 4°C and room temperature. If centrifuging at 4°C, bring the plate back to room temperature prior to the dispense.

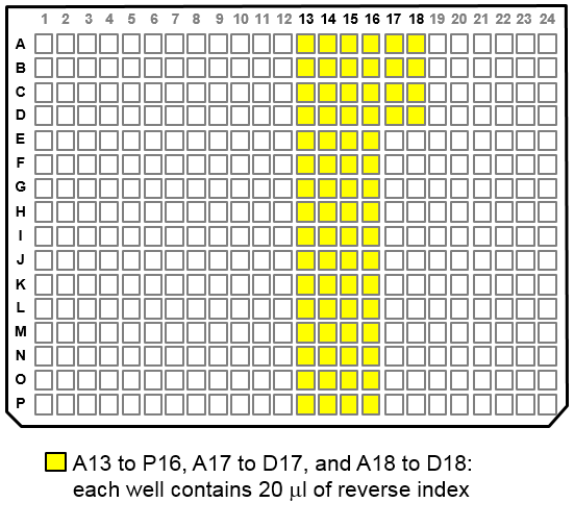


Figure 43. SMART-Seq ICELL8 reverse indexing primers. The 72 reverse indexes shown in yellow are prealiquoted into a 384-well plate (20 µl per well). See Appendix A for barcode sequences.

Procedure

1. Remove the chip from the ICELL8 Chip Cycler (Section V.G, Step 14). Centrifuge the chip at 3,220*g* for 3 min at 4°C.
2. Peel off the RC Film from the chip. Place the chip on the Dispensing Platform and make sure that the chamfered corner is facing the bottom right. Make sure that the **Tip Clean** procedure was done 3–4 times.
3. Remove the RC Film from the 384-well plate containing the reverse indexing primers (Section V.F, Step 10) and place the plate in the ICELL8 MSND.
4. On the ICELL8 MSND software screen, under the *FLA* tab, type the chip number in the "Chip ID" field.
5. Under the *FLA* tab, click the browse button next to the Filter file field to load the `<Chip Number>.CSV` filter file.

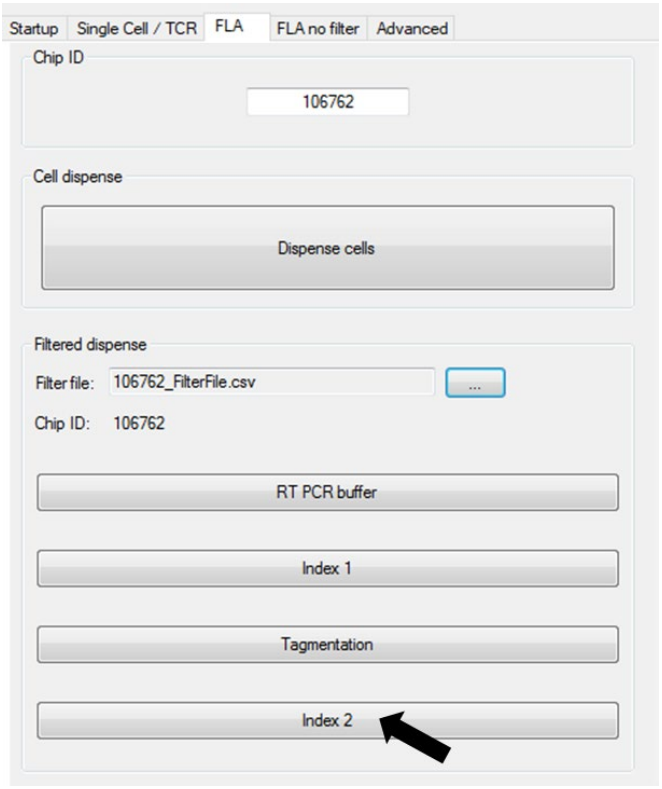


Figure 44. Load the PredispensedFilter.csv file into the ICELL8 MSND software and choose [Index 2].

6. Click [Index 2] and carefully go through all questions prompted and then click [OK]. The ICELL8 MSND will dispense 35 nl of reverse indexing primer into each nanowell.
7. After dispensation is completed, remove the chip from the Dispensing Platform and gently blot for two seconds with a fresh Blotting Paper and a Blotter (Figure 11).
8. Seal the blotted chip with an RC Film using a film sealing roller (Section V.B, Figure 12). Make sure that the chip is securely sealed to avoid well-to-well contamination.
9. On the ICELL8 MSND, perform the **Tip Clean** procedure 3–4 times
10. Centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.

11. Place the chip into the ICELL8 Chip Cycler with a heated-lid temperature of 72°C to perform Library PCR 1.
12. Resume the Library PCR 1 program:

77°C	12 sec
72°C	3 min
99°C	11 sec
95.5°C	1 min
6 cycles:	
100°C	20 sec
99°C	10 sec
53.3°C	5 sec
58°C	15 sec
71°C	5 sec
67.5°C	2 min
4°C	forever

SAFE STOPPING POINT: The chip can be left in the ICELL8 Chip Cycler at 4°C overnight.

I. Protocol: Library Extraction from the Chip

This protocol extracts the library amplified by Library PCR 1 from the chip.

Before you start

- Set the centrifuge(s) used for spinning the chip to 4°C.
- This protocol requires components from the ICELL8 Collection Kit – L: Collection Fixture – L, Collection Tube, and Collection Film.

Procedure

1. Remove the chip from the ICELL8 Chip Cycler. Centrifuge the chip at 3,220*g* for 3 min at 4°C.
2. Open the collection kit and label the Collection Tube with the engraved chip number. Assemble the collection module by attaching the Collection Tube to the Collection Fixture.

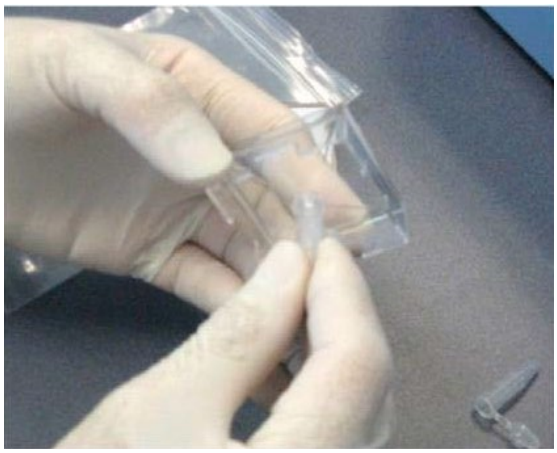


Figure 45. Assembling the collection module.

3. Carefully peel off the RC Film from the chip (Figure 46, below).

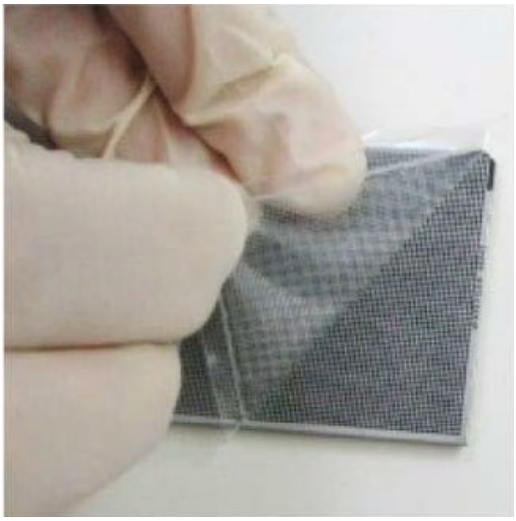


Figure 46. Removing the RC Film from the chip. Start from one corner and apply even pressure to pull.

4. With the nanowells facing down, place the chip into the assembled collection module (Collection Tube plus Collection Fixture; Figure 47, below). Surface tension will hold the liquid in the nanowells.

Figure 47. Placing the chip into the collection module.

5. Seal the chip and the top of the collection module with a supplied Collection Film (Figure 48, below).

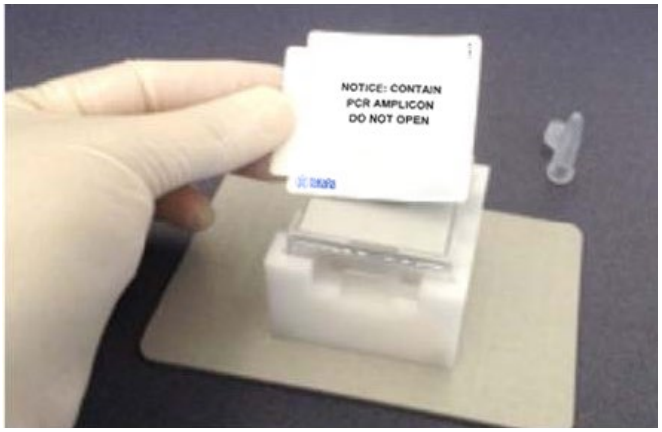


Figure 48. Securing the collection module with Collection Film.

6. Using a balance or blank chip, assemble another collection module. Centrifuge both collection modules at 3,220*g* (minimum 2,600*g*) for 10 min at 4°C.
7. Carefully remove the Collection Tube containing the extracted cDNA library (Figure 49, below).

IMPORTANT: Measure the volume of the collected eluate. The volume should be no less than 60% of the expected value. The expected volume (in µl) can be calculated with this formula:

[(5,184 wells x 35 nl/well) + (Number of candidates x 35 nl/well x 4 dispenses)] / [1,000 nl/µl]

For example, if 1,000 nanowells were selected for processing, the expected volume would be 321 µl. Therefore, you should expect to collect at least 60% of 321 µl, or 193 µl of eluate.

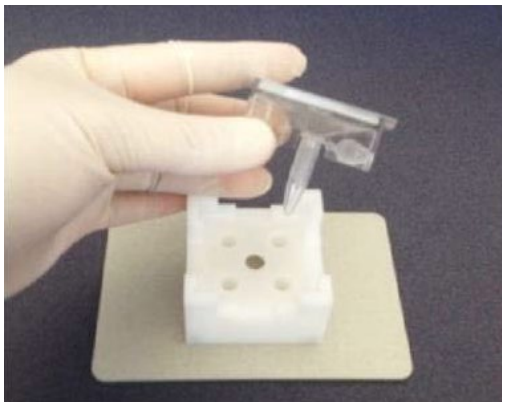


Figure 49. Removing the Collection Tube containing the eluate.

8. Attach the supplied second Collection Tube to the Collection Fixture and seal the entire module. Discard the module in a biohazard waste bin. DO NOT discard the collection module containing the balance or blank chip; retain it as a balance module for future extractions.

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

J. Protocol: Purification of the Extracted Library

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

Before you start

- Equilibrate an aliquot of AMPure XP beads to room temperature for ~30 min prior to use.

NOTE: 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 2 ml of 80% (v/v) ethanol. Use an anhydrous ethanol stock.
- This protocol requires Elution Buffer from the SMART-Seq ICELL8 Reagent Kit.
- Use only half volume of the extracted library and store the rest of half volume in a freezer as the backup.

Procedure

1. Transfer half of the volume of the extracted library to 0.2 ml PCR tube(s) and store the remaining half in a freezer as backup.

   NOTE: Add no more than 100 µl of library per tube. If you have more than 100 µl, split it into multiple tubes.

2. Add 1:1 volume of well-vortexed AMPure XP Beads. (For example, add 100 µl of AMPure XP beads to 100 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 binds to the beads.
5. Place the tube on the SMARTer-Seq Magnetic Separator - PCR Strip for ~5 min or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.

   NOTE: During the ~5 min 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. While the tube is sitting on the magnetic separator, pipette out the supernatant.
7. Keep the tube on the magnetic separator. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads. Wait for 30 seconds and carefully pipette out the supernatant containing contaminants. DNA will remain bound to the beads during the washing process.
8. Repeat Step 7 one more 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 seconds, and then remove all remaining ethanol by using 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: If you overdried the beads, you will see cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be less.

12. Once the beads are dried, add 50 µl of 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 separator for 1 minute or longer until the solution is completely clear.

    NOTE: During the incubation on the magnet, there may be a small population 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 clear supernatant containing purified library to a clean 0.2 ml nuclease-free nonsticky tube.
15. Add 50 µl of AMPure XP beads to the purified library.
16. Repeat Step 3–14 using 14 µl of Elution Buffer at Step 12.

K. Protocol: Library Amplification (Library PCR 2)

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

Before you start

- Preprogram the PCR thermal cycler with the Library PCR 2 program (Step 3) before the experiment.
- Run and hold the program before the reaction.
- This protocol requires the following components from the SMART-Seq ICELL8 Reagent Kit: Terra PCR Direct Polymerase Mix (pink cap), 5X Primer Mix (green cap), and SeqAmp CB PCR Buffer (clear cap).

Procedure

1. Take out all reagents out from the freezer and thaw the 5X Primer Mix. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
2. Add all reagents in the order below to the purified library after Library PCR 1 (Section V.J, Step 16):

14 µl	Purified library after Library PCR 1
25 µl	SeqAmp CB PCR Buffer (clear)
10 µl	5X Primer Mix (green)
1 µl	Terra PCR Direct Polymerase Mix (pink)
50 µl	**Total volume**

3. Place the tube in a preheated thermal cycler with a heated lid and run the following Library PCR 2 program:

98°C		2 min
7 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.

L. Protocol: Purification of the Sequencing-Ready Library

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

Before you start

- Equilibrate an aliquot of AMPure XP beads to room temperature for ~30 min prior to use.

NOTE: 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 of fresh 80% (v/v) ethanol from a stock of anhydrous ethanol.
- This protocol requires Elution Buffer provided with the SMART-Seq ICELL8 Reagent Kit.

Procedure

1. Remove the PCR tube from the thermal cycler (Section V.K, Step 3). Spin it down briefly.
2. Add 50 µl (1:1 v/v) of well-vortexed AMPure XP 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 binds 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 ~5-minute 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. While the tube is sitting on the magnetic stand, pipette out the supernatant.
7. Keep the tube on the magnetic stand. Add 200 µl of freshly made 80% ethanol to each sample without disturbing the beads. Wait for 30 seconds and carefully pipette out the supernatant containing contaminants. DNA will remain bound to the beads during the washing process.
8. Repeat Step 7 one more 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 seconds, 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: If you overdried the beads, you will see cracks in the pellet. If this occurs, the DNA may not elute well from the beads and recovery may be less.

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 minute or longer until the solution is completely clear.

    NOTE: During the incubation on the magnet, there may be a small population 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 clear supernatant containing purified library to a clean 1.5 ml PCR tube.

SAFE STOPPING POINT: The samples can be stored at –20ºC.

M. Protocol: Library Validation and Quantification

To determine whether library production and purification were successful, we recommend analyzing and quantifying the final libraries using the Agilent 2100 Bioanalyzer and the High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626). Alternatively, the libraries can be quantified by qPCR using the Takara Bio NGS Library Quantification Kit. 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, normalize 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. See the user manual for the Agilent High Sensitivity DNA Kit for instructions.

   NOTE: Be careful not to transfer beads with your sample.
4. Use the Bioanalyzer results to determine library quality. See Figure 50, below, for an example of a typical Bioanalyzer profile for an NGS library that has been successfully purified and size selected.
   - For libraries <850 base pairs as determined by the Bioanalyzer, use the Qubit concentration in combination with the average size of the library to calculate the molar library concentration.
   - For libraries >850 base pairs as determined by the Bioanalyzer, use qPCR to accurately measure the concentration of the sequencing library and ensure optimal loading on the sequencer. Use the Library Quantification Kit (Takara Bio, Cat. No. 638324). Refer to the "Library Quantification Kit User Manual" on the takarabio.com website for instructions and use the average size as determined by the Bioanalyzer to calculate the molar library concentration.
5. Store the sequencing library at –20°C until ready for sequencing. Please review Appendix C for sequencing guidelines.
6. Please review Appendix D for data analysis guidelines

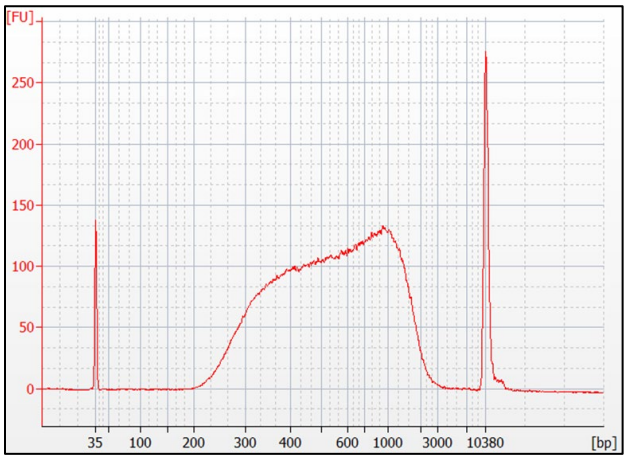


Figure 50. Example electropherogram from a library prepared from K-562 nuclei and quantified using the Agilent 2100 Bioanalyzer.

Appendix A: Forward and Reverse Indexing Primers

A shortened name is used for simplicity instead of the full name of each index. For example, "Forward Index 1" below stands for "SMART-Seq ICELL8 Forward Index 1." Sample sheets will be required in case Illumina's bcl2fastq Conversion Software is used for demultiplexing the sequencing reads. Please refer to Appendix D for details.

Table 4. SMART-Seq ICELL8 Indexing Primer Set – A and SMART-Seq ICELL8 Indexing Primer Set – B forward indexing primers 1–72.

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 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 34	B7	AACCTCAG	AACCTCAG	CTGAGGTT
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 5. SMART-Seq ICELL8 Indexing Primer Set – A reverse indexing primers 1–72.

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 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 40	H15	CCGGTTAG	CTAACCGG
Reverse Index 41	I15	CCTCGACG	CGTCGAGG
Reverse Index 42	J15	CGAAGCTG	CAGCTTCG
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 6. SMART-Seq ICELL8 Indexing Primer Set – B reverse indexing primers 73–144.

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 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 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 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 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: RT-PCR Cycling Guidelines

While 14 cycles of PCR during the RT-PCR step will work to provide good transcript identification and sensitivity for many cell types, different cell types may have different optimal cycle numbers during RT-PCR. For example, cells with high mRNA content (like cell lines) and low mRNA content (like primary cells) may have different behaviors at 14 cycles of PCR.

After bioinformatic analysis, if a cell type exhibits high percentages of exonic reads (≥80%), the cycle number may be reduced. If instead a cell type exhibits low percentages of exonic reads (50–80%), then the cycle number may be increased.

Appendix C: Guidelines for Library Sequencing

A. Final Sequencing Library Structure

Libraries generated using the SMART-Seq full-length protocol on the ICELL8 system have standard Illumina adaptors 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 51, below. For the complete list of sequences of forward and reverse indexing primers, please refer to Appendix A.

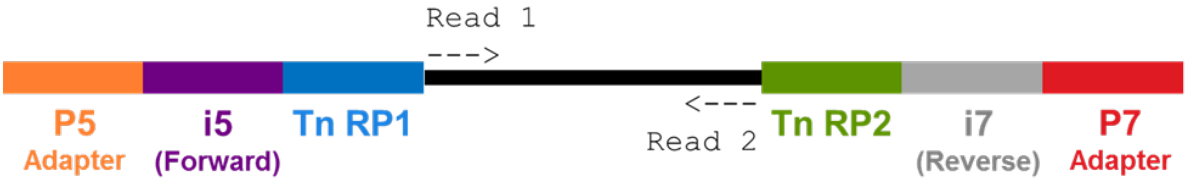


Figure 51. The structure of a final sequencing library. The final library is composed of Tn RP1 and Tn RP2 (Illumina Nextera read primer 1 and 2 sequences added by the TDE1 enzyme), i5 and i7 indexes, and P5 and P7 adapters.

B. Compatible Illumina Platforms

SMART-Seq full-length libraries generated with the ICELL8 system can be run on the following Illumina platforms with either single-end or paired-end sequencing (Takara Bio validated libraries with MiSeq, MiniSeq, NextSeq and NovaSeq):

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

C. Recommendations for Library Sequencing

Samples should be pooled to a final concentration of 4 nM. Refer to Illumina documentation for instructions on denaturing and diluting libraries.

1. Loading Concentration and PhiX Recommendations

For libraries <850 base pairs as determined by the Bioanalyzer (Section V.M, Step 4), use the Qubit concentration in combination with the average size of the library to calculate the molar concentration of the library. For libraries >850 base pairs as determined by the Bioanalyzer, use the qPCR concentration (Section V.M, Step 4).

For NextSeq instruments, we recommend a loading concentration of 1.6 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, particular sample types sometimes do 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 50,000–100,000 reads/cell. However, identification of rare genes or alternative splicing transcript isoforms may need higher sequencing depth. We usually recommend more than 300,000 reads per cell. Table 7 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 7. 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 (N x 10^6)	Reads per cell*; 1,000 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 libraries generated with the ICELL8 system 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 8, 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 full-length libraries (e.g., 1 x 100, 1 x 150, and 1 x 250).

Table 8. Sequencing run parameters.

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

Appendix D: Demultiplexing and Data Analysis

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

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

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

   Significant advantages of using CogentAP:

   - 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 system) as the input without the need to manually handle barcodes and sample information
   - CogentAP automatically performs a reverse-complement check and correction of barcodes
   - There is no limit on the number of barcodes one can process
   - Output files generated by CogentAP can be loaded into Cogent NGS Discovery Software (CogentDS), our R-kit for extended analysis, to create classic t-SNE analysis popular in scRNASeq.

   Installation information and complete user guides for both CogentAP and CogentDS are available through the ICELL8 software portal 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 in order to use the software. Sample sheets can be created by Illumina Experiment Manager https://support.illumina.com/sequencing/sequencing_software/experiment_manager.html.

   SMART-Seq ICELL8 indexes are unique sequences that are different from Illumina indexes. Before demultiplexing, the sample sheets created in the Illumina Experiment Manager (above) have to be customized with SMART-Seq ICELL8 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 SMART-Seq indexes of the wells selected during an experiment can be obtained from the well-list file generated by the CellSelect Software on the ICELL8 instrument. 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, such as the CogentAP software.

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