Takara Bio USA, Inc.

Shasta™ Whole-Genome Amplification Kit - 2 Chip User Manual for the ICELL8® cx Single-Cell System

Cat. No. 640286
for ICELL8 cx CELLSTUDIO™ v2.6 Software
(062824)

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

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

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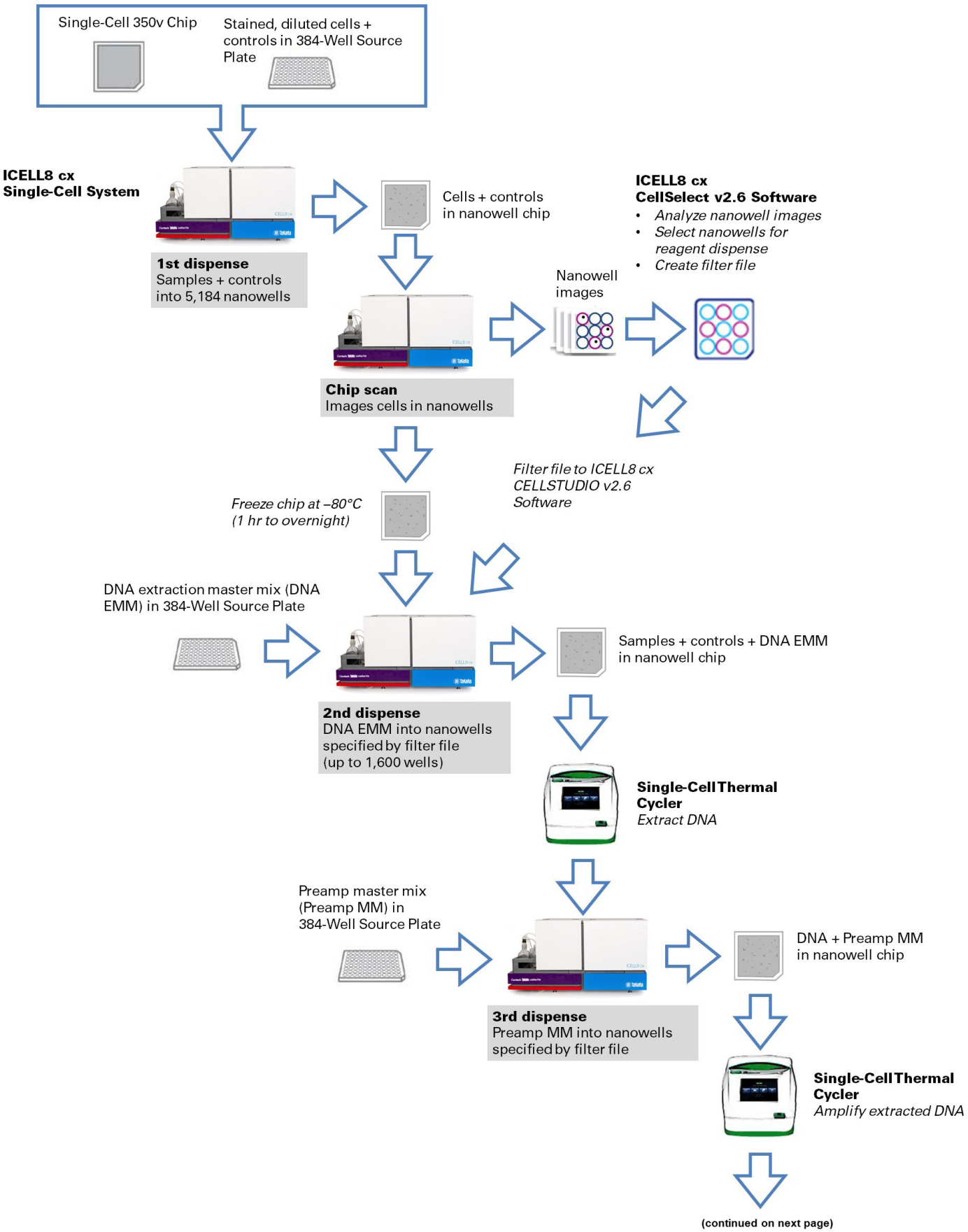
I. Introduction

The **Shasta Whole-Genome Amplification Kit - 2 Chip** (Shasta WGA, Cat. No. 640286) protocol generates high-quality whole-genome amplification (WGA) libraries from single cells isolated on the ICELL8 cx Single-Cell System (Cat. No. 640188 or 640189). By leveraging the capabilities of the ICELL8 cx system along with PicoPLEX® technology, this protocol provides reproducible, reliable, and cost-effective detection of copy number variants (CNVs), single nucleotide variants (SNVs), indels, and small structural variants from single cells.

The workflow (Figure 1, below) begins with staining and dilution of cell samples and the preparation of positive and negative controls. The cells and controls are then dispensed into a Single-Cell 350v Chip using the ICELL8 cx system and ICELL8 cx CELLSTUDIO Software (v2.6.43 or later). Up to eight different samples can be dispensed in a single run, which is completed in approximately 15 min. During this time, cells are maintained in the humidity- and temperature-controlled environment provided by the ICELL8 cx instrument.

After sample and control dispense, the 5,184 nanowells are imaged by the ICELL8 cx system with both blue and red wavelength filters, and ICELL8 cx CellSelect® Software (v2.6.52 or later) is used to analyze the resulting images. Initial candidate identification is performed automatically, based on the size and morphology of the cells and nuclei stained with Hoechst 33342 (blue) with additional differentiation by propidium iodide (red). Users may further refine candidate identification by configuration adjustments to the automated threshold detection (auto-tune) or by visual triage of the images. After selection of candidate wells, CellSelect software generates a filter file to be imported into CELLSTUDIO software to control downstream reagent dispenses.

For library construction, the ICELL8 cx system dispenses DNA extraction reagents in up to 1,600 candidate wells of the Single-Cell chip designated by the filter file. The chip is run through DNA extraction on the ICELL8 cx Thermal Cycler (referred to as thermal cycler throughout this manual). Following DNA extraction, preamplification mix containing proprietary quasi-random primers is dispensed. The quasi-random primers bind to selective sites on the genomic DNA, which is then preamplified in a linear manner. DNA is then amplified using forward and reverse indexing primers, generating the final library construct. The resulting libraries are extracted from the chip and purified. After validation steps, the libraries are ready for sequencing on Illumina® platforms. Figure 1 (over the next two pages) shows a workflow of the application starting from prepared cells to creation of sequencing-ready libraries.



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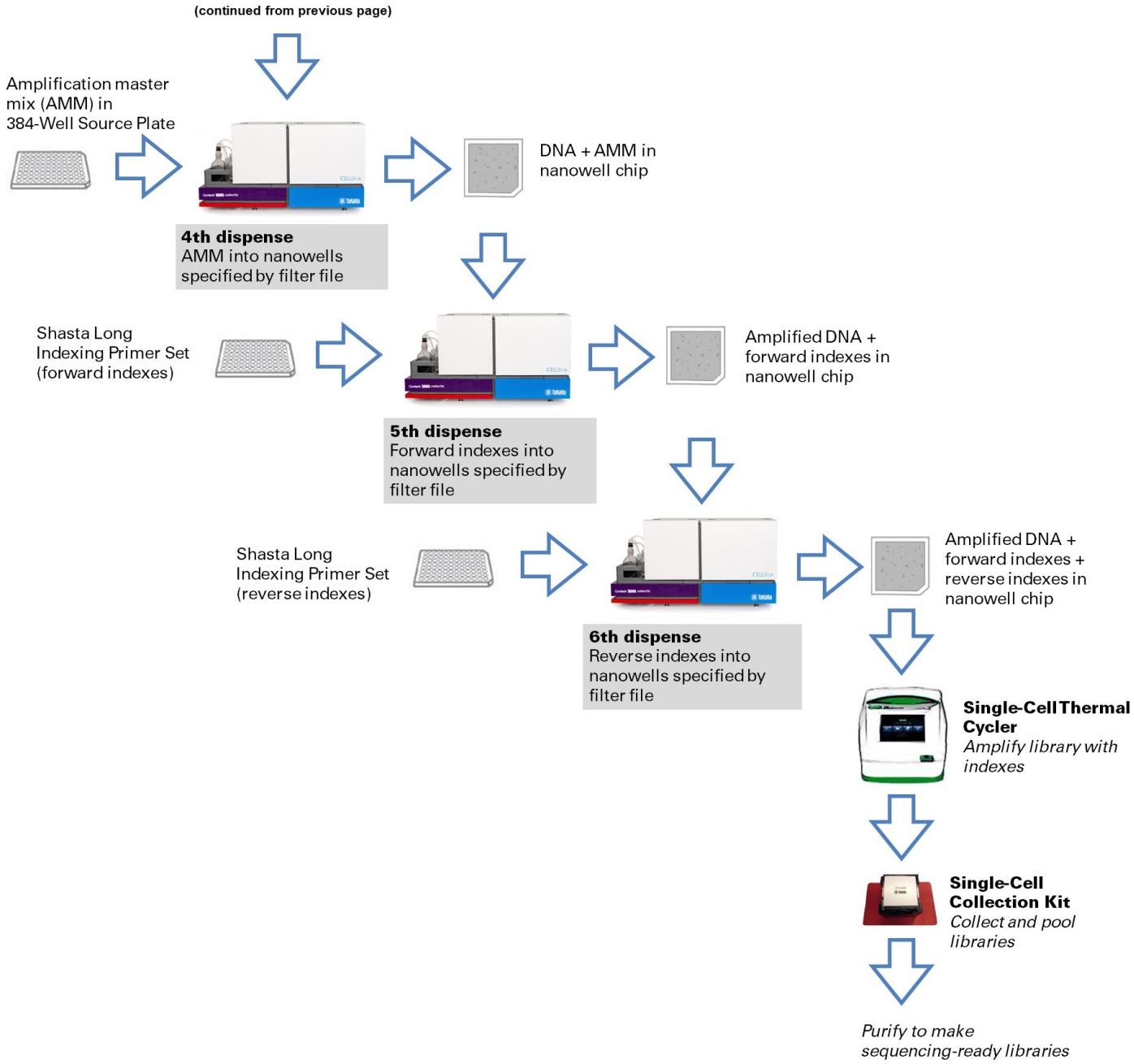

Figure 1. Shasta Whole-Genome Amplification Kit on the ICELL8 cx Single-Cell System workflow.

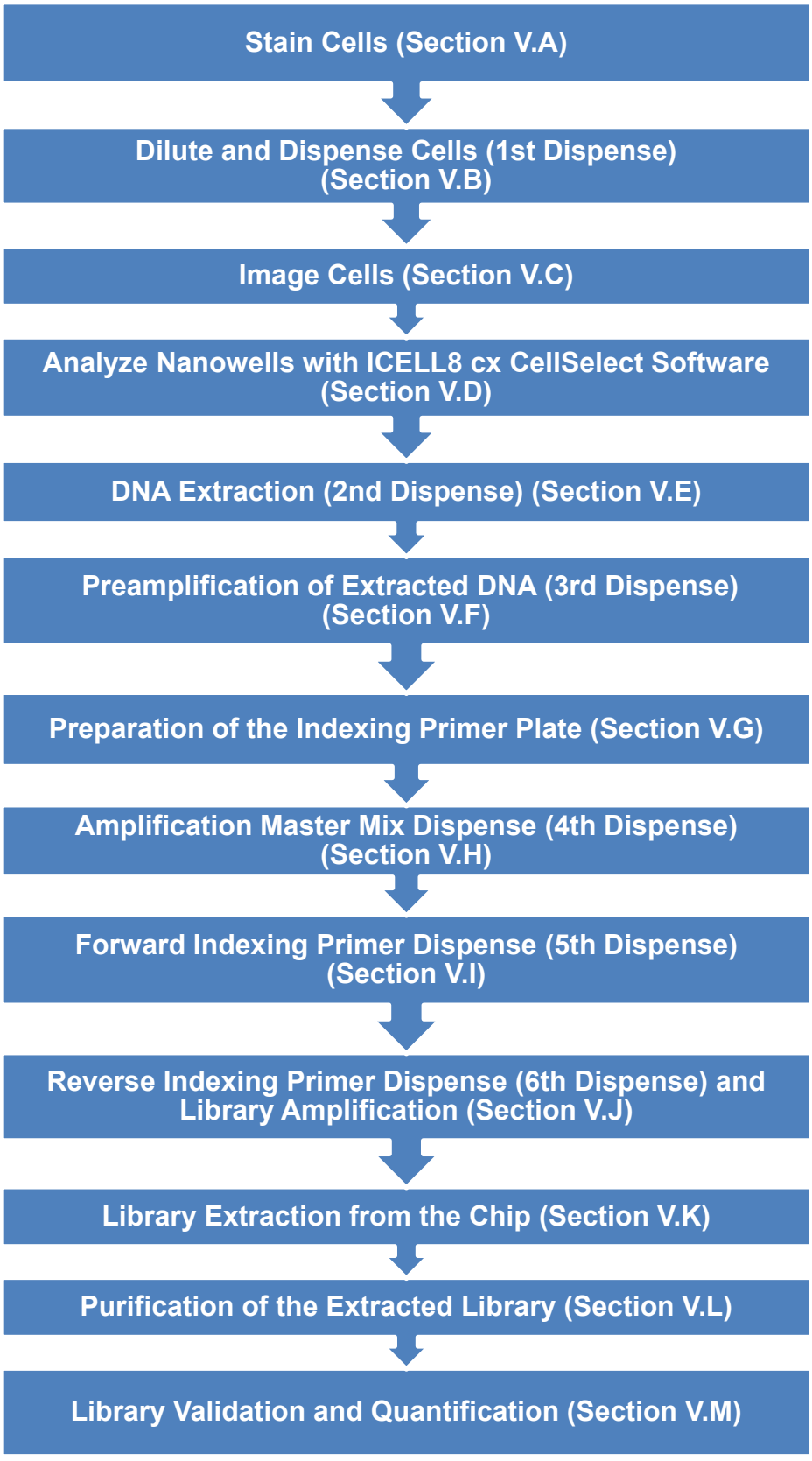

Figure 2. Protocols in the Shasta Whole-Genome Amplification Kit on the ICELL8 cx Single-Cell System workflow.

II. List of Components

The Shasta Whole-Genome Amplification application workflow requires use of an ICELL8 cx Single-Cell System (Cat. No. 640188 or 640189) and the Shasta Whole-Genome Amplification Kit - 2 Chip (Cat. No. 640286).

Table 1. Shasta Whole-Genome Amplification Kit - 2 Chip components.

Shasta Whole-Genome Amplification Kit - 2 Chip	640286 (2 chips)
Shasta Whole-Genome Amplification Reagents (Cat. No. 640287; Store at –20°C)*	
DNA Extraction Buffer	2 x 240 µl
DNA Extraction Enzyme	2 x 10 µl
PreAmp Buffer	2 x 240 µl
PreAmp Enzyme	2 x 10 µl
Amplification Buffer	2 x 177.5 µl
Amplification Enzyme	2 x 10 µl
Second Diluent (100X)	2 x 25 µl
Elution Buffer	2 x 350 µl
Nuclease-Free Water	2 x 500 µl
1X TE Buffer	2 x 12 ml
Shasta Long Indexing Primer Set - A (Cat. No. 640283) (Store at room temperature)	**2**
Shasta Long Indexing Primer Set - A (0.05 nmol /well)†	2 x 1
Plate Sealing Film	2 x 1
Single-Cell 350v Chip (Cat. No. 640019) (Store at room temperature)	**2**
Single-Cell 350v Chip, 5,184 Wells, 2.2 mm (350 nl)	2 x 1
Single-Cell Loading Kit (Cat. No. 640206) (Store at room temperature)	**2**
Chip Freezing Film	2 x 1
RC Film	2 x 6
Blotting Paper	2 x 7
Single-Cell Collection Kit (Cat. No. 640212) (Store at room temperature)	**2**
Single-Cell Collection Fixture	2 x 1
Collection Tube (2.0 ml)	2 x 2
Collection Film	2 x 1

*Enough reagents are included to dispense up to 1,600 wells per each Single-Cell 350v Chip. Not sold separately.
†Each well contains a specific forward or reverse primer. Index sequences listed in Appendix D.

III. Additional Materials Required

Required general lab supplies

- Personal protective equipment (PPE): powder-free gloves, safety glasses, lab coat, sleeve protectors, etc.
- Nanodispenser 384-Well Source Plate and Seals (Takara Bio, 20/pack, Cat. No. 640018; 120/pack, Cat. No. 640037)
- 384-Well Plate Seal Applicator, included with the ICELL8 cx instrument

- Film Sealing Roller for PCR Plates ("film sealing roller") (Bio-Rad, Cat. No. MSR0001)
- Minicentrifuges for 1.5 ml tubes
- 384-well plate orbital shaker with 3 mm mixing orbit (e.g., Southwest Science SBT1500, Benchmark Orbi-Shaker MP series)
- Vortex mixer
- Centrifuges, rotors, and adapters. Recommended:
  - Eppendorf 5810R with Microplate Buckets (VWR, Cat. No. 53513-874), ≥2,600*g*, room temperature and 4ºC operation
  - Kubota 3740 with rotor SF-240 for cell preparation
- Nuclease-free LoBind 1.5 ml microcentrifuge tubes (Eppendorf)
- Conical tubes, 50 ml and 15 ml sizes
- 25 ml reagent reservoirs (Thermo Fisher Scientific, Cat. No. 809311)
- 5 ml flip-cap tubes
- Single-channel pipettes: 2 µl, 10 µl, 20 µl, 200 µl, and 1,000 µl
- Multichannel pipette: 200 µl
- Filter pipette tips: 20 µl, 200 µl, and 1,000 µl
- Wide-bore pipette tips: 200 µl and 1,000 µl
- Serological pipettes and controller
- Nuclease-decontamination solution
- Exhaust hood system with UV

For staining and dispensing cells:

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

For dissociating adherent cells:

- 1X PBS (no Ca^{2+}, Mg^{2+}, phenol red, or serum, pH 7.0–7.3; Thermo Fisher Scientific, Cat. No. 14190144 or an equivalent PBS)
- TrypLE Express (Thermo Fisher Scientific, Cat. No. 12604021)
- Appropriate cell culture medium

For cell counting

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

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

For preparing Control gDNA

- 1X TE Buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA)
- Control gDNA. For human cell-based experiments, we recommend Human Genomic DNA (Takara Bio, Cat. No. 636401) or an appropriate gDNA sample from the Coriell Institute for Medical Research (e.g., NA12878)

For library purification

- NucleoMag NGS Clean-up and Size Select (Takara Bio; 5 ml size: Cat. No. 744970.5; 50 ml size: Cat. No. 744970.50; 500 ml size: Cat. No. 744970.500). The AMPure XP PCR purification kit (Beckman Coulter; 5 ml size: Cat. No. A63880; 60 ml size: Cat. No. A63881) is an appropriate substitute.

  NOTES:
  - The kit has been specifically validated with the beads listed above. Substitutions may lead to unexpected results.
  - Beads need to come to room temperature before the container is opened. We strongly recommend aliquoting the beads into 1.5 ml tubes upon receipt and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (~30 min). Aliquoting is also instrumental in decreasing the chances of bead contamination.

- Molecular grade anhydrous ethanol
- Magnetic Separator compatible with 1.5 ml tubes

For library validation and quantification

- Agilent 2100 Bioanalyzer instrument or similar
- Agilent High Sensitivity DNA Kit (110 samples; Agilent Technologies, Cat. No. 5067-4626)
- Qubit 2.0 Fluorometer (Thermo Fisher Scientific) or similar
- Qubit 1X dsDNA High Sensitivity (HS) and Broad Range (BR) Assay Kit (Thermo Fisher Scientific, Cat. No. Q33230 or Q33231)
- Library Quantification Kit (Takara Bio, Cat. No. 638324) and DNA Standards for Library Quantification (Takara Bio, Cat. No. 638325)

IV. General Considerations

A. Protocol Best Practices

- The kit contains two sets of reagents and is designed for performing two tests (one chip each). Use one set of reagents and consumables each time and dispose of leftovers. Do not reuse the leftovers.
- Perform all experimental procedures in sterile environments with the proper personal protective equipment (PPE). Use designated UV hoods with proper ventilation for manipulating cells and setting up molecular biology reactions. Decontaminate gloves with nuclease-decontamination solution, water, and ethanol. Change gloves routinely.
- Minimize the exposure time of unsealed chips, reservoirs, reagents, and other consumables to the open air. Wearing laboratory sleeve protectors may reduce the likelihood of introducing contaminants from exposed hands and arms.

- The assay is very sensitive to variations in pipette volume. Please make sure that all pipettes are calibrated for reliable reagent delivery and that nothing adheres to the outsides of the tips when dispensing liquids.
- 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 Shasta WGA protocol.
- Because of the large volume or viscosity of mixtures subject to purification using NucleoMag NGS Clean-up and Size Select beads, each round of purification requires a very strong magnet. Place the samples on the magnetic separation device for ~5 min or longer until the liquid appears completely clear and there are no beads left in the supernatant.
- UV-treat reagent reservoirs, seals, pipettes, filter tips, and compatible reagents prior to use.

B. Safety

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

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

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

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

C. ICELL8 cx System Application Notes

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

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

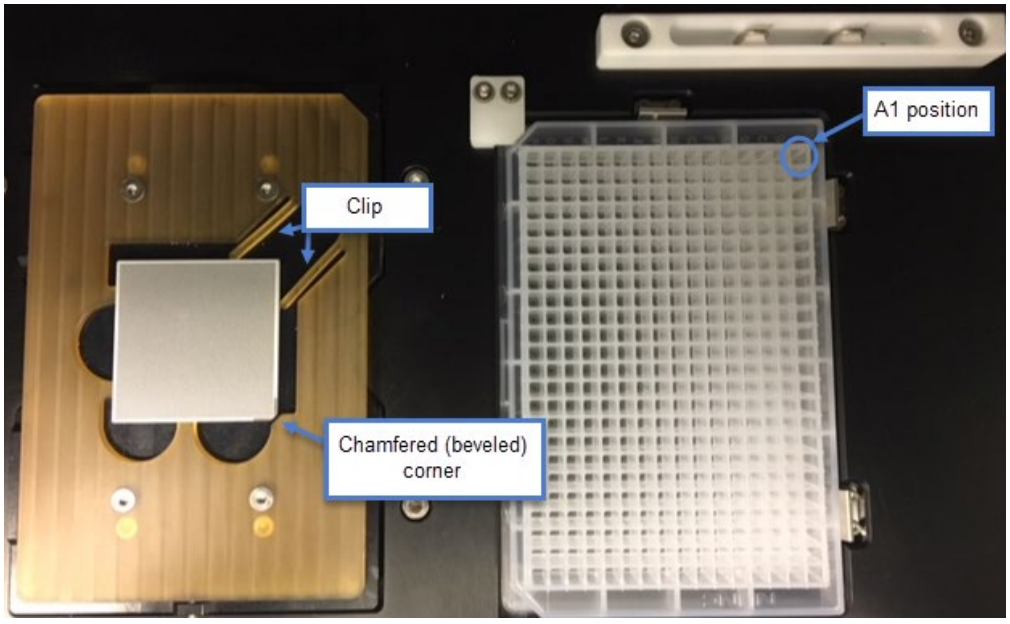

Figure 3. ICELL8 cx Single-Cell System Stage Module. **(Left)** chip nest. **(Right)** source plate nest.

D. Single-Cell 350v Chip

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

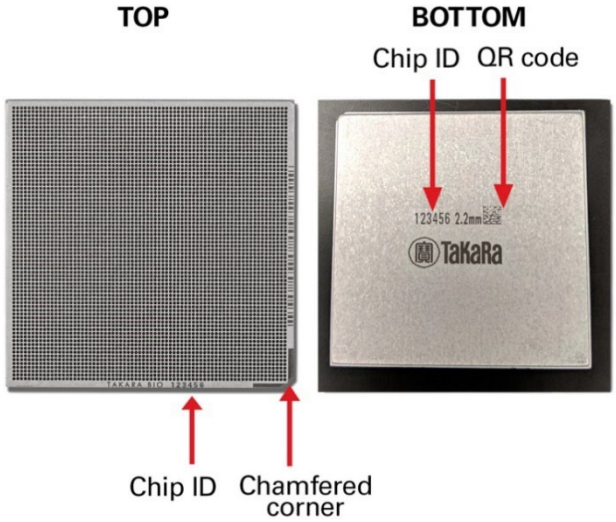

Figure 4. Single-Cell 350v Chip features. **(Left)** Top view of the chip. Note the chamfered (beveled) corner at the bottom right. The "TaKaRa" logo and the chip ID (unique to each chip) are engraved on the chip border, near the chamfered corner. **(Right)** Bottom view of the chip. The chip ID is also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

E. Software

The instructions in this manual are written for use with CELLSTUDIO v2.6.43 (or later) and CellSelect v2.6.52 (or later) Software. Please refer to the ICELL8 cx Single-Cell System User Manual and the ICELL8 cx CellSelect v2.6 Software User Manual for more detailed information.

Please contact technical support if you need to upgrade your ICELL8 software.

V. Procedure

A. Protocol: Stain Cells

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

1. Cell and Chip Handling Notes

- This protocol requires healthy cell culture suspension(s). Nonadherent cells, such as K-562 cells, can directly start with this procedure. As a general guideline, a minimum of 100,000 cells should be stained per Single-Cell chip.
  For adherent cells, such as 3T3 cells, refer to Appendix A for trypsinization and preparing the 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 and minimize bubble formation and frothing.
- Wear nitrile or powder-free gloves to reduce imaging artifacts.
- Centrifugation speed and times listed in the protocol may need to be modified for different cell types.

2. Before You Start

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

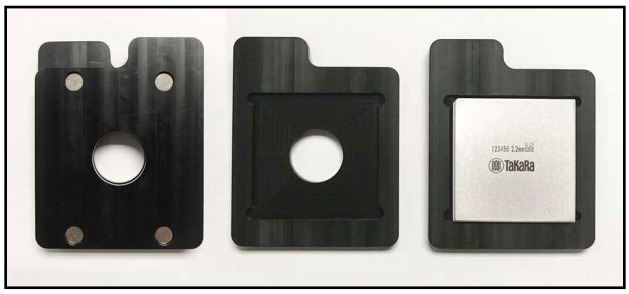

Figure 5. Nanodispenser Chip Holder.

- Chill some of the 1X PBS to 4°C.
- Set the chip centrifuge to 4°C.
- Dilute Control gDNA using 1X TE Buffer to 10 ng/µl for use in the next protocol (Section V.B). Keep the dilution on ice.

NOTE: Quantify the Control gDNA via Qubit or NanoDrop before dilution to get an accurate concentration.

3. Staining Cells in Suspension

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

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

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

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

   - K-562 or 3T3 cells: 300*g* for 3 min.
   - PBMCs or similarly sized cells: 500*g* for 3 min.

9. Gently remove the tube from the centrifuge without disturbing the cell pellet.
10. Carefully decant the supernatant without disturbing the cell pellet.
11. Wipe remaining fluid from the top of the tube using a fresh Kimwipe such that it is gently removed.
12. Gently add 1 ml of prechilled 1X PBS to the side wall of the tube.
13. Use a wide-bore 1 ml pipette tip to gently mix the cell suspension by slowly pipetting up and down ~5 times. DO NOT vortex or overagitate the cells.
14. Count the cells using your preferred method. Take two readings for each stained cell sample and average the results.
15. Keep the prepared cell suspension on ice. Proceed to the next protocol (Section V.B).

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

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

1. Required Components

- From the Shasta Whole-Genome Amplification Kit - 2 Chip:
  - Shasta Whole-Genome Amplification Reagents
    - Second Diluent (100X)
  - Single-Cell Loading Kit
    - Blotting Paper, RC Film
- From the Nanodispenser 384-Well Source Plate and Seal: one (1) 384-Well Source Plate and one (1) plate seal
- Control gDNA (diluted to 10 ng/µl in the previous protocol, Section V.A.2)
- 384-Well Plate Seal Applicator and film sealing roller

2. Before You Start

- Confirm that initialization and setup procedures for the ICELL8 cx system have been completed (see "Before You Start" in Section V.A.2, above).
- Confirm the empty Nanodispenser Chip Holder (Figure 5, above) is frozen at –80°C.
- Aliquot 300–500 µl of 1X PBS (Ca^{2+} and Mg^{2+} free, pH 7.4) on ice for positive and negative controls.
- Thaw Second Diluent (100X) on ice. Once thawed, keep on ice for the remainder of the protocol.
- Use the concentration of stained cell suspension measured at the end of the previous protocol (Section V.A.3) and the information in Table 2 (below) to calculate the volumes of stained cell suspension and 1X PBS that should be combined for a final concentration of 1.4 cells/50 nl (28,000 cells/ml) in a total volume of 1 ml.

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

3. Procedure

a) Prepare the Single-Cell chip

1. Open the packet containing the sealed Single-Cell 350v Chip.

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

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

b) Prepare diluted stained cell suspension

3. Briefly vortex the Second Diluent. Spin the tubes briefly to collect contents at the bottom.

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

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

5. To the 1.5 ml microcentrifuge tube from the previous step (containing Second Diluent and 1X PBS), add the calculated volume of stained cell suspension that will yield a final concentration of 1.4 cells/50 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 yet.

NOTES:

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

c) *Prepare positive and negative controls*

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

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 gDNA should be 15 pg/50 nl.

Table 2. Sample preparation guidelines*.

Components	Negative control	Positive control	Diluted stained cell suspension	Volume per source well (for each sample)†
Second Diluent (100X)	1.0 µl	1.0 µl	10 µl	1.0 µl
Control gDNA (10 ng/µl)	–	3.0 µl	–	–
Stained cell suspension	–	–	Dilute to 1.4 cells/50 nl†	Dilute to 1.4 cells/50 nl†
1X PBS (Ca^{2+} and Mg^{2+} free)	99.0 µl	96.0 µl	Up to 1,000 µl	Up to 100 µl
Total	**100 µl**	**100 µl**	**1,000 µl‡**	**100 µl§**

*This table is the primary recommended guideline for making diluted stained cell suspensions.

†Sufficient stained-cell suspension should be included such that the final concentration in the 1,000 µl volume of diluted stained cell suspension is 1.4 cells/50 nl (i.e., 28,000 cells/ml).

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

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

d) Prepare sample dispense source plate

7. Add positive control and negative control to the 384-Well Source Plate as indicated in the following steps and in Figure 6 (below):
   - Add 25 µl of prepared negative control to well A24.
   - Add 25 µl of prepared positive control to well P24.
8. Using a wide-bore 1 ml pipette tip, gently mix the diluted stained cell suspension prepared in Step 5 of this protocol (above) by slowly pipetting up and down ~5 times. DO NOT vortex or overagitate the cells.

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

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

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

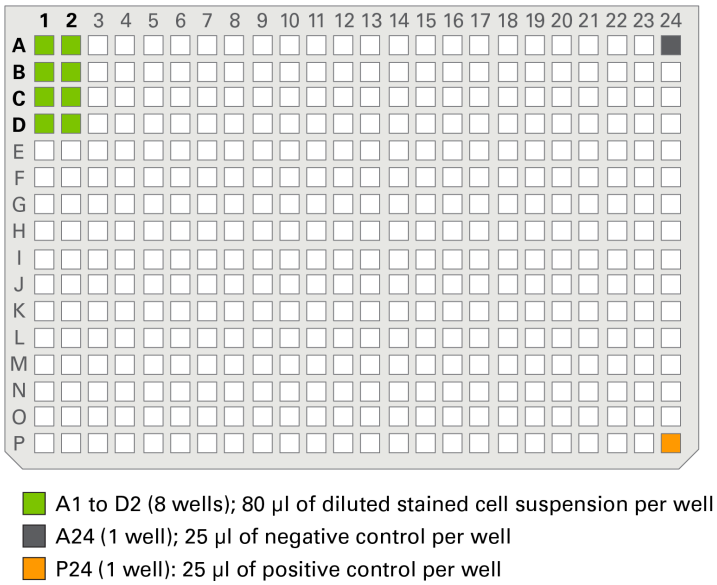

Figure 6. Setting up the 384-Well Source Plate for dispensing cell samples and controls.

10. Place the 384-Well Source Plate in the ICELL8 cx Single-Cell System with the A1 corner positioned at the top-right corner of the plate nest. The beveled corners of the 384-Well Source Plate should be on the left side (refer to Figure 3 or the ICELL8 cx Single-Cell System User Manual, Section X.B).
11. In CELLSTUDIO software, click the [Dispense Cells and Controls (50 nl)] button (Figure 7).

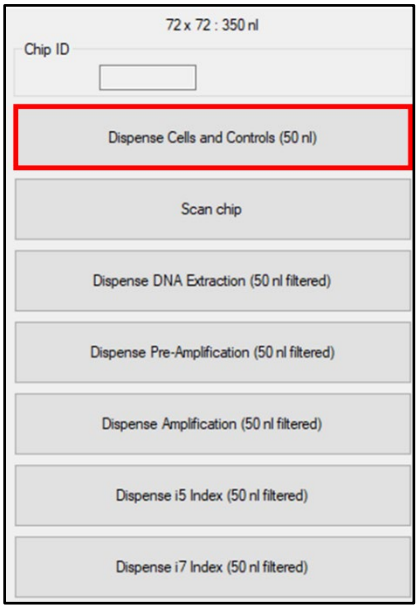

Figure 7. Use CELLSTUDIO software to dispense cells into the Single-Cell 350v Chip.

A "Select index set" *Workflow* window will display asking you to indicate which Indexing Primer Plate you are using (Figure 8). Choose "Shasta_Long_SetA" from the drop-down menu and then click [Done]. If this window doesn't pop up, "Shasta_Long_SetA" has been selected as the default.

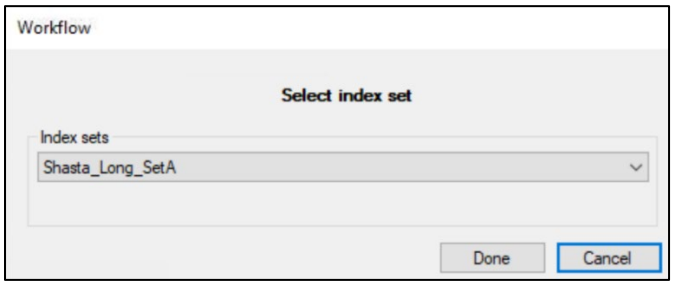

Figure 8. *Select index set* workflow prompt.

12. Follow the subsequent software prompts and check the orientation of the source plate and Single-Cell 350v Chip to ensure they are correctly loaded on the dispensing platform.

    During these checks, when prompted, remove the seal on the Single-Cell 350v Chip and from the 384-Well Plate, if a seal was used.

13. Click [Done] to proceed.

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

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

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

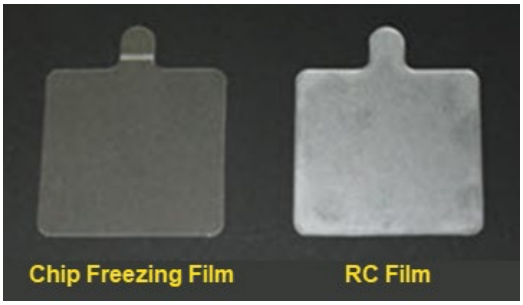

Figure 9. Chip Freezing and RC Films required for the dispenses. The Chip Freezing Film (left) has three layers and a green dot sticker on it. The RC Sealing Film (right) has a translucent backing. Please follow the instructions in each subprotocol regarding preparation and handling of the films.

16. Remove the liner from the RC Film and apply the exposed side of the film to the blotted chip (Figure 10, left, below).
17. Seal the blotted chip with the film using a film sealing roller (Figure 10, right, below).

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

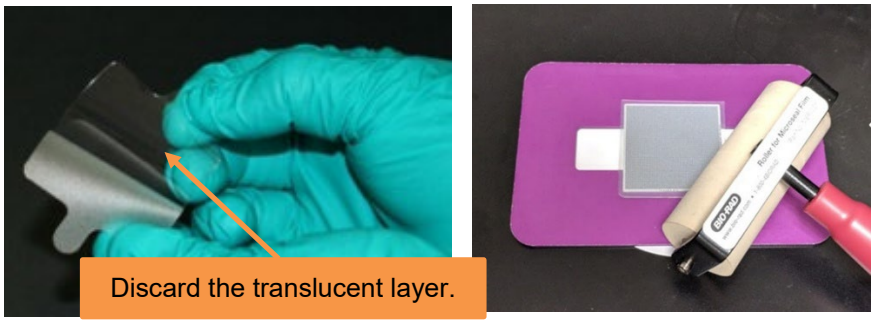

Figure 10. Preparing and adhering the RC Film. RC Film is composed of two layers: one clear and the other translucent. **(Left)** When sealing a chip, remove the translucent layer and discard it. Put the remaining layer on the chip. **(Right)** Tightly adhere the RC Film using the film sealing roller.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

18. Place the sealed chip on a Nanodispenser Chip Centrifuge Spinner (included with the ICELL8 cx instrument accessories) and centrifuge the sealed chip at 300*g* for 5 min at 4°C with full acceleration and full brake (Figure 11, below). If you have one chip, balance the centrifuge with the supplied Balance Chip or a blank Single-Cell 350v Chip. Proceed to the next protocol (Section V.C) once the centrifugation is complete.

Figure 11. Nanodispenser Chip Centrifuge Spinner.

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

C. Protocol: Image Wells

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

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

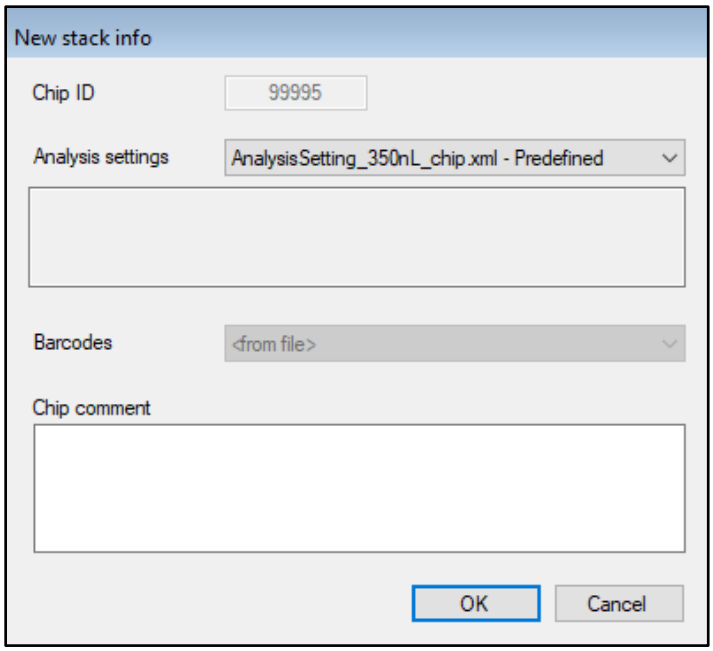

Figure 12. *New stack info* dialog window.

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

   c. Continue with the ICELL8 cx system user manual Section X.E procedure from Steps 7–12.
   d. At Step 13, remove the liner from only one side of the Chip Freezing Film (the side that does not have the green dot sticker) and apply the exposed sticky side of the film to the chip, sealing carefully with the plate seal applicator (similar to Figure 10, above). Make sure that the film has adhered completely and evenly on the chip. Remove the side with the green dot sticker.
2. Place the sealed chip into an empty Nanodispenser Chip Holder that has been prechilled at –80°C (Figure 5). The chip holder should click closed and should close evenly, indicating a proper magnetic seal.
3. Freeze the chip and chip holder at –80°C for a minimum of 1 hr before proceeding to DNA extraction (Section V.E).

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

D. Protocol: Analyze Nanowells with ICELL8 cx CellSelect Software

Either accept the automatic cell candidacy suggested by CellSelect Software or, if desired, manually inspect the selected nanowells in the software to exclude or include one or more candidate wells. Please refer to the ICELL8 cx CellSelect v2.6 Software User Manual for more information about the manual triage process.

NOTES:

- A filter file **MUST** be used in this workflow. As noted in the table footnote in Section II, only enough reagents are included in the kit to dispense to ~1,600 wells per chip. Please triage your nanowells accordingly.
- We recommend using automated threshold detection (auto-tune) to determine candidate wells for the downstream dispenses. Refer to Appendix F of the ICELL8 cx CellSelect v2.6 Software User Manual for more details.
- If you opt out of generating the filter file in CellSelect software, it must be manually created. See Appendix D, "Advanced Filter File Configuration," in the ICELL8 cx Single-Cell System User Manual for detailed instructions about constructing the file.

E. Protocol: DNA Extraction (2nd Dispense)

IMPORTANT: Do not begin this step unless you are prepared and have the time to perform the rest of the protocol up through Section V.K, "Library Extraction from the Chip." There are no safe stopping points in Section V.E–Section V.J.

1. Required Components

- From the Shasta Whole-Genome Amplification Kit - 2 Chip:
  - Shasta Whole-Genome Amplification Reagents
    - DNA Extraction Buffer, DNA Extraction Enzyme
  - Single-Cell Loading Kit
    - Blotting Paper, RC Film
- From the Nanodispenser 384-Well Source Plate and Seal: one (1) 384-Well Source Plate and one (1) plate seal
- 384-Well Plate Seal Applicator and film sealing roller

2. Before You Start

- If needed, perform a once-a-day warmup on the ICELL8 cx instrument.
- Set the centrifuge(s) used for spinning the Single-Cell chip and 384-Well Source Plate to 4°C.
- Preprogram the ICELL8 cx Thermal Cycler with the DNA Extraction program (Step 10) before the experiment. Make sure the lid temperature is set at 72ºC. Run and immediately hold the program so the thermal cycler is preheated before the reaction.
- Thaw DNA Extraction Buffer on ice. Gently mix and spin down after thawing. Keep on ice.
- Keep DNA Extraction Enzyme in storage at –20°C until just prior to use. Spin down and keep on ice at all times.

3. Procedure

1. Remove the Nanodispenser Chip Holder containing the Single-Cell 350v Chip from the –80°C freezer. Thaw the chip in the chip holder until it reaches room temperature (about 10 min) to lyse the sample(s).
2. While the chip is thawing, prepare the DNA extraction master mix in a 1.5 ml tube.

   NOTE: Remove DNA Extraction Enzyme from the freezer for this step and add to the mix.

   DNA extraction master mix:

240 µl	DNA Extraction Buffer
10.0 µl	DNA Extraction Enzyme
250.0 µl	**Total volume**

   Pipette-mix and spin the tube briefly in a minicentrifuge to collect contents.
3. Take the thawed chip out of the chip holder. Use a Kimwipe to dry any liquid on the chip surface, and then centrifuge the chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4°C. Keep the chip on ice until ready for the DNA extraction dispense.
4. Pipette 60 µl of DNA extraction master mix into the 384-Well Source Plate wells A3, B3, C3, and D3 highlighted in Figure 13, below.

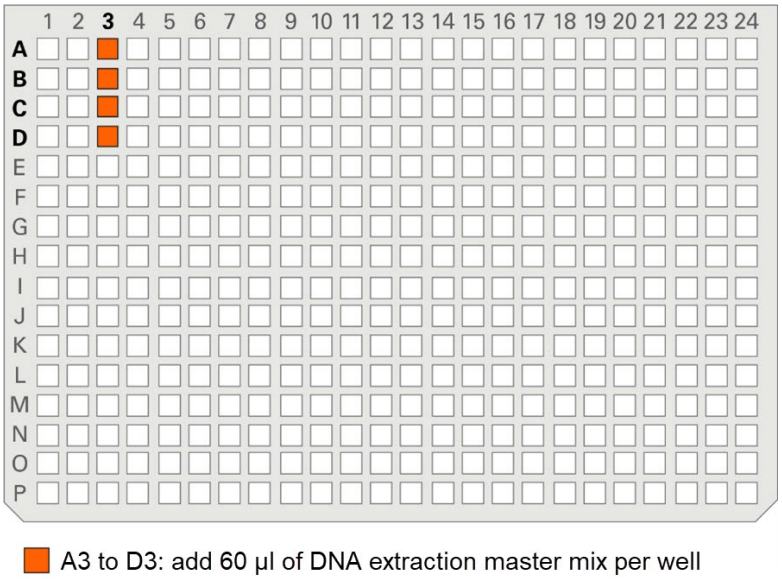

Figure 13. Aliquot 60 µl of DNA extraction master mix into wells A3–D3 of the 384-Well Source Plate. Wells are highlighted in orange.

5. Seal the 384-Well Source Plate with a 384-Well Source Plate Seal using the plate seal applicator.
6. Centrifuge the source plate at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.
7. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:
   a. Start in Section X.F, Step 5, with loading the 384-Well Source Plate into the plate nest.
   b. At Step 8, click the [Dispense DNA Extraction (50 nl filtered)] button (Figure 14).

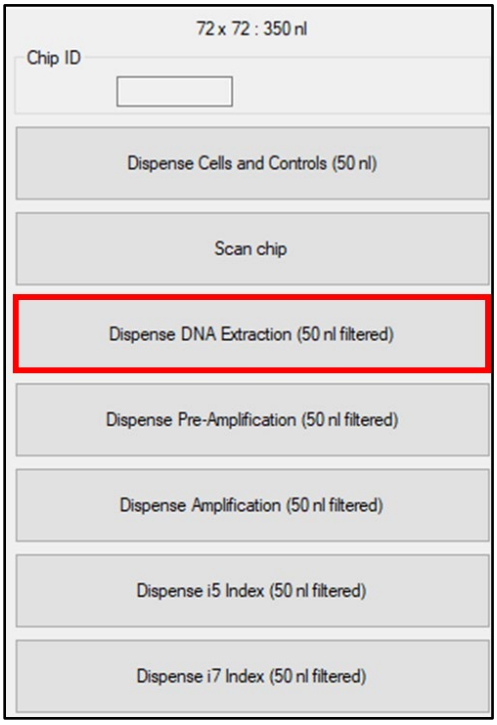

Figure 14. The [Dispense DNA Extraction (50 nl filtered)] step in CELLSTUDIO software.

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

d. For Step 15, seal with RC Film.

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

8. During centrifugation, perform the [Tip Clean] procedure 3 times on the instrument.
9. Make sure the thermal cycler lid temperature is at 72°C and the block temperature is at 75°C. After centrifugation, place the chip into the thermal cycler.
10. Resume the DNA Extraction program:

75°C	10 min
95°C	4 min
4°C	forever

NOT A SAFE STOPPING POINT: Immediately proceed to Section V.F, "Preamplification of Extracted DNA."

F. Protocol: Preamplification of Extracted DNA (3rd Dispense)

1. Required Components

- From the Shasta Whole-Genome Amplification Kit - 2 Chip:
  - Shasta Whole-Genome Amplification Reagents
    - PreAmp Buffer, PreAmp Enzyme
  - Single-Cell Loading Kit
    - Blotting Paper, RC Film
- From the Nanodispenser 384-Well Source Plate and Seal: one (1) 384-Well Source Plate and one (1) plate seal
- 384-Well Plate Seal Applicator and film sealing roller

2. Before You Start

- If needed, perform a once-a-day warmup on the ICELL8 cx instrument.
- Set the centrifuges used for spinning the Single-Cell chip and 384-Well Source Plate to 4°C.
- Thaw PreAmp Buffer on ice. Gently mix and spin down after thawing. Keep on ice.
- Keep PreAmp Enzyme in storage at –20°C until just prior to use. Spin down and keep on ice at all times.

3. Procedure

1. Remove the chip from the thermal cycler (Section V.E, Step 10). Centrifuge the chip at 3,220*g* for 3 min at 4°C. Keep the chip on ice until ready for Preamplification Dispense.
2. During centrifugation, program the thermal cycler with the Preamp program (Step 9). Make sure the lid temperature is set at 72ºC. Run and immediately hold the program to preheat the thermal cycler.
3. Prepare the preamp master mix by mixing the following components in a 1.5 ml tube. Gently pipette up and down to mix, and spin down on a minicentrifuge.

Preamp master mix:

240.0 µl	PreAmp Buffer
10.0 µl	PreAmp Enzyme
250.0 µl	**Total volume**

4. Pipette 60 µl of preamp master mix into the 384-Well Source Plate wells A11, B11, C11, and D11 highlighted in Figure 15, below.

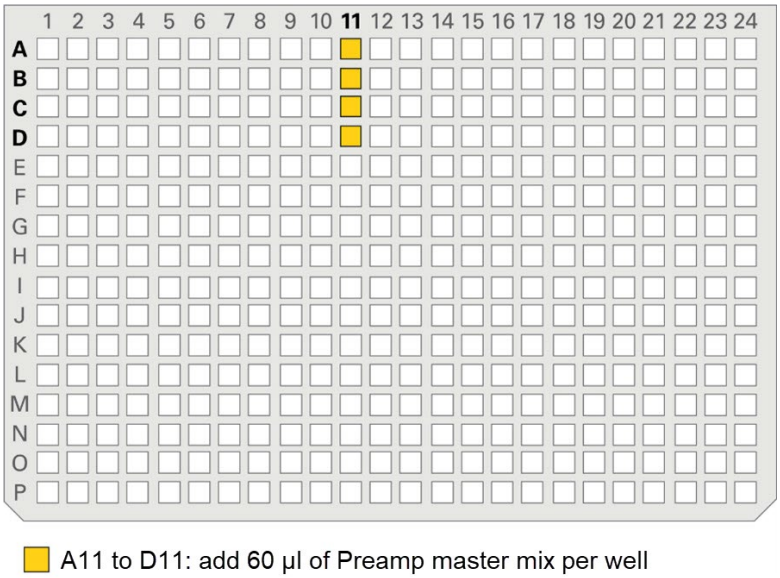

Figure 15. Aliquot 60 µl of preamp master mix into wells A11–D11 of the 384-Well Source Plate. Target wells are highlighted in yellow.

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

6. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:
   a. Start in Section X.F, Step 5, with loading the 384-Well Source Plate into the plate nest.
   b. At Step 8, click the [Dispense Pre-Amplification (50 nl filtered)] button (Figure 16).

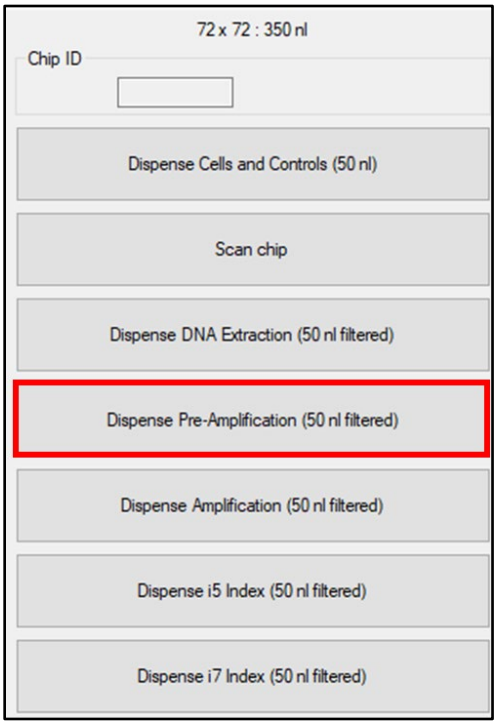

Figure 16. Use CELLSTUDIO software to dispense the preamp master mix.

   c. Follow Steps 9–14 as written in Section X.F.
   d. For Step 15, seal with RC Film.
   e. For Step 16, centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.
7. During centrifugation, perform the [Tip Clean] procedure 3 times on the instrument.
8. Make sure the thermal cycler lid temperature is preheated at 72°C and the block temperature is preheated at 95°C. After centrifugation, place the chip into the thermal cycler.

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

	95°C	2 min
12 cycles:		
	95°C	15 sec
	15°C	50 sec
	25°C	40 sec
	35°C	30 sec
	65°C	20 sec
	75°C	20 sec
	4°C	forever

NOT A SAFE STOPPING POINT:

– It is strongly recommended that the next step (Section V.G, " Preparation of the Shasta Long Indexing Primer Plate") be performed while the Preamp program is running to guarantee sufficient time to complete the primer preparation process.

– After the preamp program has reached 4°C, immediately proceed to Section V.H, "Amplification Master Mix Dispense."

G. Protocol: Preparation of the Shasta Long Indexing Primer Plate

72 forward indexing primers and 72 reverse indexing primers are resuspended with 1X TE Buffer for the 5th and 6th dispenses.

IMPORTANT: It is important to proceed with the forward indexing primer dispense right after the amplification dispense (Section V.H).

1. Required Components

- From the Shasta Whole-Genome Amplification Kit - 2 Chip:
  - Shasta Long Indexing Primer Set - A
    - Shasta Long Indexing Primer Set - A, Plate Sealing Film
  - Shasta Whole-Genome Amplification Reagents
    - 1X TE Buffer
- 384-Well Plate Seal Applicator
- 25 ml reagent reservoirs

2. Before You Start

- Thaw 1X TE Buffer at room temperature
- Set the centrifuge to room temperature

3. Procedure

1. Take the Shasta Long Indexing Primer Set - A plate out of its packaging.

   NOTE: Do not open the sealing foil yet.

2. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 10 min at room temperature to dislodge the lyophilized pellets from the bottom of the plate.
3. Carefully remove sealing foil from the primer plate.

4. Add 10 ml of thawed 1X TE Buffer to a 25 ml reagent reservoir.
5. Using a multichannel pipette, aspirate 1X TE Buffer from the reagent reservoir and add into primer plate wells using the following scheme:
   - 33.3 µl into wells A5–P8, A9–D9, and A10–D10 (Figure 17, pink)
   - 33.3 µl into wells A13–P16, A17–D17, and A18–D18 (Figure 17, blue)

NOTE: When dispensing the 1X TE Buffer, aim the pipette tip at the bottom of the well and dispense without introducing any small droplets. Apply steady pressure to the plunger until the first stop (avoid the droplet becoming static).

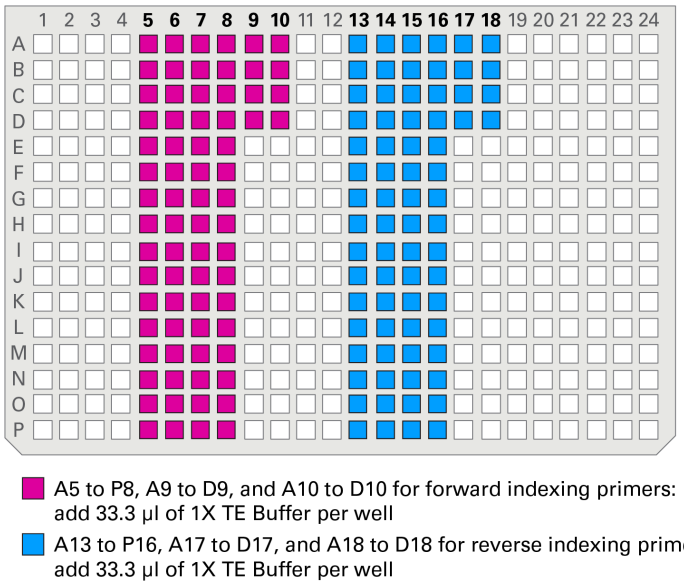

Figure 17. Schematic of the Shasta Long Indexing Primer Plate. The plate is predispensed with 72 forward and 72 reverse lyophilized indexing primers. Add 1X TE Buffer to wells of forward indexing primers in pink (33.3 µl per well) and reverse indexing primers in blue (33.3 µl per well). See Appendix D for barcode sequences.

6. Seal the plate with the Plate Sealing Film using a plate seal applicator.
7. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 10 min at room temperature to avoid condensation on the film.
8. Shake the plate with a plate shaker with 3 mm orbit at 700 rpm for 10 min at room temperature.
9. Centrifuge the plate at 3,220*g* (minimum 2,600*g*) for 10 min at room temperature to avoid condensation on the film.
10. Keep the plate at room temperature until the dispense of forward indexing primers (5th dispense, Section V.I).

NOT A SAFE STOPPING POINT: Immediately after the preamp program from the previous section (Section V.F) has reached 4°C, proceed to section V.H, "Amplification Dispense."

H. Protocol: Amplification Master Mix Dispense (4th Dispense)

1. Required Components

- From the Shasta Whole-Genome Amplification Kit - 2 Chip:
  - Shasta Whole Genome Amplification Reagents
    - Nuclease-Free Water, Amplification Buffer, Amplification Enzyme
  - Single-Cell Loading Kit
    - Blotting Paper, RC Film
- From the Nanodispenser 384-Well Source Plate and Seal: one (1) 384-Well Source Plate and one (1) plate seal
- 384-Well Plate Seal Applicator and film sealing roller

2. Before You Start

- Set the centrifuges used for spinning the Single-Cell chip and 384-Well Source Plate to 4°C.
- Thaw Nuclease-Free Water and Amplification Buffer on ice. Gently mix and spin down after thawing and keep on ice.
- Keep Amplification Enzyme in storage at –20°C until just prior to use. Spin down and keep on ice at all times.

3. Procedure

1. Remove the chip from the thermal cycler (Section V.F, Step 9). Centrifuge the chip at 3,220*g* for 3 min at 4°C. Keep the chip on ice until ready for amplification dispense.
2. Prepare the amplification master mix by mixing all components in the order listed in the table below in a 1.5 ml tube. Gently pipette up and down to mix, and spin down on a minicentrifuge.

Amplification master mix:

177.5 µl	Amplification Buffer
10.0 µl	Amplification Enzyme
62.5 µl	Nuclease-Free Water
250.0 µl	**Total volume**

3. Pipette 60 µl of the amplification master mix into the 384-Well Source Plate wells M3, N3, O3, and P3 as highlighted in Figure 18, below.

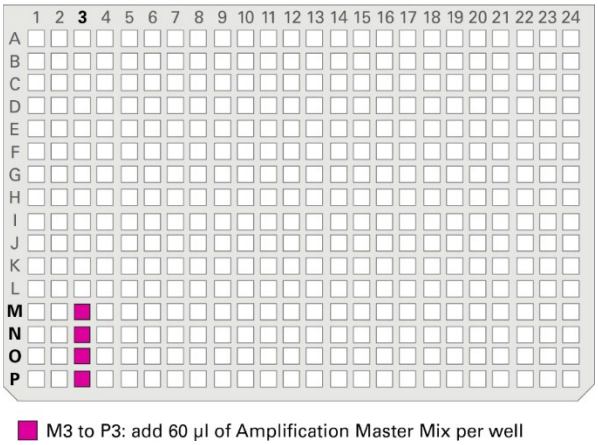

Figure 18. Aliquot 60 µl of the amplification master mix into wells M3–P3 of the 384-Well Source Plate. Target wells are highlighted in pink.

4. Seal the 384-Well Source Plate with a 384-Well Source Plate Seal using the plate seal applicator.
5. Centrifuge the 384-Well Source Plate at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.
6. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:
   a. Start in Section X.F, Step 5, with loading the 384-Well Source Plate into the plate nest.
   b. At Step 8, click the [Dispense Amplification (50 nl filtered)] button (Figure 19).

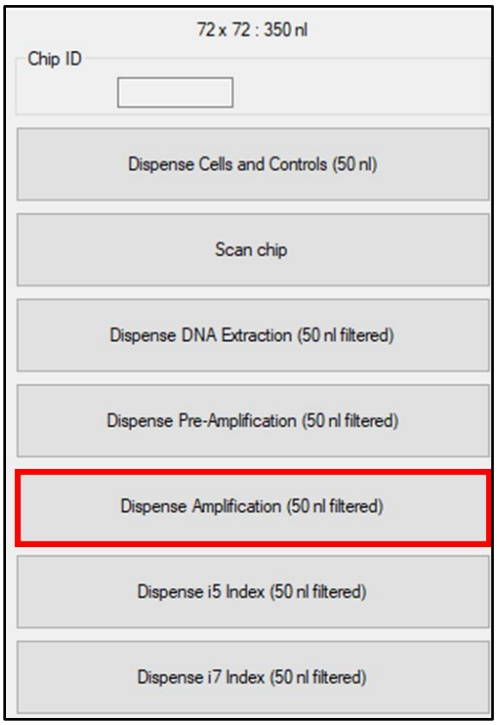

Figure 19. Use CELLSTUDIO software to dispense the amplification master mix.

   c. Follow Steps 9–14 as written in Section X.F.
   d. For Step 15, seal with RC Film.
   e. For Step 16, centrifuge the sealed chip at 3,220*g* (minimum 2,600*g*) for 3 min at 4ºC.
7. During centrifugation, perform the [Tip Clean] procedure 3 times on the instrument.

NOT A SAFE STOPPING POINT: Immediately continue to Section V.I, "Forward Indexing Primer Dispense."

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

72 forward indexing primers are dispensed from the Shasta Long Indexing Primer Plate - A (prepared in Section V.G) and used during library amplification (Section V.J).

1. Required Components

- From the Shasta Whole-Genome Amplification Kit - 2 Chip:
  - Shasta Long Indexing Primer Plate - A (prepared in Section V.G)
  - Single-Cell Loading Kit
    - Blotting Paper, RC Film
- A razor blade
- Film sealing roller

2. Before You Start

Visually check the prepared Shasta Long Indexing Primer Plate - A for bubbles in the wells. Bubbles should not be present given shaking and centrifuge steps earlier (Section V.G, Steps 7 & 8), but if bubbles are observed:

- Prior to continuing, centrifuge again for 3,220*g* (minimum 2,600*g*) for 3 min at room temperature -or-
- Remove the bubbles gently by pipette after removing the seal (Step 2, below)

3. Procedure

1. Using a razor blade, cut the plate seal on the Shasta Long Indexing Primer Plate - A into two halves between columns 11 and 12 (Figure 20).

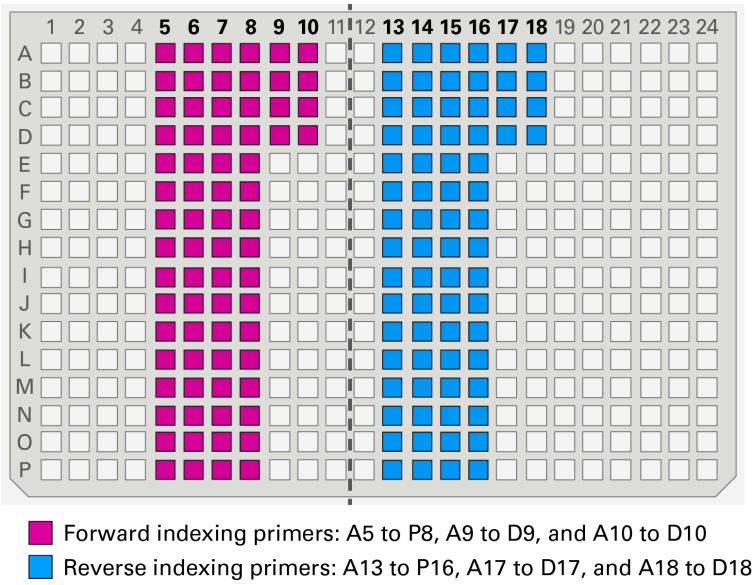

Figure 20. Plate map of the Indexing Primers, indicating the cut line for use in the 5th dispense. The indexes (shown in pink and blue) were reconstituted in Section V.G. The razor cut placement is shown by the black dotted line.

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

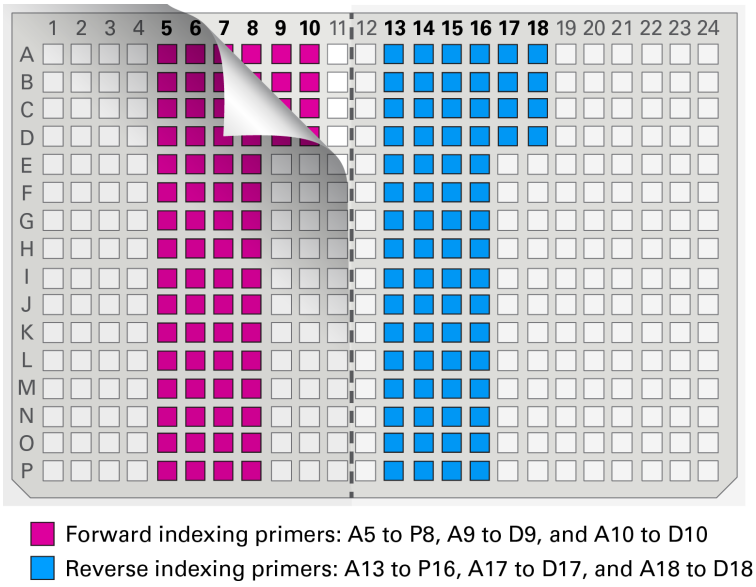

Figure 21. Peeling the plate seal from the forward indexing primers. The forward indexing primers are shown in pink. The plate seal should remain on the right-half of the plate, covering the reverse indexing primers (blue).

3. Refer to the ICELL8 cx Single-Cell System User Manual, Section X.F "Dispense Reagents and/or Indexes into the Chip" for detailed information about this protocol, with the following guidelines:
   a. Start in Section X.F, Step 5, with loading the indexing primer plate into the plate nest.
   b. At Step 8, click the [Dispense i5 Index (50 nl filtered)] button (Figure 22).

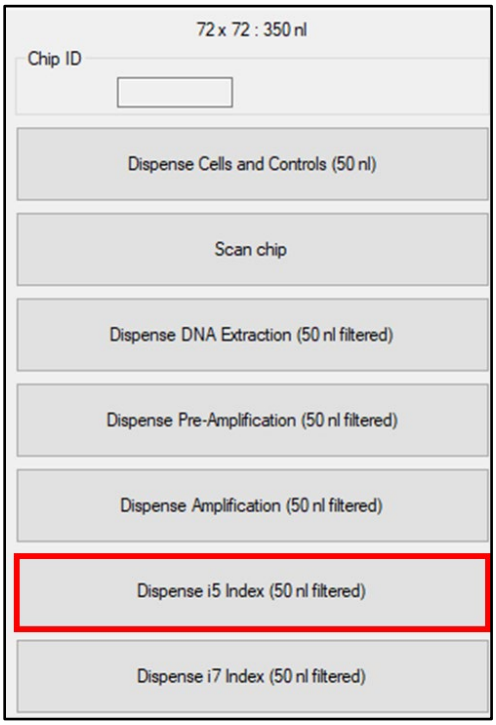

Figure 22. Use CELLSTUDIO software to dispense i5 Indexes.

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

d. For Step 15, seal with RC Film.

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

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

NOT A SAFE STOPPING POINT: Immediately proceed to the next step (Section V.J) for the dispense of the reverse indexing primers.

J. Protocol: Reverse Indexing Primer Dispense (6th Dispense) and Library Amplification

72 reverse indexing primers are dispensed from the Shasta Long Indexing Primer Plate - A (prepared in Section V.G) and the libraries amplified.

1. Required Components

- From the Shasta Whole-Genome Amplification Kit - 2 Chip:
  - Shasta Long Indexing Primer Plate - A (modified in Section V.I)
  - Single-Cell Loading Kit
    - Blotting Paper, RC Film
- Film sealing roller

2. Before You Start

Preprogram the thermal cycler with the amplification program (Step 5). Make sure the lid temperature is set at 72ºC. Run and immediately hold the program to preheat the thermal cycler.

3. Procedure

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

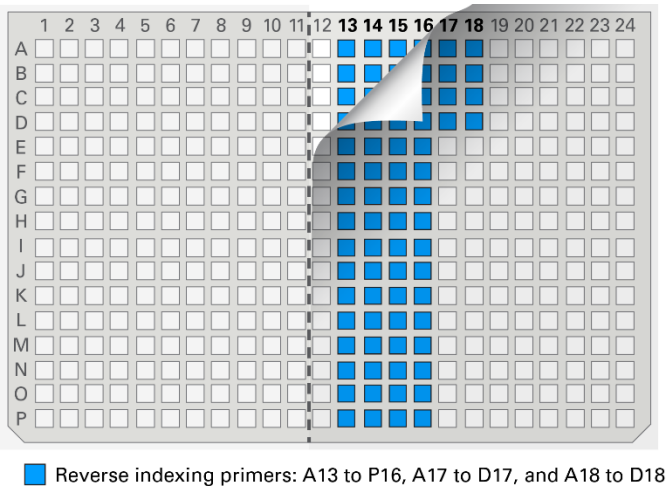

Figure 23. Peeling the plate seal from the reverse indexing primers. Remove the remaining seal from the right-side of the plate to uncover the reverse index wells.

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

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

   a. Start in Section X.F, Step 5, with loading the indexing primer plate into the plate nest.

   b. At Step 8, click the [Dispense i7 Index (50 nl filtered)] button (Figure 24).

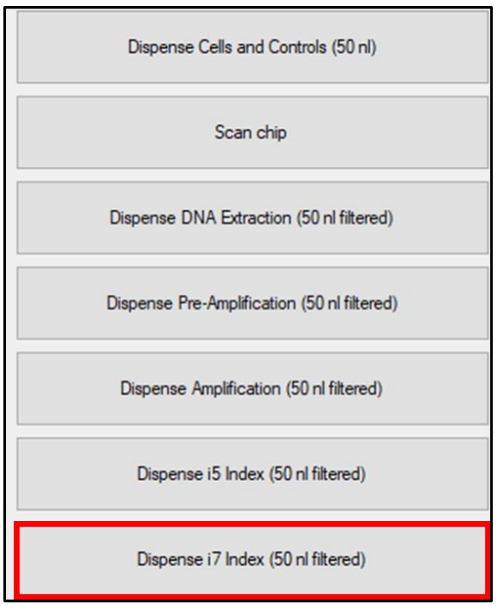

Figure 24. Use CELLSTUDIO software to dispense i7 Indexes.

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

   d. For Step 15, seal with RC Film.

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

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

4. Make sure the thermal cycler lid temperature is preheated to 72°C and the block temperature to 95°C. After centrifugation, place the chip into the thermal cycler.

5. Once the chip is loaded into the thermal cycler, resume the amplification program:

	95°C	4 min
4 cycles:		
	95°C	20 sec
	63°C	20 sec
	72°C	20 sec
7 cycles:		
	95°C	20 sec
	72°C	20 sec
	4°C	forever

IMPORTANT: For the best results, we recommend extracting the library from the chip (Section V.K) immediately after the amplification program is completed. If necessary, however, the chip can be left in the thermal cycler at 4ºC overnight.

K. Protocol: Library Extraction from the Chip

This protocol extracts the amplified library from the Single-Cell chip.

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

SAFE STOPPING POINT: The eluate can be frozen indefinitely at –20°C, although library performance will degrade over a long period of time.

L. Protocol: Purification of the Extracted Library

We recommend a double-sided size selection and bead clean-up for the Shasta WGA library. Refer to an explanation of double-sided size selection from the Illumina technical support page: https://support.illumina.com/bulletins/2020/07/library-size-selection-using-sample-purification-beads.html.

1. Required Components

- From the Shasta Whole-Genome Amplification Kit - 2 Chip:
  - Shasta Whole-Genome Amplification Reagents
    - Elution Buffer
- Prealiquoted tube of NucleoMag NGS Clean-up and Size Select (see note in Section III)
- Molecular grade anhydrous ethanol

2. Before You Start

- Equilibrate a prealiquoted 1.5 ml tube of NucleoMag NGS Clean-up and Size Select beads to room temperature for ~30 min prior to use.
- Immediately prior to use, vortex the beads until they are well dispersed. The color of the liquid should appear homogeneous. Confirm that there is no remaining pellet of beads at the bottom of the tube. Mix well to disperse before adding the beads to your reactions. The beads are viscous, so pipette them slowly.
- Prepare fresh 80% (v/v) ethanol before each cleanup (2 ml each). Use an anhydrous ethanol stock.

3. Procedure

1. If the library has been kept frozen after extraction from the chip in Section V.K, thaw the library on ice before measuring the volume. If this is directly after extraction, proceed to Step 2.
2. Measure the total volume of the extracted library from the chip (from Section V.K).
3. Pipette half of the extracted library to a new 1.5 ml tube for downstream purification. Make a note of the halved volume of extracted library being used for the subsequent steps.

   Example: If the total volume of the extracted library measured in Step 2 is 400 µl, you should have a 200 µl aliquot you will be purifying in this protocol.

NOTE: Freeze and store the other half of the extracted library at –20ºC in case further analysis is needed.

4. Add 0.5:1 volume of well-vortexed NucleoMag NGS Clean-up and Size Select beads to the collection tube and the aliquoted half of the extracted library.

   Example: Continuing the example from Step 3, you would add 100 µl of magnetic beads to the 200 µl aliquot of the extracted library.

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

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

8. When the liquid appears clear, keep the tube on the magnetic separator and transfer the supernatant to a new 1.5 ml tube. Discard the tube with the beads.
9. Add an additional volume of well-vortexed magnetic beads to the supernatant collected in Step 8. The total bead-to-library volume ratio (including the original addition of beads) should be 0.8:1 of the original extracted library volume.

   Example: Continuing the example of using a 200 µl aliquot of extracted library, the calculation would be:

   0.8:1 = 160 µl magnetic beads:200 µl extracted library

   Therefore, add **60 µl** (160 µl − 100 µl (Step 4)) magnetic beads at this step.

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

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

12. Keep the tube on the magnetic separator and pipette out the supernatant. Add 1 ml of freshly made 80% ethanol to the tube without disturbing the beads. Wait for 30 sec and carefully pipette out the supernatant containing contaminants. DNA will remain bound to the beads during the washing process.
13. Repeat Step 12 one time.
14. Spin down the tube briefly to collect the liquid at the bottom of the tube.
15. Place the tube on the magnetic separator for 30 sec and then remove all remaining ethanol with a pipette.

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

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

    NOTE: Check the pellet frequently during this time and continue to Step 17 when it is dry enough. Please refer to our visual guide for pellet dry-down on our website if you'd like additional assistance with this determination.

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

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

17. Once the beads are dried, remove the samples from the magnetic separator and add 50 µl of Elution Buffer to cover the beads.
18. Incubate the tube with the beads and Elution Buffer at room temperature for 2 min to rehydrate.
19. 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 min or longer until the solution is completely clear.

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

20. Transfer the clear supernatant containing the purified, sequencing-ready library to a clean 1.5 ml tube.

SAFE STOPPING POINT: The sequencing-ready library may be stored at –20°C.

M. Protocol: Library Validation and Quantification

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

Procedure

1. Measure the concentration of the purified, sequencing-ready library using 2 µl of the library, a Qubit fluorometer, and the Qubit 1X dsDNA High Sensitivity (HS) and Broad Range (BR) Assay Kit (Thermo Fisher Scientific, Cat. No. Q33230 or Q33231). Refer to the Qubit assay kit user manual for sample prep instructions.
2. Based on the Qubit measurement, dilute 2 µl of the library to 2.0–4.0 ng/µl with 1X TE Buffer, based on your preferred concentration and the input range accepted by your instrument.
3. Use 1 µl of diluted library 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.

4. Use the Bioanalyzer results to determine library quality and average size for qPCR. An example of a typical Bioanalyzer profile for an NGS library that has been successfully purified and size selected is shown in Figure 25 (next page).

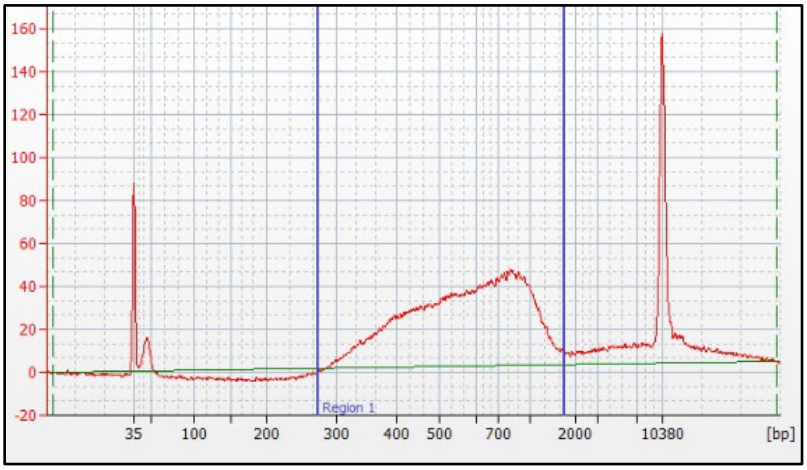

Figure 25. An electropherogram example from a library quantified using the Agilent 2100 Bioanalyzer. Library was prepared from GM22601 cells.

5. Run qPCR using the Library Quantification Kit. Refer to the Library Quantification Kit User Manual for instructions. Use the average size as determined by the Bioanalyzer results to calculate the molar library concentration.
6. Use the qPCR result to determine the final library quantity for sequencing.

SAFE STOPPING POINT: Store the sequencing library at –20°C until ready to sequence. See Appendix C for sequencing guidelines.

VI. Demultiplexing and Data Analysis

We recommend using Illumina's bcl2fastq or bcl-convert software for demultiplexing. Please refer to the user guides for the two software for more details.

- bcl2fastq software User Guide: https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html
- bcl-convert software User Guide: https://emea.support.illumina.com/sequencing/sequencing_software/bcl-convert.html

A custom sample sheet must be generated to use the software. The template and reference documentation for creating sample sheets can be found at https://support.illumina.com/downloads/sample-sheet-v2-template.html. The sample sheet should be customized with our forward and reverse indexes (detailed sequences are listed in Appendix D).

Please note that forward indexes (i5) in the sample sheet are dependent on Illumina sequencers. With some sequencers, the indexes must be entered in the sample sheet with reverse-complement. The indexes of the wells selected during an experiment can be obtained from the well-list file generated by the ICELL8 cx CellSelect Software.

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

Appendix A. Preparing Adherent Cell Types from a 75 cm Culture Flask*

*Adjust volumes accordingly for different-sized flasks.

A. Before You Start

- Prewarm the following to 37°C:
  - 11 ml 1X PBS
  - 4 ml TrypLE Express
  - 8 ml of cell culture medium for your sample

B. Dissociate Cells via Trypsinization

1. Carefully remove culture media from a 75 cm flask containing adherent cells using a serological pipette.
2. Add 10 ml of prewarmed 1X PBS by dispensing on the side walls of the flask. DO NOT pour PBS directly onto cells.
3. Wash the cells by tilting the flask gently. DO NOT mix by pipetting.
4. Remove the PBS from the cells using a serological pipette.
5. Add 3 ml of prewarmed TrypLE Express to the flask to dissociate the cells.

   IMPORTANT: 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 prewarmed cell culture medium. Do not vortex or overagitate cells.
7. Pellet the cells by centrifugation at 100*g* for 3 min at room temperature.

   NOTES:
   - Optimal centrifugation speed and time may vary depending on the cell type being analyzed. For K-562 or 3T3 cells, centrifuge at 300*g*. For smaller cells or cells that are not pelleting at 300*g*, centrifuge speed can be increased up to 500*g*.
   - Avoid over-centrifugation or pelleting into a firm pellet or clump.
8. Gently add 1 ml of prewarmed 1X PBS to the side wall of the tube.
9. 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.
10. Return to Section V.A.3, "Staining Cells in Suspension," starting from Step 1.

Appendix B. Reduced Cell Dispense Volumes

If your samples are not sufficient to prepare at least 1 ml of stained cell suspension (with a concentration of 28,000 cells/ml), it is possible to prepare a smaller volume of at least **500 µl**. 60 µl of the stained cell suspension will then be distributed to each of the eight source wells. See Table 3 for the formulas to follow.

Table 3. Modified sample preparation guidelines for reduced cell-dispense volumes.

Components	Negative control	Positive control	Diluted stained cell suspension	Volume per source well (for each sample)*
Second Diluent (100X)	1.0 µl	1.0 µl	5 µl	0.8 µl
Control gDNA (10 ng/µl)	–	3.0 µl	–	–
Stained cell suspension	–	–	Dilute to 1.4 cells/50 nl*	Dilute to 1.4 cells/50 nl*
1X PBS (Ca^{2+} and Mg^{2+} free)	99.0 µl	96.0 µl	Up to 500 µl	Up to 80 µl
Total	**100 µl**	**100 µl**	**500 µl†**	**80 µl‡**

*Sufficient stained cell suspension should be included such that the final concentration in the 500 µl volume of diluted stained cell suspension is 1.4 cells/50 nl (i.e., 28,000 cells/ml).

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

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

Appendix C. Guidelines for Library Sequencing

A. Final Sequencing Library Structure

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

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

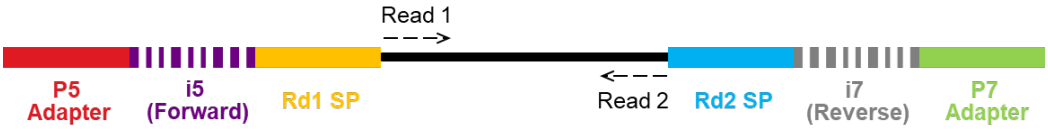

Figure 26. Structure of the final sequencing library. The final library is composed of the DNA insert, Rd1 SP and Rd2 SP (Illumina Read 1 and Read 2 Sequencing Primer binding sites), i5 and i7 indexes, and P5 and P7 adapters.

B. Compatible Illumina Platforms

Libraries generated from the Shasta WGA application can be run on the following Illumina platforms with either single-end or paired-end sequencing:

- MiSeq®
- NextSeq® 500/550, 1000/2000
- HiSeq® 2000/2500, 3000/4000
- HiSeq X series (Rapid Run and High Output)
- NovaSeq™ 6000
- NovaSeq X/X Plus

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

C. Recommendations for Library Sequencing

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

1. Loading Concentration and PhiX Recommendations

The Shasta WGA protocol generates libraries ready for cluster amplification and sequencing on Illumina NGS platforms using standard Illumina reagents. However, due to low-diversity bases in the preamplification primers, a higher effective concentration of PhiX or other high complexity library is required for optimal sequencing results. Follow Illumina loading instructions and the recommendations in Table 4 for optimal clustering and sequencing performance.

Table 4. Recommended loading concentrations by Illumina platform.

Platform	Library Loading Concentration (qPCR)	Recommended % PhiX
MiSeq V3	~12 pM	15%
NextSeq 500/550	~1.7 pM	20%
NextSeq 1000/2000	~1,000 pM*	20%
NovaSeq X/X Plus	~150 pM	20%

*On-board denature.

2. Sequencing Depth Recommendations

The sequencing depth is dependent on the purpose of the study. We recommend ≥1 x 10^6 paired reads/cell for the reliable identification of segmental CNVs with an average bin size of 500 kb to 1 Mb. We were able to achieve the identification of ≥25 Mb gain or loss using an even shallower sequencing depth of about 250,000 paired reads/cell with a bin size of 1 Mb. However, for the detection of single nucleotide polymorphisms (SNPs), we would recommend more than 3.5 x 10^7 paired reads/cell or using the pseudo-bulk approach.

Table 5 provides sequencing depth recommendations for an experiment in which 1,000 single cells are selected. Please refer to the "Illumina sequencing platforms" page (https://www.illumina.com/systems/sequencing-platforms.html) for more details.

Table 5. Sequencing depth recommendations. The platform recommendation is based on an estimation of 1,000 cells. Therefore, it varies based on the number of single cells sequenced as well as on the purpose of the study.

Platform	Maximum reads per run (x 10^6)	Flow cell type	# of flow cells per run	# of lanes per flow cell	Maximum reads per lane (x 10^6)	Reads per cell* (x 10^3)†
NextSeq 500/550	400	High-output	1	4	-	400
NextSeq 1000/2000	400	P2	1	1	-	400
	1,200	P3	1	1	-	1,200
	1,800	P4	1	1	-	1,800
NovaSeq 6000	1,600	S1	1 or 2	2	800	1,600
	4,100	S2	1 or 2	2	2,050	2,050
	10,000	S4	1 or 2	4	2,500	10,000
NovaSeq X Plus	1,600	1.5B	1 or 2	2	800	1,600
	10,000	10B	1 or 2	8	1,250	10,000
	26,000	25B	1 or 2	8	3,250	26,000

*The required number of reads per cell depends on the goal of the experiment.
†Formula used: (# of reads per cell) = (maximum reads per run or lane) / (# of cells).

3. Sequencing Run Parameters

Shasta WGA libraries use standard Illumina sequencing primers and do not need custom primers. Dual indexes (8 cycles for each index) ***must*** be sequenced, and we recommend the number of cycles shown in Table 6, below. However, cycles of Read 1 and Read 2 can be flexible (e.g., 2 x 50, 2 x 100, 2 x 150) depending on your experiments.

Single-end sequencing can also be performed with Shasta WGA libraries (e.g., 1 x 100, 1 x 150).

Table 6. Sequencing run parameters.

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

*For phasing and prephasing calculations, add one additional cycle to Read 1 and Read 2 than the number of cycles you wish to analyze. For the run parameters in Table 6, set up the run as 76 cycles for Read 1, eight cycles for the i7 Index, eight cycles for the i5 Index, and 76 cycles for Read 2.

Appendix D. Shasta Long Indexing Primer Set - A Barcodes

In Tables 7 & 8 below, a shortened name is used for simplicity instead of using the full name of each index. For example, "Forward Index 1" below stands for " Shasta Long Indexing Primer Set - A, Forward Index 1".

Sample sheets will be required if Illumina's bcl2fastq conversion software is used for demultiplexing the sequencing reads. Please refer to Section VI, "Demultiplexing and Data Analysis," for details.

Table 7. Shasta Long Indexing Primer Set - A, forward indexing primers 1–72 barcodes. Continued over the next two pages.

Forward indexing (i5) primers			Forward indexes (i5) on sample sheet	
Index number	Well position	Index sequence in primers	MiSeq, HiSeq 2000/2500, NovaSeq	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 indexing (i5) primers			Forward indexes (i5) on sample sheet	
Index number	**Well position**	**Index sequence in primers**	**MiSeq, HiSeq 2000/2500, NovaSeq**	**NextSeq, HiSeq 3000/4000, HiSeq X**
Forward Index 19	C6	AACCGAAC	AACCGAAC	GTTCGGTT
Forward Index 20	D6	AACCGCCA	AACCGCCA	TGGCGGTT
Forward Index 21	E6	AATAAGGA	AATAAGGA	TCCTTATT
Forward Index 22	F6	ACCTTATT	ACCTTATT	AATAAGGT
Forward Index 23	G6	TGGTCCTG	TGGTCCTG	CAGGACCA
Forward Index 24	H6	CAACGAGG	CAACGAGG	CCTCGTTG
Forward Index 25	I6	CCAATGGA	CCAATGGA	TCCATTGG
Forward Index 26	J6	CGCCTATG	CGCCTATG	CATAGGCG
Forward Index 27	K6	CTCTCCAA	CTCTCCAA	TTGGAGAG
Forward Index 28	L6	GGCTTGAA	GGCTTGAA	TTCAAGCC
Forward Index 29	M6	GTTAAGTT	GTTAAGTT	AACTTAAC
Forward Index 30	N6	TCAAGTAT	TCAAGTAT	ATACTTGA
Forward Index 31	O6	TCGCGGAT	TCGCGGAT	ATCCGCGA
Forward Index 32	P6	TGAGTCCT	TGAGTCCT	AGGACTCA
Forward Index 33	A7	AGTTGAAC	AGTTGAAC	GTTCAACT
Forward Index 34	B7	AACCTCAG	AACCTCAG	CTGAGGTT
Forward 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 indexing (i5) primers			Forward indexes (i5) on sample sheet	
Index number	**Well position**	**Index sequence in primers**	**MiSeq, HiSeq 2000/2500, NovaSeq**	**NextSeq, HiSeq 3000/4000, HiSeq X**
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 8. Shasta Long Indexing Primer Set - A, reverse indexing primers 1–72 barcodes. Continued over the next two pages.

Reverse indexing (i7) primers			Reverse indexes (i7) on sample sheet
Index number	**Well position**	**Index sequence in primers**	**MiSeq, 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	TCTTGCCT	AGGCAAGA
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 indexing (i7) primers			Reverse indexes (i7) on sample sheet
Index number	**Well position**	**Index sequence in primers**	**MiSeq, NextSeq, HiSeq 2000/2500, HiSeq 3000/4000, HiSeq X, NovaSeq**
Reverse Index 24	H14	TTCGATGA	TCATCGAA
Reverse Index 25	I14	TTGATCCA	TGGATCAA
Reverse Index 26	J14	AACCTGCC	GGCAGGTT
Reverse Index 27	K14	AACGCATC	GATGCGTT
Reverse Index 28	L14	AACGCCAT	ATGGCGTT
Reverse Index 29	M14	AACGCGCA	TGCGCGTT
Reverse Index 30	N14	AAGAATGG	CCATTCTT
Reverse Index 31	O14	AAGACGCT	AGCGTCTT
Reverse Index 32	P14	ACCAACCG	CGGTTGGT
Reverse Index 33	A15	ACCGAATG	CATTCGGT
Reverse Index 34	B15	ACTCGCTA	TAGCGAGT
Reverse Index 35	C15	AGAAGAGC	GCTCTTCT
Reverse Index 36	D15	AGAATCTC	GAGATTCT
Reverse Index 37	E15	ATGCTTAG	CTAAGCAT
Reverse Index 38	F15	CAGACCTT	AAGGTCTG
Reverse Index 39	G15	CCGCTAGG	CCTAGCGG
Reverse Index 40	H15	CCGGTTAG	CTAACCGG
Reverse Index 41	I15	CCTCGACG	CGTCGAGG
Reverse Index 42	J15	CGAAGCTG	CAGCTTCG
Reverse 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 indexing (i7) primers			Reverse indexes (i7) on sample sheet
Index number	Well position	Index sequence in primers	MiSeq, NextSeq, HiSeq 2000/2500, HiSeq 3000/4000, HiSeq X, NovaSeq
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

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