Takara Bio USA, Inc.

ICELL8® cx 3′ DE User Manual

Cat. Nos. 640167, 640199, 640197, 640212
for ICELL8 cx CELLSTUDIO™ v2.5 Software
(052522)

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I. Introduction

High-throughput differential expression analysis of single cells

The **ICELL8 cx 3′ DE workflow** enables Illumina® sequencing and differential gene expression analysis—via 3′ end counting of transcripts—of single cells isolated on the **ICELL8 cx Single-Cell System** (Cat. No. 640188, 640189).

The workflow (Figure 1, below) begins with staining and dilution of cell samples and the preparation of positive and negative controls using components of the ICELL8 cx 3′ DE Reagent Kit (Cat. No. 640167), followed by the dispensing of the cells and controls into the uniquely barcoded 5,184 nanowells of the ICELL8 cx 3′ DE Chip (Cat. No. 640199) using the ICELL8 cx Single-Cell System. 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 cx Single-Cell System.

Following the dispensing of the samples and controls, the ICELL8 cx Single-Cell System is used to acquire images of the 5,184 nanowells with both blue and red wavelength filters, enabling visualization of Hoechst 33342 and propidium iodide or EthD-III staining, respectively (Hoechst staining is used to visualize and identify candidate cells based on size/morphology criteria, while propidium iodide or EthD-III staining is used to identify dead cells). Following image acquisition, images are analyzed either automatically or manually using ICELL8 cx CellSelect® v2.5 Software, which identifies nanowells containing single, viable cells that meet user-defined criteria. Upon identification and selection of candidate nanowells, ICELL8 cx CellSelect v2.5 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.

Following freezing of the ICELL8 cx 3′ DE Chip, the chip is returned to the ICELL8 cx Single-Cell System and RT-PCR reagents are distributed to nanowells selected by the software. The ICELL8 cx 3′ DE Chip is then transferred to a thermal cycler (included with Cat. No. 640188), which is programmed to perform first-strand synthesis and amplification of cDNA in a single run. During this process (Figure 2, below), the oligo(dT) primer preprinted into each nanowell of the ICELL8 cx 3′ DE Chip prime first-strand synthesis, adding an adapter and nanowell-specific barcode to the 3′ ends of first-strand cDNA, while a 5′ SMART® (**S**witching **M**echanism **A**t 5′ end of **R**NA **T**emplate) primer and template-switching technology are used to add an adapter to the 3′ ends of full-length cDNAs. The added adapter then serves as a priming site for the ICELL8 3′ DE primer during the PCR amplification portion of the program, allowing for unbiased amplification of full-length cDNA. The barcoded cDNAs from each nanowell are pooled, concentrated, and purified off-chip using Agencourt AMPure XP beads. Illumina sequencing libraries are prepared using the Nextera® XT kit, employing PCR to enrich for cDNA derived from mRNA 3′ ends, followed by a limited-cycle PCR amplification step that results in incorporation of Illumina index sequences. Following subsequent purification, size selection, and validation steps, libraries are ready for sequencing on Illumina platforms.

By leveraging the capabilities of ICELL8 cx Single-Cell System along with SMART technology, the ICELL8 cx 3′ DE workflow provides an efficient, cost-effective solution for high-throughput differential expression analysis of single cells.

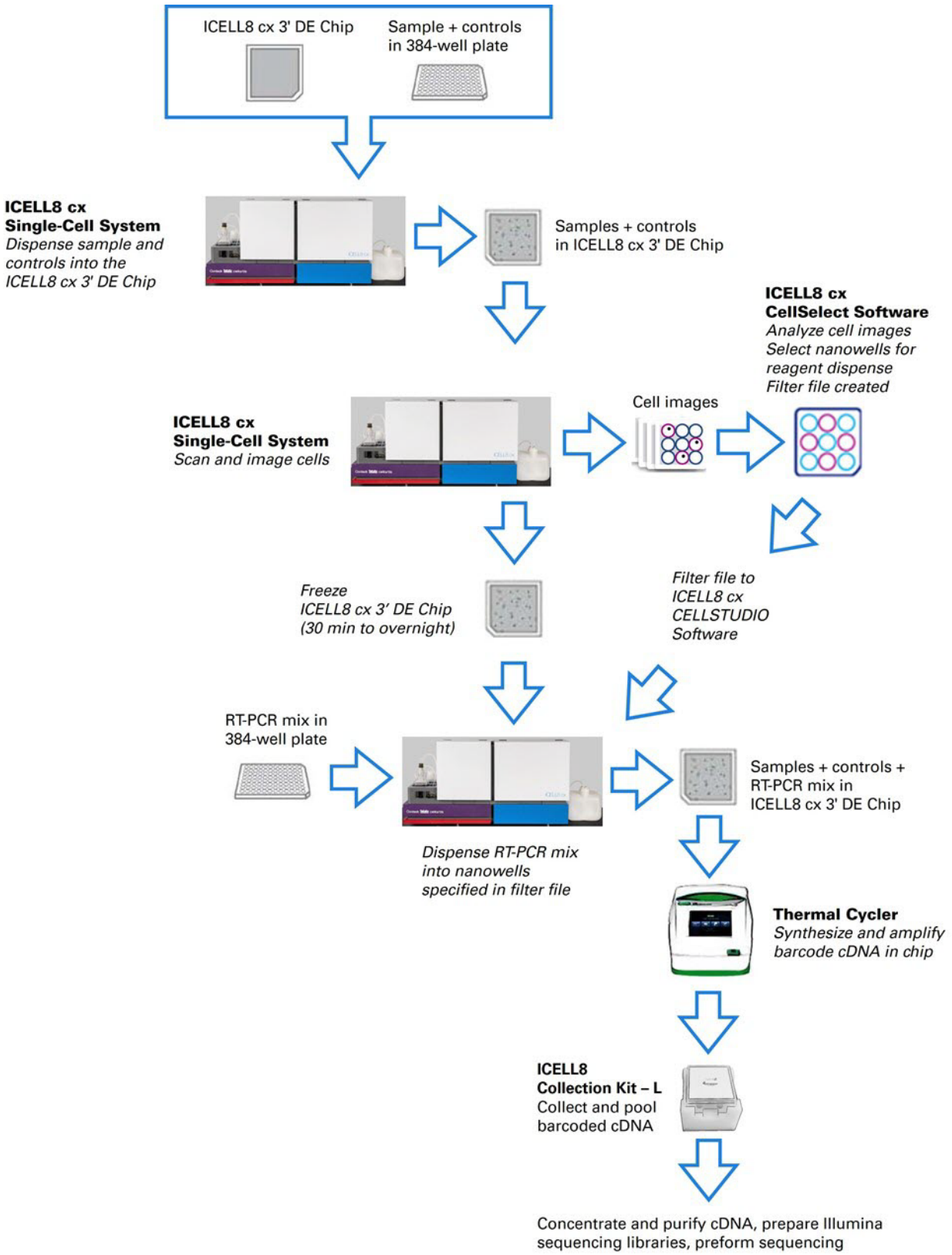

Figure 1. Diagram for the ICELL8 cx 3′ DE workflow.

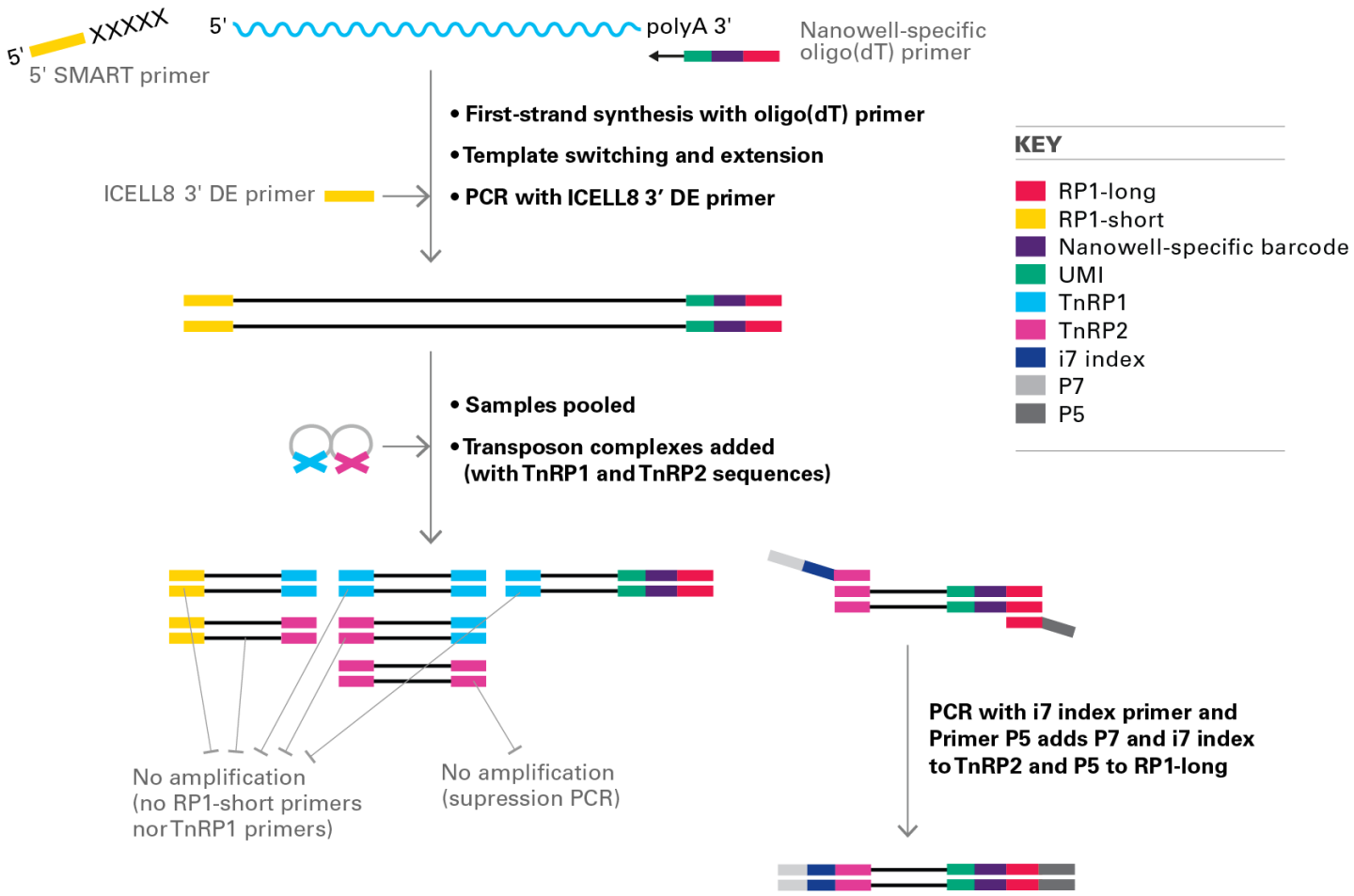

Figure 2. cDNA synthesis and sequencing library construction with the ICELL8 cx 3′ DE Chip and ICELL8 cx 3′ DE Reagent Kit. First-strand synthesis is primed by oligo(dT) primers, which include nanowell-specific barcode sequences, that are preprinted into each nanowell of the ICELL8 cx 3′ DE Chip. The oligo(dT) primers also include random nucleotide sequences of fixed length that contribute to library complexity during initial cycles of the sequencing reaction but do not yield meaningful data for performing differential expression analysis with the ICELL8 cx 3′ DE Chip and ICELL8 3′ DE Reagent Kit. Upon reaching the 5′ end of the RNA template, the MMLV-derived SMARTScribe™ Reverse Transcriptase adds a stretch of nontemplated nucleotides that hybridize with complementary nucleotides included in the 5′ SMART primer (provided in the ICELL8 3′ DE Oligo Mix). The template-switching SMARTScribe enzyme then incorporates additional nucleotides onto the 3′ end of the first-strand cDNA using an adapter sequence included in the 5′ SMART primer as a template. Adapter sequences incorporated at both ends of the first-strand cDNA then serve as binding sites for the ICELL8 3′ DE primer (also included in ICELL8 3′ DE Oligo Mix) during subsequent rounds of PCR amplification. Upon completion of the RT-PCR reaction, double-stranded cDNA is extracted from the nanowell, pooled together, and purified using AMPure XP beads. Purified cDNA is then fragmented and tagged in a transposase-based reaction using the Illumina Nextera XT kit, and the “tagmented” cDNA is then subjected to a limited-cycle PCR reaction that results in incorporation of Illumina adapters and indexes. Following subsequent purification, size selection, and validation steps, NGS libraries are ready for sequencing on Illumina platforms.

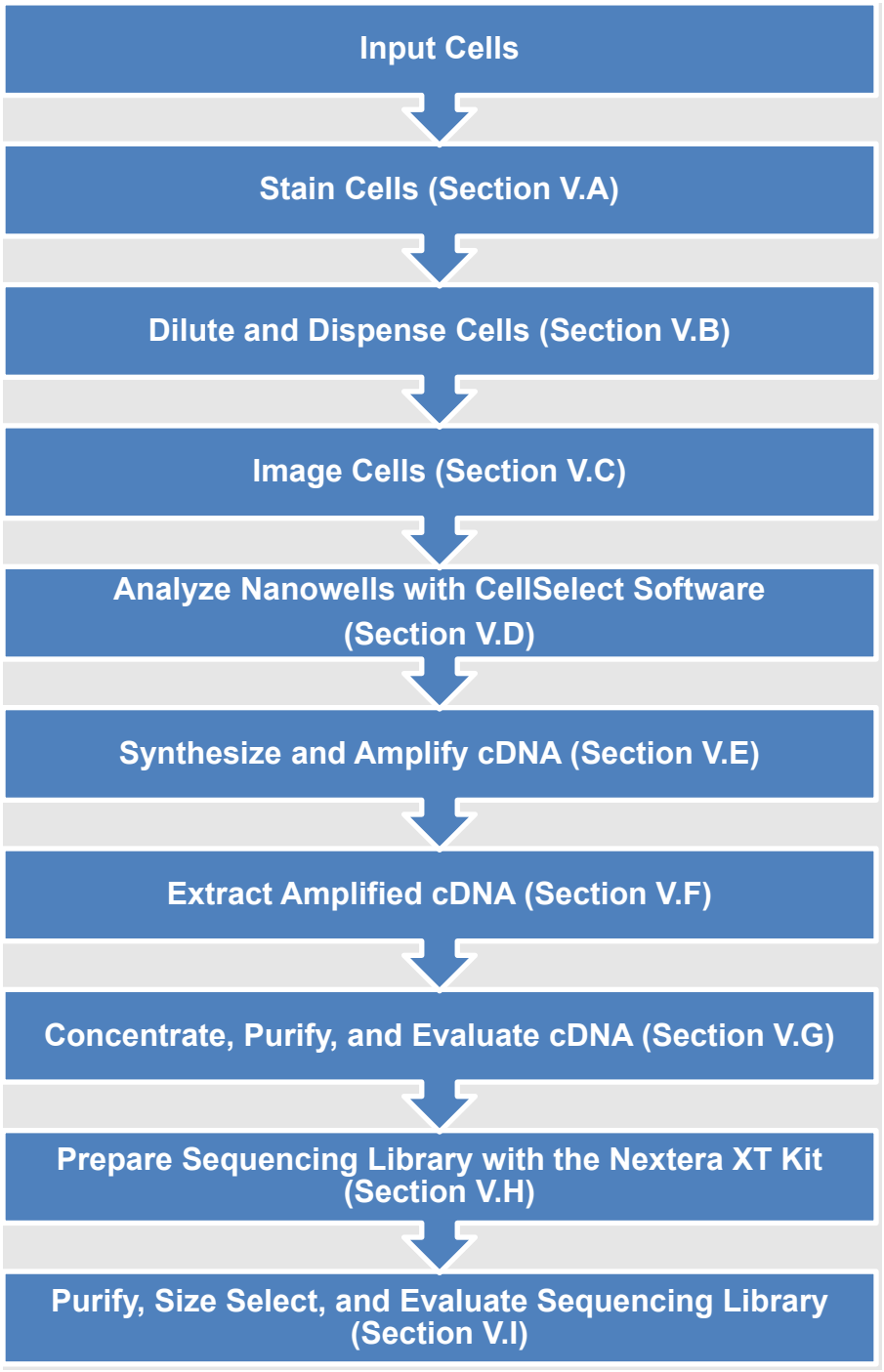

Figure 3. Protocols included in this user manual.

II. List of Components

The ICELL8 cx 3′ DE workflow requires use of an ICELL8 cx Single-Cell System (Cat. No. 640188, 640189) and consists of the ICELL8 3′ DE Reagent Kit (Cat. No. 640167), the ICELL8 cx 3′ DE Chip (640199), the ICELL8 cx Loading Kit (640197), and the ICELL8 Collection Kit – L (640212). Each of these products must be purchased separately:

ICELL8 3′ DE Reagent Kit (Cat. No. 640167)	Cap color	Volume
Package 1 (Store at –70°C)		
Control K-562 RNA (1 µg/µl)	Yellow	5 µl
Package 2 (Store at –20°C)		
$MgCl_2$ (1 M)	Blue	5 µl
5X First-Strand Buffer	Red	65 µl
SeqAmp™ PCR Buffer (2X)	Green	35 µl
SMARTScribe Reverse Transcriptase (100 U/µl)	Purple	30 µl
SeqAmp DNA Polymerase	Green	10 µl
Triton X-100 (10%)	Blue	20 µl
ICELL8 3′ DE Oligo Mix	Pink	5 µl
DTT (100 mM)	Orange	10 µl
GC Melt (5 M)*	Clear	60 µl
dNTP Mix (25 mM each)	White	25 µl
Second Diluent (100X)	Light blue	15 µl
ICELL8 Fiducial Mix (1X) (not used)		25 µl
RNase Inhibitor (40 U/µl)	White	15 µl
Primer P5 (5 µM)	Brown	10 µl
Nuclease-Free Water		500 µl

*At times, precipitate may be observed in the GC Melt reagent. This precipitate does not affect the performance of the kit. The precipitate can be dissolved rapidly by mixing at room temperature or warming at 37°C for a few min.

ICELL8 cx 3′ DE Chip (Cat. No. 640199, store at room temperature)

ICELL8 cx Loading Kit (Cat. No. 640197, store at room temperature)

Component	Quantity per kit
Blotting Paper	2
Optical Imaging Film	1
TE Sealing Film	1
SmartChip® Intermediate Film	1

ICELL8 Collection Kit (Cat. No. 640212, store at room temperature)

Component	Quantity per kit
Collection Fixture – L	1
Collection Tube (2.0 ml)	2
Collection Film	1

Details on the components included in these products are available for download at takarabio.com.

III. Additional Materials Required

The following reagents and materials are required but not supplied with the ICELL8 cx 3′ DE workflow products or the ICELL8 cx Single-Cell System:

- Personal protective equipment (PPE; e.g., powder-free gloves, safety glasses, lab coat, sleeve protector)
- Nuclease-decontamination solution
- Nuclease-free water
- Centrifuges and rotors for conical tubes and plates (e.g., Eppendorf 5810R with swinging plate buckets, ≥2,600*g*, room temp and 4°C operation; Kubota 3740 with rotor SF-240 for cell prep)
- 15-ml conical tubes
- 5-ml flip-cap tubes
- Nuclease-free: 0.2-ml PCR tubes and nonstick 1.5-ml 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
- Minicentrifuges for 1.5-ml tubes and 0.2-ml tubes or strips
- Vortex mixer
- Exhaust hood system with UV

For staining and dispensing cells (all cell types):

- 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)
- ICELL8 384-Well Source Plate and Seal (Takara Bio, Cat. Nos. 640192, 640018, or 640037)
- Molecular-biology-grade/PCR-grade/nuclease-free water

For adherent cells:

- TrypLE Express (Life Technologies, Cat. No. 12604-021)
- Cell culture media, particular to your sample

For primary cells (such as PBMCs):

- Live/Dead Cell Staining Kit II (PromoCell, Cat. No. PK-CA707-30002)
  NOTE: Only EthD-III from the kit is needed for this protocol

For cDNA concentration and quantification:

- DNA Clean & Concentrator-5 - Capped Columns (50 preps; Zymo Research, Cat. No. D4013)
- Qubit dsDNA HS Assay Kit (100 assays; Thermo Fisher Scientific, Cat. No. Q32851)
- Qubit Fluorometer (Thermo Fisher Scientific)
- Agilent 2100 Bioanalyzer (Sections V.F and V.H): High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)
- Molecular-biology-grade/PCR-grade/nuclease-free water

For cDNA and sequencing library purification:

- 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.
- 70% ethanol: prepared fresh from anhydrous ethanol for each experiment

For sequencing library preparation and validation:

- Nextera XT DNA Library Preparation Kit (24 samples; Illumina, Cat. No. FC-131-1024)
- Nextera XT Index Kit (24 indexes, 96 samples; Illumina, Cat. No. FC-131-1001)
- KAPA Library Quantification Kit (for Illumina platforms; KAPA Biosystems, Cat. No. KK4854)
- Thermal cycler with block for 0.2-ml tubes
- Agilent 2100 Bioanalyzer (Sections V.F and V.H): High Sensitivity DNA Kit (Agilent, Cat. No. 5067-4626)

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

IV. General Considerations

A. 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 10^5 cells/ml prior to starting the protocol.

B. 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 amplification reagents and 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.
- UV treat reagent reservoirs, seals, pipettes, filter tips, and compatible reagents prior to use. **DO NOT UV treat preprinted chips.**

C. 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.

D. 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 the chip oriented with the chamfered (beveled) corner positioned towards the lower-right corner of the chip nest (Figure 4, left).
- 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 source plate nest. (Figure 4, right).

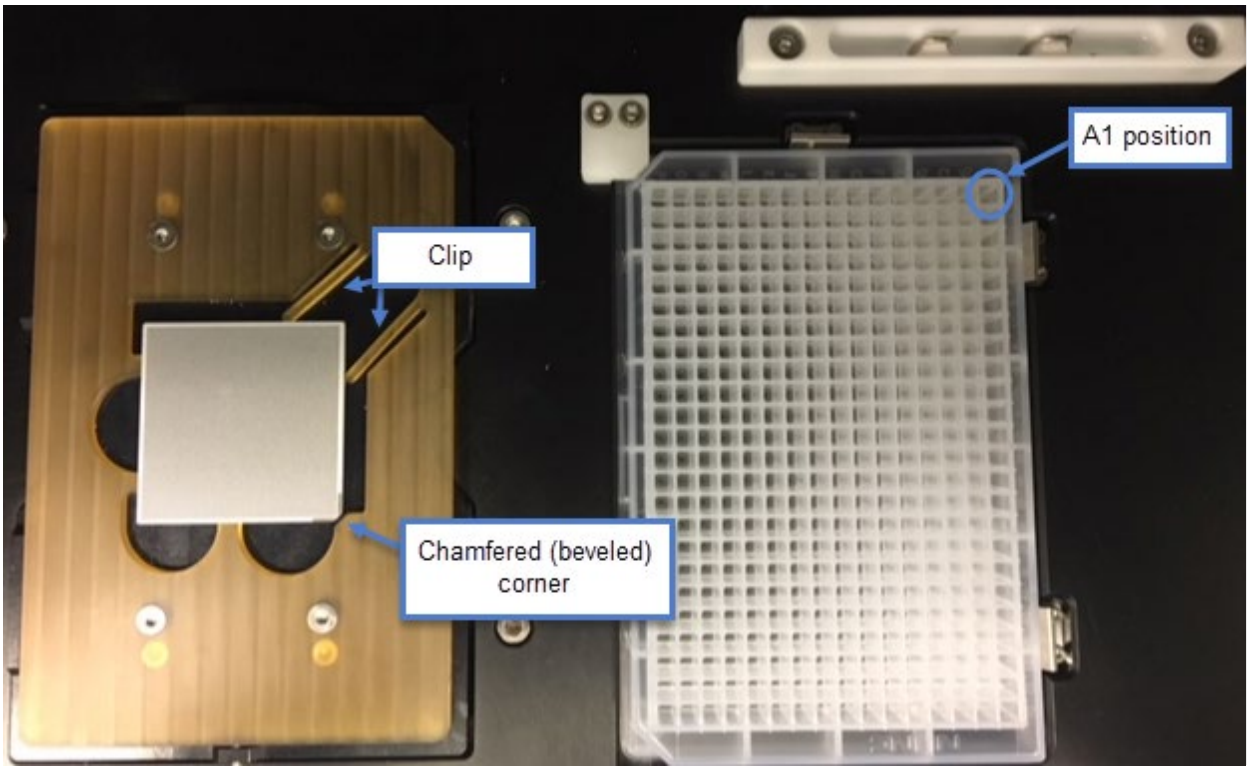

Figure 4. ICELL8 cx Single-Cell System stage module. **(Left)** chip nest. **(Right)** source plate nest.

E. ICELL8 cx 3′ DE Chip

- Each ICELL8 cx 3′ DE Chip is engraved with a unique number. You can use this number to link your chip images and other experimental record files.
- Oligos containing nanowell-specific barcodes were preprinted into each nanowell of the ICELL8 cx 3′ DE Chip; the printing lot number is stamped onto the chip (Figure 5, below). A total of 5,184 unique nanowell barcodes are available; each nanowell barcode was printed once into the chip.

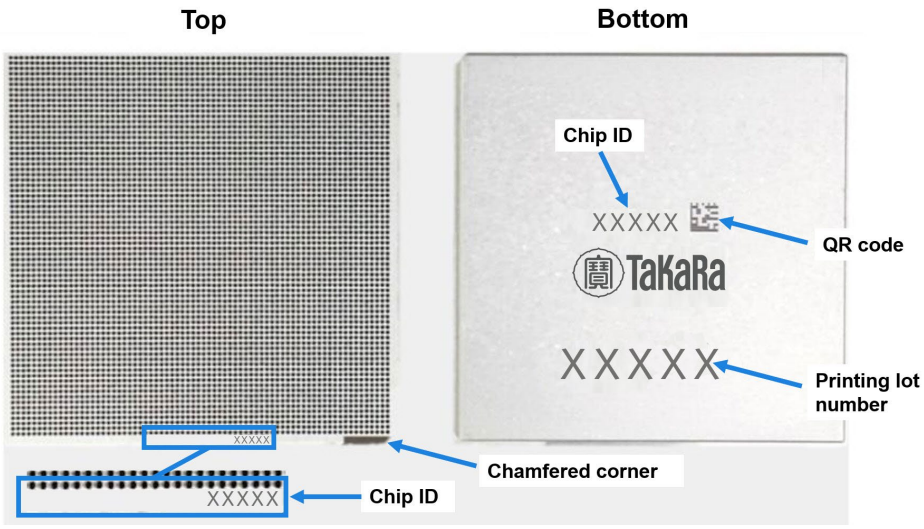

Figure 5. ICELL8 cx 3′ DE Chip features. (Left). Top view of the chip. In addition to the 5,184 nanowells, note the chamfered (notched) corner at the bottom right. "TaKaRa" and the chip ID (unique to each chip) are engraved near the chamfered corner. **(Right)** Bottom view of the chip. The chip ID is 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. The lot number corresponding to the printing of the barcode- and oligos is also engraved on this side of the chip.

F. Software

The instructions in this manual are written for use with CELLSTUDIO and CellSelect v2.5 Software. Please refer to the ICELL8 cx Single-Cell System User Manual and the ICELL8 cx CellSelect v2.5 Software User Manual for more detailed information.

V. Procedure

A. Protocol: Stain Cells

In this protocol, sample cells are stained with Hoechst 33342 and propidium iodide or ethidium homodimer dyes that enable imaging, analysis, and selection of candidates suitable for downstream analysis following dispensation into the ICELL8 cx 3′ DE Chip.

- If working with cells in suspension, start from Section V.A.1
- If working with adherent cells, start from Section V.A.2 and then proceed to Section V.A.1.
- If working with primary cells such as PBMCs, start from Section V.A.3 and then proceed to Section V.A.1

Prerequisite:

- Several milliliters of healthy cell culture suspension freshly resuspended in PBS.
- Maintain cell density between 1 x 10^5 and 7.5 x 10^6 cells/ml.

Before you start:

- Perform the steps to prepare the ICELL8 cx system for use, if it has not been run yet for the day. Refer to the ICELL8 cx Single-Cell System User Manual (Section VIII, "Protocol: Prepare the ICELL8 cx System") for more details.
- Prefreeze ICELL8 Chip Holder(s) (Figure 6, below) at –80°C.

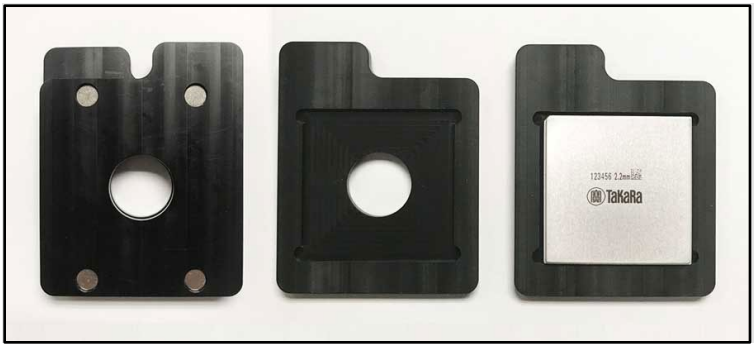

Figure 6. ICELL8 Chip Holder.

- Set the chip centrifuge to 22°C.
- Thaw Second Diluent (100X) and Nuclease-Free Water on ice. Once thawed, keep on ice for the remainder of the protocol.
- Prewarm to 37°C:
  - 1X PBS (Ca^{2+} and Mg^{2+} free, pH 7.4)

  If using adherent cells, also prewarm to 37°C:
  - TrypLE Express
  - Cell culture medium for your sample
- Dilute Control K-562 RNA (1 µg/µl) to 10 ng/µl for use in the next protocol (Section V.B, Table 1) as indicated in the following steps and keep the dilution on ice:

  a. Dilute to 50 ng/µl by mixing 38 µl of nuclease-free water with 2 µl of Control K-562 RNA (1 µg/µl) in a sterile nuclease-free microcentrifuge tube.

  b. Dilute to 10 ng/µl by mixing 20 µl of nuclease-free water with 5 µl of the Control K-562 RNA diluted to 50 ng/µl in the previous step a.

NOTES:

- Return Control K-562 RNA (1 µg/µl) stock solution to storage at –70°C.
- Diluted Control K-562 RNA should be kept on ice at all times.

Cell and chip handling notes:

- Keep cells at 37°C with 5% CO_2 in a cell culture incubator when not performing manipulations.
- Some cell lines may require trypsinization to achieve a single-cell suspension.
- 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.

1. Preparation of Stained Suspension Cells (e.g., Human K-562 cells)

1. Prepare a 1:2 mixture of Hoechst 33342 and propidium iodide. Combine 80 µl of Hoechst 33342 and 160 µl of propidium iodide per ml of cells to be stained. An example using 2 ml of cells is described below (e.g., prepare 480 µl of premixed dye solution).

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

2. Transfer ~2.1 ml of suspension cells in PBS 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 480 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 prewarmed 1X PBS to stained cells. For the example described here, 2 ml of 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 for 3 min at room temperature.

   NOTES:
   - Optimal centrifugation speed and time may vary depending on the cell type being analyzed. For K-562 or 3T3 cells, centrifuge at 100*g*. For smaller cells, such as PBMCs, or cells that are not pelleting at 100*g*, centrifuge speed can be increased up to 500*g*.
   - Avoid over-centrifugation or pelleting into a firm pellet or clump.

9. Gently 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 prewarmed 1X PBS 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 1.2×10^5–5.0×10^5 cells/ml. If you end up with a concentration lower than 1.2×10^5 cells/ml, re-pellet 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. Preparation of Adherent Cells in 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 prewarmed TrypLE Express 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 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 100*g*. For smaller cells or cells that are not pelleting at 100*g*, centrifuge speed can be increased up to 500*g*.
   - Avoid over-centrifugation or pelleting into a firm pellet or clump.

8. Gently add 10 ml of prewarmed 1X PBS to the tube side wall.
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. Follow the procedure for Preparation of Stained Suspension Cells (Section V.A.1, above) starting from Step 1.

3. Preparation of PBMCs and Other Primary Cells

1. Prepare 20 µM EthD-III dilution in PBS following the instructions for the Live/Dead Cell Staining Kit II from PromoCell. For example, combining 20 µl of 2 mM EthD-III with 1,980 µl of PBS will result in 2 ml of 20 µM EthD-III. Vortex briefly.
2. Combine 80 µl of Hoechst 33342 and 1 ml of 20 µM EthD-III per ml of cells to be stained. An example using 1 ml of cells is described below (e.g., prepare 1,080 µl of premixed dye solution).

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

3. Transfer ~1.1 ml of suspension cells to a fresh 5-ml tube.
4. 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.
5. Add 1,080 µl of the premixed Hoechst 33342 and 20 µM EthD-III dye mix to the cells. Mix gently by inverting the tube 5 times. Do not vortex or overagitate the cells.

6. Incubate cells at 37°C for 20 min.
7. Add an equal volume of prewarmed 1X PBS to stained cells. For the example used above, 2 ml of 1X PBS is added to the 5-ml tube containing the stained cell suspension.
8. Follow the procedure for Preparation of Stained Suspension Cells (Section V.A.1, above) starting from Step 7.

B. Protocol: Dilute and Dispense Cells

In this protocol, sample cells and controls are diluted and aliquoted into a 384-well source plate and dispensed into the ICELL8 cx 3′ DE Chip using the ICELL8 cx Single-Cell System. Refer to the ICELL8 cx Single-Cell System User Manual for detailed information about instrument setup and operation.

Before you start

- Confirm that initialization and setup procedures for the ICELL8 cx Single-Cell System have been completed for the day or prepare the system if needed. (Refer to the ICELL8 cx Single-Cell System User Manual, Section VIII, "Protocol: Prepare the ICELL8 cx System", for more details).
- Pre-freeze the empty Chip Holder (Figure 6, above) at –80°C, if that was not done in the previous step (Section V.A).
- Aliquot 300–500 µl of 1X PBS (Ca^{2+} and Mg^{2+} free, pH 7.4) on ice for positive and negative controls.
- This protocol requires a 384-Well Source Plate and Seal; the following components from the ICELL8 cx 3′ DE Reagent Kit: Control K-562 RNA (diluted to 10 ng/µl in Section V.A "Before you Start", above), Second Diluent (100X), and RNase Inhibitor (40 U/µl); and from the ICELL8 cx Loading Kit: Blotting Paper and SmartChip Intermediate Film.
- Use the concentration of stained cell suspension measured at the end of the previous protocol (Section V.A) and the information in Table 1 (below) to calculate the volumes of stained cell suspension and 1X PBS that should be combined to obtain a final concentration of 1.4 cells/50 nl in a total volume of 1 ml.

Table 1. Sample Preparation Guidelines

Components	Negative Control	Positive Control	Diluted Stained Cell Suspension	For 8 Samples (Per Sample Amounts)
Second Diluent (100X)	1.0 µl	1.0 µl	10.0 µl	1.0 µl
RNase Inhibitor (40 U/µl)	1.0 µl	1.0 µl	10.0 µl	1.0 µl
Control K-562 RNA (10 ng/µl)	–	2 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)	98.0 µl	96 µl	Up to 1,000.0 µl	Up to 100 µl
Total	100.0 µl	100.0 µl	1,000.0 µl**	100 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 1.4 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 7, below).

Procedure

Prepare diluted stained cell suspension

1. Vortex Second Diluent and RNase Inhibitor 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, and Second Diluent indicated in the corresponding column of Table 1 (above). 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, and Second Diluent), 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 cx Cell Dilution Tool spreadsheet provided by the Field Application Specialist during training). DO NOT use the 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 1 (above).

   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/50 nl (equivalent to the total RNA content from 1 cell).

Prepare cell dispense source plate

Refer to the ICELL8 cx Single-Cell System 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 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.

6. 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 7 (below).

NOTES:

- Make sure to take each aliquot from the center of the tube containing the diluted stained cell suspension.
- Be careful not to splash liquid into neighboring wells.
- Make sure not to introduce bubbles when adding the cell suspension to the 384-well source plate.
- Do not vortex or spin down the 384-well source plate.
- Do not tap plate. If any bubbles are present, remove using a pipette tip.

7. Add Positive and Negative Controls to the 384-well source plate as indicated in the following steps and in Figure 7 (below):
    - Add 25 µl of prepared Positive Control to well P24.
    - Add 25 µl of prepared Negative Control to well A24.

NOTES:

- Be careful not to splash liquid into neighboring wells.
- Make sure not to introduce bubbles when adding reagents to the 384-well source plate.
- Do not vortex or spin down the 384-well source plate.
- Do not tap plate. If any bubbles are present, remove using a pipette tip.

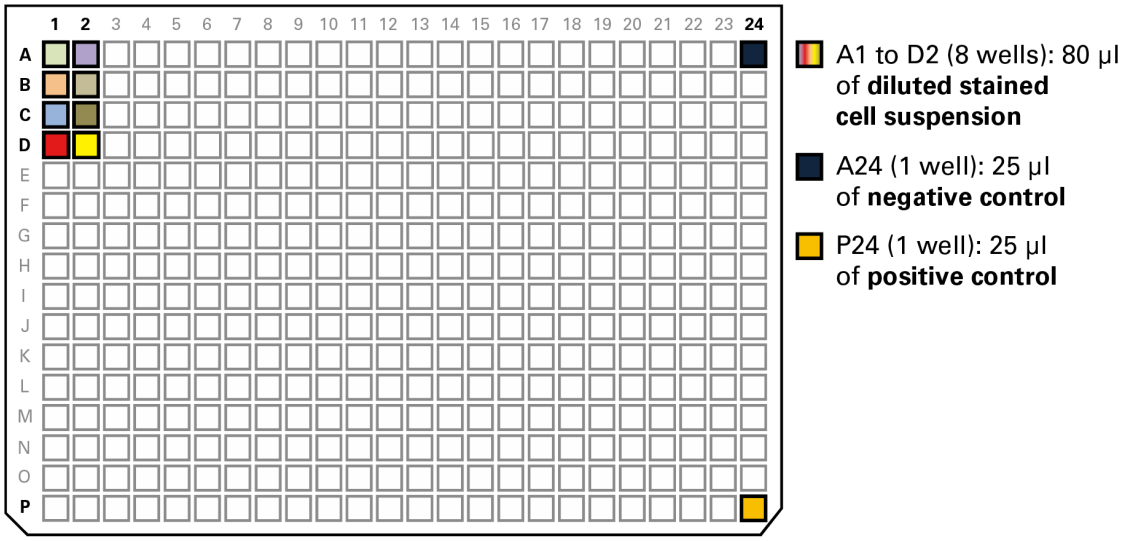

Figure 7. Setting up the 384-well source plate for dispensing cell samples and controls.

8. Seal the 384-well source plate with the Plate Seal. Do not centrifuge the plate.
9. Open the packet containing the sealed ICELL8 cx 3′ DE Chip.
10. 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 chuck of the chip nest (refer to the ICELL8 cx Single-Cell System User Manual, Section X.A).

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

12. In CELLSTUDIO Software, click the [Dispense cells and Controls (50 nl)] button (Figure 8, below).

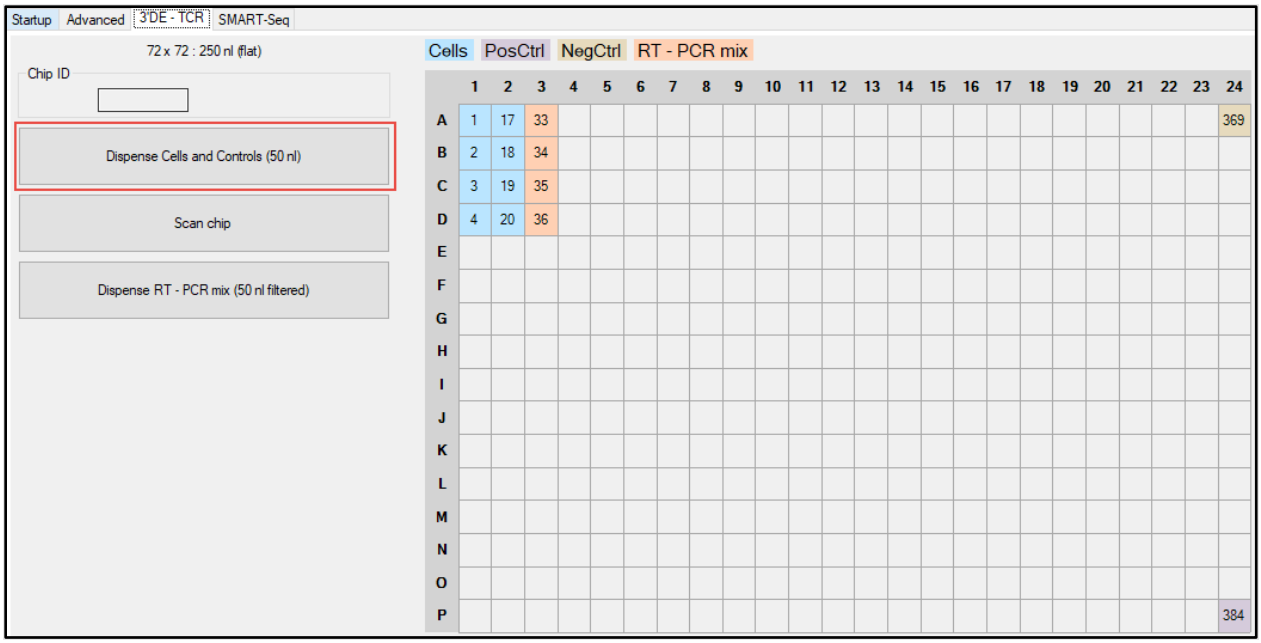

Figure 8. Using CELLSTUDIO Software to dispense cells and controls into the ICELL8 cx 3′ DE Chip.

13. Follow the software prompts and check the orientation of the source plate and ICELL8 cx 3′ DE Chip to ensure they are correctly loaded on the dispensing platform. Click [Done] to proceed. 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, using them as a guideline for navigating the 3′ DE workflow.

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

15. Seal the loaded chip with the SmartChip Intermediate Film (Figure 9, left).

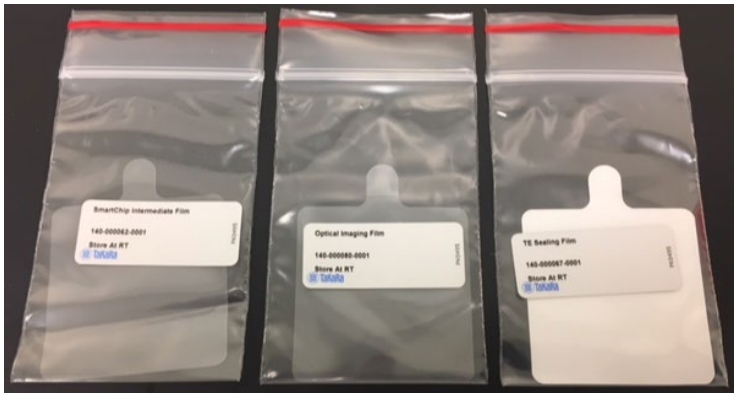

Figure 9. TE Sealing Film, Optical Imaging Film, and SmartChip Intermediate Film. **(Left)** SmartChip Intermediate Film is single-sided with a clear backing. **(Center)** Optical Imaging Film is double-sided and is provided between two layers of clear backing. **(Right)** TE Sealing Film is provided with a white backing.

Remove the liner from the Intermediate Film and apply the exposed side of the film to the blotted chip.

16. Seal the blotted chip with the film using a film applicator or sealing roller (Figure 10, below).

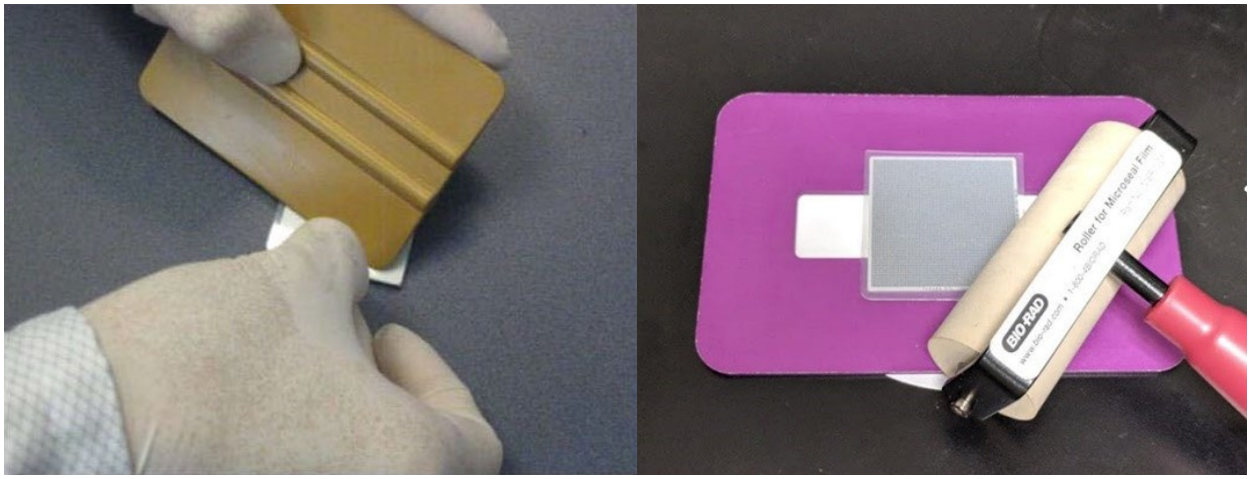

Figure 10. Sealing the blotted chip. Seal the blotted chip using **(Left)** a film applicator or **(Right)** a film sealing roller.

17. Place the sealed chip on a centrifuge spin plate and centrifuge the sealed chip at 300*g* for 5 min at 22°C with full acceleration and full brake (Figure 11, below). If you have one chip, balance the centrifuge with the supplied Balance Chip or a blank chip. Proceed to the next protocol (Section V.C) once the centrifugation is complete.

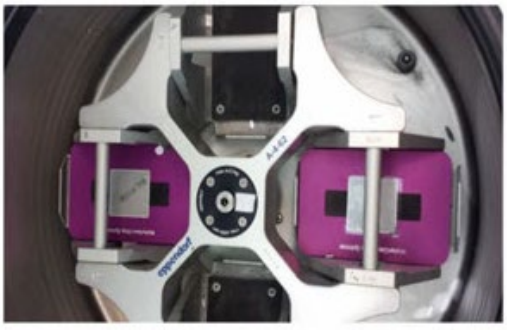

Figure 11. Centrifuge Chip Spinner.

C. Protocol: Image Cells

In this protocol, images of all 5,184 nanowells of the ICELL8 cx 3′ DE Chip are acquired.

NOTE: This section is only necessary when the sample source is single cells.

If starting with nucleic acids or your sample does not require imaging:

- The `<chipID>_WellList.txt` file that may be imported into Takara Bio bioinformatics software will be generated by CELLSTUDIO Software.
- Skip the remainder of this section and go to Section V.E.

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 "Analysis settings" should have the value '`AnalysisSetting_250nL_chip.XML`' selected and greyed out. For "Barcodes", choose '`3'DE.xml`' from `C:\ProgramData\Takara\CellSelect\AssayMaps\`.

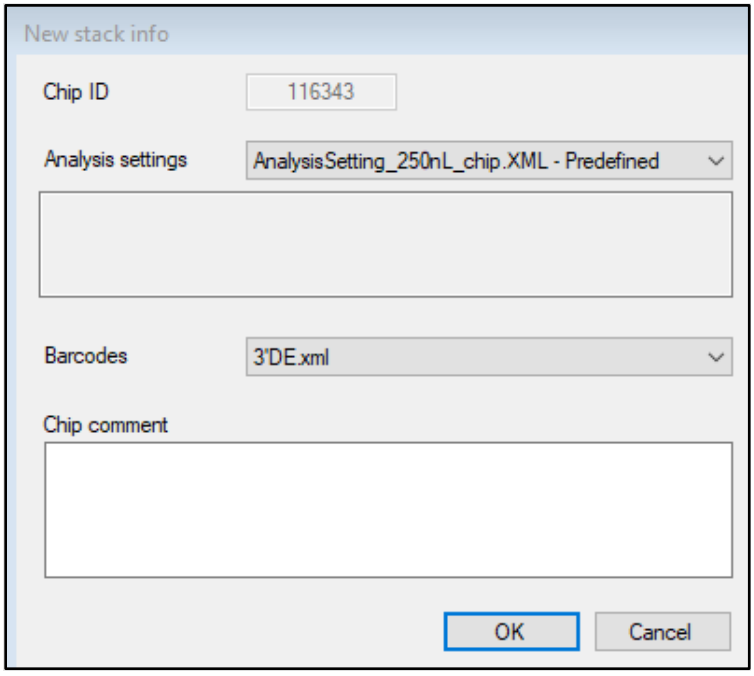

Figure 12. *New stack info* dialog window.

   c. Continue with the procedure from steps 7–12.
   d. At Step 13, remove the liner from only one side of the Optical Imaging film and apply the exposed sticky side of the imaging film to the chip, sealing carefully (similar to Figure 10, above).
2. Place the imaged chip into an empty Chip Holder that has been pre-chilled at –80°C. The chip holder should click closed and should close evenly, indicating a proper magnetic seal. Make sure that the Imaging Film is well sealed over the chip.

3. Freeze cells at –80°C for a minimum of 30 min before proceeding to RT-PCR (Section V.E, below).

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

D. Protocol: Analyze Nanowells with ICELL8 cx CellSelect v2.5 Software

If using cells which were imaged in the previous step, manually inspect the selected nanowells in CellSelect Software to exclude or include one or more candidate wells. Please refer to the ICELL8 cx CellSelect v2.5 Software User Manual for more information about this process.

NOTE: We recommend using automated threshold detection (auto-tune) to determine candidate wells for the RT-PCR dispense. Refer to Appendix F of the ICELL8 cx CellSelect v2.5 Software User Manual for more details.

E. Protocol: Synthesize and Amplify cDNA

In this protocol, reagents for cDNA synthesis and amplification are dispensed into selected wells of the ICELL8 cx 3′ DE Chip using the ICELL8 cx Single-Cell System, and RT-PCR is performed in-chip. Products from the RT-PCR reaction are then extracted from the chip and pooled together for further processing.

For this protocol, you will need the following reagents from the ICELL8 cx 3′ DE Reagent Kit:

GC Melt (5 M), dNTP Mix (25 mM each), $MgCl_2$ (1 M), DTT (100 mM), 5X First-Strand Buffer, Triton X-100 (10%), ICELL8 3′ DE Oligo Mix, SMARTScribe Reverse Transcriptase (100 U/µl), SeqAmp PCR Buffer (2X), and SeqAmp DNA Polymerase**.**

Before you start:

- Confirm that initialization and setup procedures for the ICELL8 cx Single-Cell System have been completed for the day or prepare the system if needed. (Refer to the ICELL8 cx Single-Cell System User Manual, Section VIII, “Protocol: Prepare the ICELL8 cx System”, for more details).
- Set the centrifuge(s) used for spinning the chip and 384-well source plate to 4°C.
- This protocol requires an ICELL8 384-Well Source Plate and Seal; the following components from the ICELL8 cx Loading Kit: Blotting Paper, TE Sealing Film; and the following components from the ICELL8 Collection Kit – L: Collection Fixture, Collection Tube, and Collection Film.
- Thaw all reagents on ice except for the SMARTScribe Reverse Transcriptase, SeqAmp DNA Polymerase, and Triton X-100. Thaw and keep Triton X-100 at room temperature. 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 besides the Triton X-100 and enzymes.

Procedure:

1. Remove the chip holder containing the ICELL8 cx 3′ DE Chip from the –80°C freezer. Thaw the chip in the chip holder until it reaches room temperature (about 10 min) to lyse cells.
2. 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 dispensation of RT-PCR mix.
3. Combine RT-PCR reagents in a microcentrifuge tube on ice in the order listed in the recipe below, up to and including the Triton X-100. Vortex the mixture after addition of the Triton X-100 until it is completely dissolved. Keep the reagents on ice.
4. Add ICELL8 3′ DE Oligo Mix. Vortex gently and spin down. Keep the mixture on ice.
5. Add the SMARTScribe Reverse Transcriptase and SeqAmp DNA Polymerase to the mixture immediately prior to use.

   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.

56 µl	GC Melt (5 M)
24 µl	dNTP Mix (25 mM each)
3 µl	$MgCl_2$ (1 M)
9 µl	DTT (100 mM)
62 µl	5X First-Strand Buffer
33 µl	SeqAmp PCR Buffer (2X)
16 µl	Triton X-100 (10%)*
2 µl	ICELL8 3′ DE Oligo Mix
29 µl	SMARTScribe Reverse Transcriptase** (100 U/µl)
10 µl	SeqAmp DNA Polymerase**
244 µl	Total volume per reaction

*Vortex the mixture after addition of the Triton X-100 until it is completely dissolved. Keep the reagents on ice.
**Add the enzymes and mix by pipetting up and down.

6. Pipette 50 µl of the RT-PCR reaction mix into wells A3, B3, C3, and D3 of a 384-Well Source Plate, as shown in Figure 13.

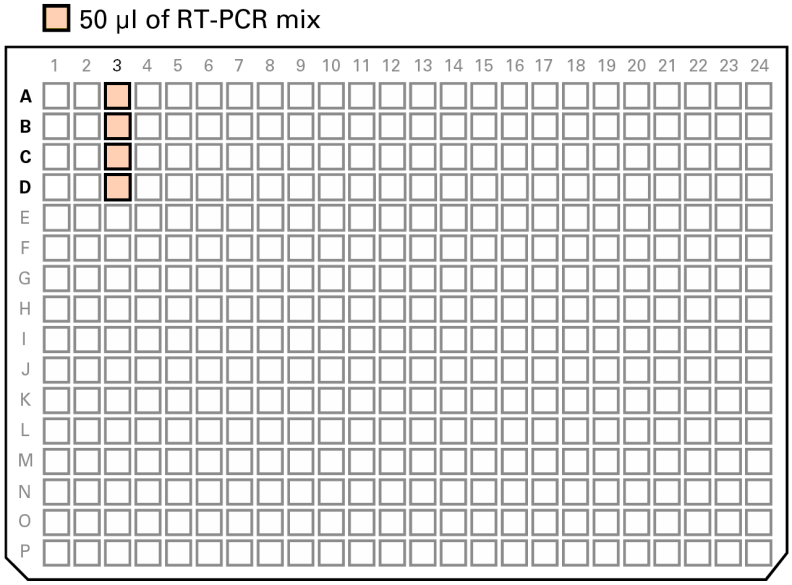

Figure 13. Setting up the source plate for dispensing RT-PCR mix.

7. Seal the 384-Well Source Plate with the 384-Well Plate Seal.
8. Centrifuge the source plate at 3,220*g* (minimum 2,600*g*) for 3 min at 4°C.
9. 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 3, with loading the source plate into the plate nest.
   b. At step 6, click the [Dispense RT - PCR mix] button (Figure 14).

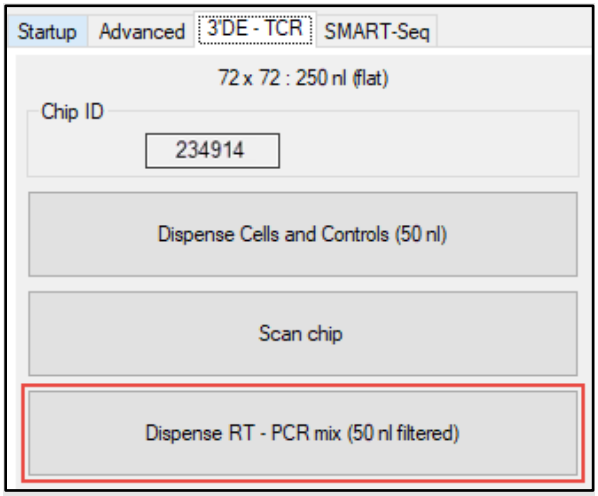

Figure 14. Using CELLSTUDIO Software to dispense the RT - PCR mix.

   c. Follow steps 7–12 as written in Section X.F.
   d. For step 13, seal with the TE Sealing Film.
   e. For step 14, centrifuge the sealed chip at 3,220*g* for 3 min at 4ºC.
   f. Perform the [Tip Clean] procedure 3–4 times (step 15).

(*Continued on the next page*)

10. Place the ICELL8 cx 3′ DE Chip into the thermal cycler to perform the RT-PCR reaction using the following program.

NOTES:

- The RT-PCR program described below is called "2DIS-PCR" and preinstalled on the ICELL8 cx Thermal Cycler provided with the ICELL8 cx System (Cat. No. 640188), using temperatures that are calibrated individually for each machine and that may vary slightly from the temperatures indicated below. Do not alter the preinstalled program or substitute a different program without first consulting with a member of the Field Support Team.
- If you are using the ICELL8 cx Single-Cell System JPN (Cat. No. 640189), use a thermal cycler of your choice. See the manufacturer's user manual for further instructions on thermal cycling the filled chip.

50°C	3 min
4ºC	5 min
42ºC	90 min
2 cycles:	
50°C	2 min
42°C	2 min
70°C	15 min
95ºC	1 min
24 cycles:	
98ºC	10 sec
65ºC	30 sec
68ºC	3 min
72ºC	10 min
4°C	forever

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

F. Protocol: cDNA Extraction from the Chip

This protocol extracts the amplified cDNA from the chip.

Refer to the ICELL8 cx Single-Cell System User Manual, Section XI "Protocol: Extract library from the chip" for the procedure. The collected volume should be around 85% of the Maximum Potential Volume.

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

G. Protocol: Concentrate, Purify, and Evaluate cDNA

In this protocol, extracted cDNA is concentrated using the Zymo DNA Clean & Concentrator-5 kit and purified using a 0.6X proportion of AMPure XP beads. The purified cDNA is then quantified with the Qubit fluorometric assay and analyzed with an Agilent Bioanalyzer.

Before you start:

- Make sure that ethanol was added to the wash buffer provided with the Zymo DNA Clean & Concentrator-5 kit prior to first use.
- 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 70% (v/v) ethanol from a stock of anhydrous ethanol.
- This protocol requires the Nuclease-Free Water provided with the ICELL8 cx 3′ DE Reagent Kit.

Procedure:

Concentrate cDNA

1. Transfer the cDNA eluent obtained in the previous protocol to a 1.5-ml nuclease-free microcentrifuge tube and measure the volume of extracted eluent with a pipette tip.
2. Use the DNA Clean & Concentrator-5 kit to purify the cDNA product by following the manufacturer's protocol:
   a. Add 5 volumes of DNA binding buffer to 1 volume of cDNA.
   b. Transfer the mixture (~700 µl at a time) to a Zymo-Spin Column affixed with a Collection Tube.
   c. Centrifuge at maximum speed (≥10,000*g*) for 30 seconds. Discard the flowthrough. Repeat Steps 2b and 2c until the entire volume of eluted cDNA is captured on the Zymo-Spin Column.
   d. Add 200 µl of DNA Wash Buffer to the column. Centrifuge for 30 seconds.
   e. Repeat the previous step (Step 2d), for a total of 2 washes.
   f. Spin the column at maximum speed (≥10,000*g*) for 1 minute to dry the column matrix.
   g. Transfer the column to a 1.5-ml nuclease-free microcentrifuge tube. Add 16 µl of Nuclease-Free Water directly to the column matrix and incubate at room temperature for 1 minute. Centrifuge at maximum speed (≥10,000*g*) for 30 seconds to elute cDNA. You should end up with ~15 µl of eluent.
   h. Repeat the previous step (Step 2g) using the same microcentrifuge tube to collect the eluent. You will end up with ~30 µl of total eluent (15 µl + 15 µl from two elutions).

Purify cDNA with AMPure beads

IMPORTANT: Be careful to avoid bead carryover into the purified cDNA sample.

3. Add 18 µl (0.6X) of DNA beads to 30 µl of cDNA obtained in the previous step (Step 2h, above).
4. Vortex to mix. Spin down briefly to collect all liquid.
5. Incubate the mixture at room temperature for ≥5 min and then on a magnetic stand for ≥2 min until the beads are completely separated from the solution.

6. Carefully remove the supernatant with a pipette. Discard the supernatant.
7. Wash the pellet with 200 µl of 70% (v/v) ethanol. Wait for 10 seconds.
8. To sufficiently wash the cDNA-bound magnetic beads, turn the tube such that the opposite tube side faces the magnet allowing the bead pellet to migrate from one side of the tube to the opposite side. Wait for 10 seconds and turn the tube back to its original position. Repeat this process one more time for a total of 2 cycles. Once the bead pellet has reformed at the bottom of the tube, carefully remove and discard the supernatant containing contaminants. cDNA amplicons of the desired molecular weight will remain bound to the beads during the washing process.
9. Repeat Steps 7 and 8, for a total of 2 washes.
10. Spin the tube briefly in a minicentrifuge to collect the remaining ethanol at the bottom.
11. Place the tube on the magnetic stand and remove all remaining ethanol.
12. Air-dry the washed beads at room temperature for ~5 min or until the ethanol has just evaporated and the pellet is no longer glossy. Do not over-dry the pellet.
13. Add 13 µl of Nuclease-Free Water and pipette up and down to mix. Make sure that all beads are suspended.
14. Centrifuge briefly to collect all liquid. Incubate at room temperature for 5 min and then incubate on a magnetic stand for 1 minute or longer until the solution is completely clear.
15. Carefully transfer 12 µl of clear supernatant with a pipette to a new PCR tube. Save the supernatant which now contains the purified cDNA.

Quantify cDNA with the Qubit fluorometric assay

16. Dilute the purified cDNA product 1:10 and use 2 µl of the dilution for quantitation with a Qubit Fluorometer and the Qubit dsDNA HS Assay Kit. Please refer to the user manual for the Qubit dsDNA HS Assay Kit for sample prep instructions.

Analyze cDNA quality with the Agilent Bioanalyzer

17. Based on the Qubit measurement obtained in the previous step, normalize the purified cDNA product to 1 ng/µl.
18. Use 1 µl of the normalized cDNA product along with the Agilent High Sensitivity DNA Kit to load the Agilent 2100 Bioanalyzer. Please refer to the user manual for the Agilent High Sensitivity DNA Kit for instructions.
19. Use the Bioanalyzer results to determine cDNA quality (see Figure 15, below, for an example of a typical Bioanalyzer profile for a successfully purified cDNA product).

If the Bioanalyzer profile indicates the presence of adapters, repeat the bead purification procedure and analysis of the purified cDNA (this section, Steps 3–19, above).

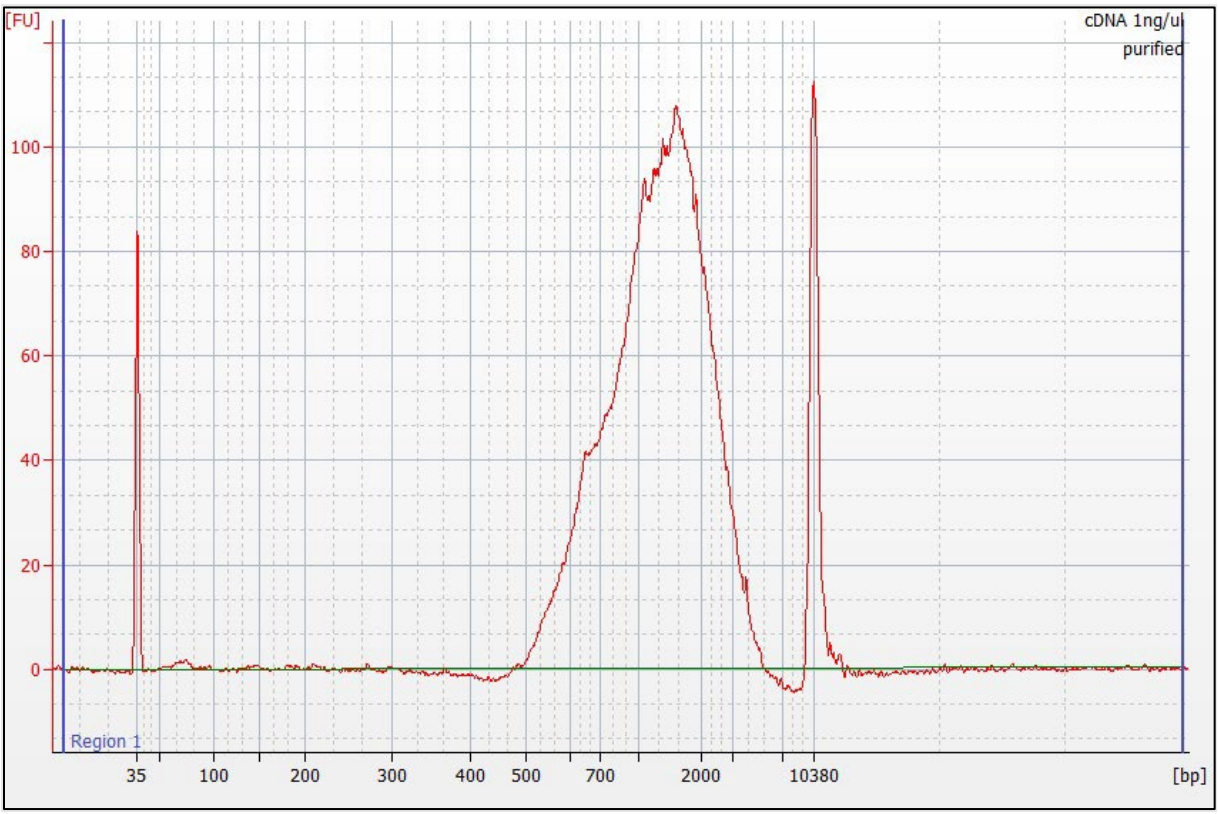

Figure 15. Typical Bioanalyzer trace for purified full-length cDNA that has been normalized to 1 ng/µl.

H. Protocol: Prepare Sequencing Library with the Nextera XT kit

In this protocol, the Nextera XT DNA Library Preparation Kit is used to add Illumina adapters and indexes to the purified cDNA via a tagmentation reaction followed by PCR.

Before you start:

- Thaw ATM (Amplicon Tagment Mix) and TD (Tagment DNA Buffer) reagents from the Nextera XT DNA Library Preparation Kit on ice.
- Thaw purified cDNA (if frozen). Dilute the cDNA to 0.2 ng/µl and use 1 ng for library preparation. Set up multiple identical Nextera XT reactions to increase the final library yield if desired.
- Make sure that the NT (Neutralize Tagment Buffer) reagent is equilibrated to room temperature and does not contain precipitate; if precipitate is observed, vortex to resuspend the particulates.
- This protocol requires the Primer P5 (5 µM) provided with the ICELL8 cx 3′ DE Reagent Kit.

Procedure:

1. Prepare the Nextera XT tagmentation mix in a 0.2-ml nuclease-free tube as indicated below:

10.0 µl	Tagment DNA Buffer (TD)
5.0 µl	Purified full-length cDNA (0.2 ng/µl)
5.0 µl	Amplicon Tagment Mix (ATM)
20.0 µl	Total volume per reaction

2. Mix and centrifuge briefly to collect all liquid. Incubate the reaction in a conventional thermal cycler using the following program:

55°C	5 min
10ºC	forever

3. Immediately upon completion of the tagmentation reaction, remove the tube from the thermal cycler and spin it briefly. Add 5 µl of Neutralize Tagment (NT) buffer and pipette up and down 5 times to mix. Briefly spin to collect contents at the bottom, and incubate the tube at room temperature for 5 min.
4. Thaw one tube of i7 index primer (orange cap) from the Nextera XT Index Kit. Replace the orange cap with a new cap after each use to avoid index contamination. Mark the i7 index tube that you have used. **DO NOT** use the i5 index primer (white cap) supplied with the Nextera XT Index Kit.
5. Thaw Primer P5 (5 µM) provided with the ICELL8 cx 3′ DE Reagent Kit.
6. Prepare the Nextera XT PCR reaction mix in a 0.2-ml nuclease-free tube as indicated below:

15.0 µl	Nextera PCR Mastermix (NPM)
5.0 µl	i7 index primer (orange cap)
5.0 µl	ICELL8 Nextera Primer P5 (5 µM)
25.0 µl	Tagmented cDNA & NT buffer mix (from Step 3)
50.0 µl	Total volume per reaction

7. Vortex to mix. Centrifuge briefly to collect all liquid. Incubate the reaction in a conventional thermal cycler using the following program:

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

I. Protocol: Purify, Size Select, and Evaluate Sequencing Library

In this protocol, tagmented cDNA is purified and size selected via a series of treatments with AMPure beads, followed by quantitation and analysis with an Agilent Bioanalyzer. The final output of this protocol is a sequencing-ready Illumina library.

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 70% (v/v) ethanol from a stock of anhydrous ethanol.
- This protocol requires the Nuclease-Free Water provided with the ICELL8 cx 3′ DE Reagent Kit.

Procedure:

Purify and size select sequencing library

1. To each 50-µl volume of PCR product from the previous protocol (Section V.H, above), add 50 µl (1X) of AMPure XP beads.
2. Vortex to mix. Spin down briefly to collect all liquid.
3. Incubate the mixture at room temperature for ≥5 min and then on a magnetic stand for ≥2 min until the beads are completely separated from the solution.
4. Carefully remove the supernatant with a pipette. Discard the supernatant.
5. Wash the pellet with 200 µl of 70% (v/v) ethanol. Wait for 10 seconds.
6. To sufficiently wash the cDNA-bound magnetic beads, turn the tube such that the opposite tube side faces the magnet allowing the bead pellet to migrate from one side of the tube to the opposite side. Wait for 10 seconds and turn the tube back to its original position. Repeat this process one more time for a total of 2 cycles. Once the bead pellet has reformed at the bottom of the tube, carefully remove and discard the supernatant containing contaminants. Library amplicons of the desired molecular weight will remain bound to the beads during the washing process.
7. Repeat Steps 5 and 6, for a total of 2 washes.
8. Spin the tube briefly in a minicentrifuge to collect the remaining ethanol at the bottom.
9. Place the tube on the magnetic stand and remove all remaining ethanol.
10. Air-dry the washed beads at room temperature for ~5 min or until the ethanol has just evaporated and the pellet is no longer glossy. Do not overdry the pellet.
11. Add 51 µl of Nuclease-Free Water and pipette up and down to mix. Make sure that all beads are suspended.
12. Centrifuge briefly to collect all liquid. Incubate at room temperature for 5 min and then incubate on a magnetic stand for 2 min or longer until the solution is completely clear.
13. Carefully transfer the clear supernatant (~50 µl) with a pipette to a new PCR tube. Save the supernatant (eluent).
14. To the eluent from the previous step (Step 13), add 25 µl (0.5X) of AMPure beads.
15. Vortex to mix. Spin down briefly to collect all liquid.
16. Incubate the mixture at room temperature for ≥5 min and then on a magnetic stand for ≥2 min until the beads are completely separated from the solution.
17. Carefully transfer the supernatant (~75 µl) to a clean PCR tube. **Save the supernatant and discard the beads**.
18. To the supernatant from the previous step (Step 17), add 10 µl (0.2X) of AMPure beads.
19. Vortex to mix. Spin down briefly to collect all liquid.
20. Incubate the mixture at room temperature for ≥5 min and then on a magnetic stand for ≥2 min until the beads are completely separated from the solution.
21. Carefully remove the supernatant with a pipette. Discard the supernatant.
22. Repeat Steps 5–9 from this protocol (above) to wash the pellet 2 times and remove the ethanol.
23. Air-dry the washed beads at room temperature for ~5 min or until the ethanol has just evaporated and the pellet is no longer glossy. Do not overdry the pellet.

24. Add 11 µl of Nuclease-Free Water and pipette up and down to mix. Make sure that all beads are suspended.
25. Centrifuge briefly to collect all liquid. Incubate at room temperature for 5 min and then incubate on a magnetic stand for 1 minute or longer until the solution is completely clear.
26. Carefully transfer the supernatant containing the purified, size selected sequencing library to a new tube. You should end up with ~10 µl of sequencing-ready library.

Evaluate sequencing library

27. Aliquot 1 µl of the final library obtained in the previous step (Step 26) for quantitation with a Qubit Fluorometer and the Qubit dsDNA HS Assay Kit. Please refer to the user manual for the Qubit dsDNA HS Assay Kit for sample prep instructions.
28. Based on the Qubit measurement obtained in the previous step, normalize the sequencing library to 1 ng/µl, and use 1 µl of it along with the Agilent High Sensitivity DNA Kit to load the Agilent 2100 Bioanalyzer. Please refer to the user manual for the Agilent High Sensitivity DNA Kit for instructions.
29. Use the Bioanalyzer results to determine library quality (see Figure 16, below, for an example of a typical Bioanalyzer profile for an NGS library that has been successfully purified and size selected).
30. Quantify the diluted library with the KAPA Library Quantification Kit (for Illumina).
31. Store the sequencing library at –20°C until ready for sequencing.

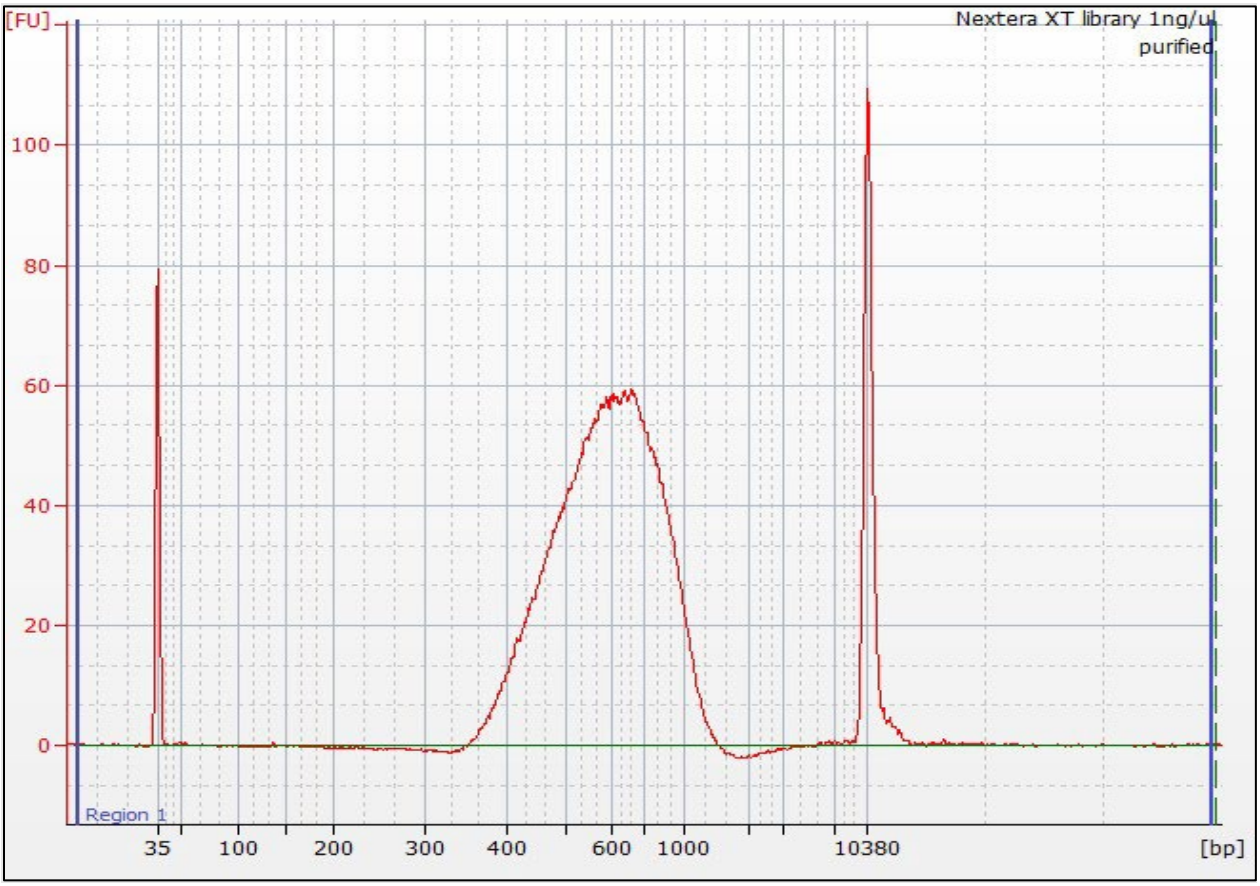

Figure 16. Typical Bioanalyzer trace for purified Nextera XT NGS library that has been normalized to 1 ng/µl.

Appendix A: In-line Indexes

The `3'DE.xml` file provided with ICELL8 cx CellSelect v2.5 Software contains the barcode sequence preprinted in each nanowell location on a chip. This allows every cell sequenced to be uniquely identified and its location on the chip known.

Appendix B: Data Analysis

The ICELL8 system is optimized for data analysis using the Cogent™ NGS Analysis Pipeline (CogentAP) and Cogent NGS Discovery Software (CogentDS).

- **CogentAP** interprets both ICELL8 and sequencing data, and outputs an HTML report. This report summarizes the complex read data into clear, simple charts.
- **CogentDS** imports sequencing data and provides secondary analysis such as Uniform Manifold Approximation and Projection (UMAP) and t-Distributed Stochastic Neighbor Embedding (t-SNE) plots for user-friendly visualization.

More information on these tools can be found on our website at takarabio.com/products/icell8-software.

Data analysis can also be performed on a variety of other platforms. A detailed but non-exhaustive list of packages can be found at https://omictools.com/rep-seq-category.

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