Takara Bio USA, Inc.

ICELL8® Chip and Reagent 3′ DE Kit User Manual

Cat. Nos. 640164, 640165, 640166
(071719)

Takara Bio USA, Inc.
1290 Terra Bella Avenue, Mountain View, CA 94043, 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

High-throughput differential expression analysis of single cells

The **ICELL8 Chip and Reagent 3′ DE Kit** (Cat. Nos. 640164, 640165, 640166) enables Illumina® sequencing and differential gene expression analysis—via 3′ end counting of transcripts—of single cells isolated on the **ICELL8 Single-Cell System** (Cat. No. 640000).

The kit workflow (Figure 1, below) begins with staining and dilution of cell samples and the preparation of positive and negative controls, followed by the dispensing of the cells and controls into the uniquely barcoded 5,184 nanowells of the ICELL8 3′ DE Chip using the ICELL8 MultiSample NanoDispenser (MSND). As many as eight different samples can be analyzed in a single run, and the dispensing process is completed in approximately 15 minutes. During this time, cells are maintained in the humidity- and temperature-controlled environment provided by the ICELL8 MSND.

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

Following freezing of the ICELL8 chip, the chip is returned to the ICELL8 MSND and RT-PCR reagents are distributed to nanowells selected by the software. The ICELL8 chip is then transferred to the ICELL8 Chip Cycler, which is programmed to perform first-strand synthesis and amplification of cDNA in a single run. During this process (Figure 2, below), the oligo(dT) primer preprinted into each nanowell of the ICELL8 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 SPRI 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 the ICELL8 Single-Cell System along with SMART technology, the ICELL8 Chip and Reagent 3′ DE Kit provides an efficient, cost-effective solution for high-throughput differential expression analysis of single cells.

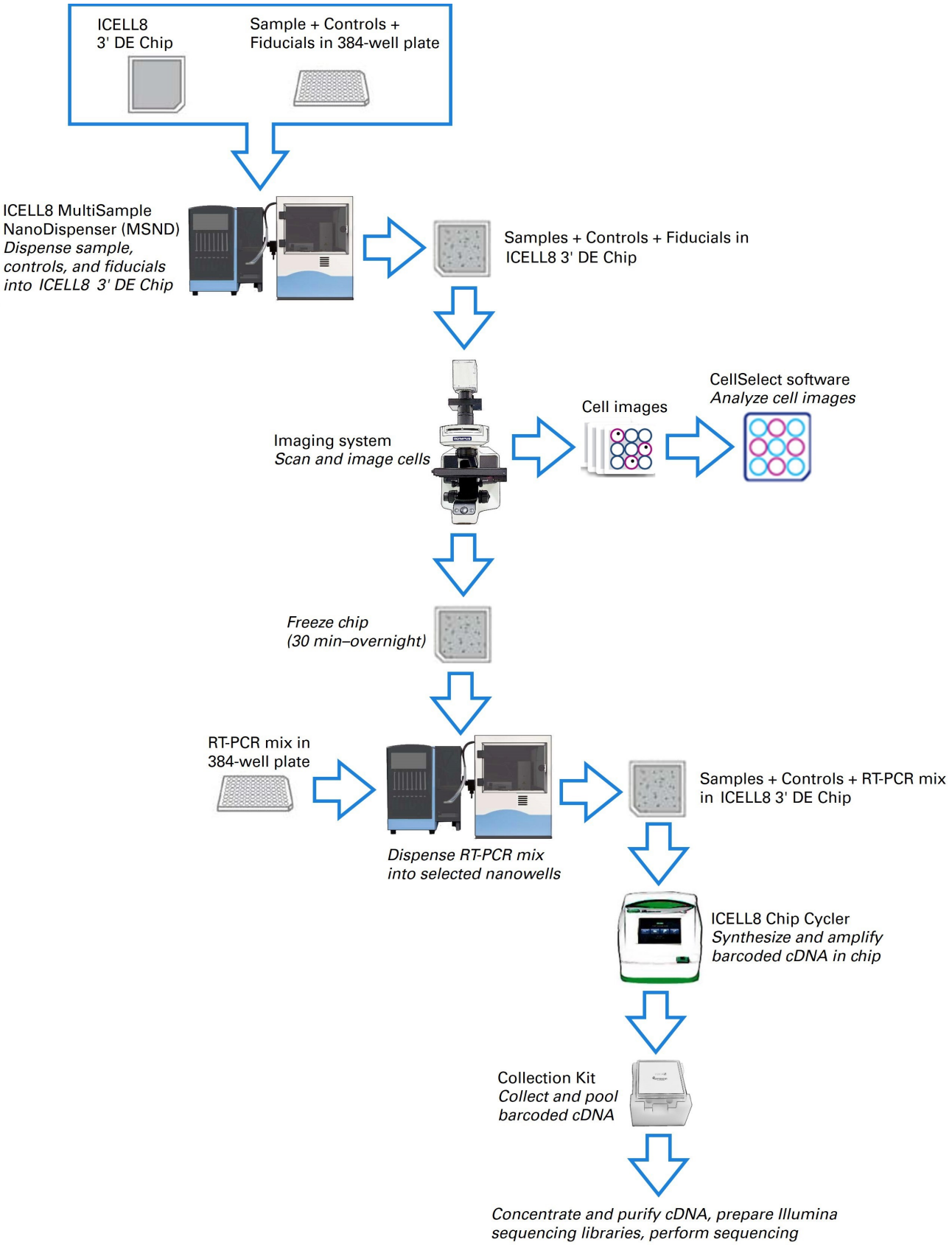


Figure 1. Workflow diagram for the ICELL8 Chip and Reagent 3′ DE Kit.

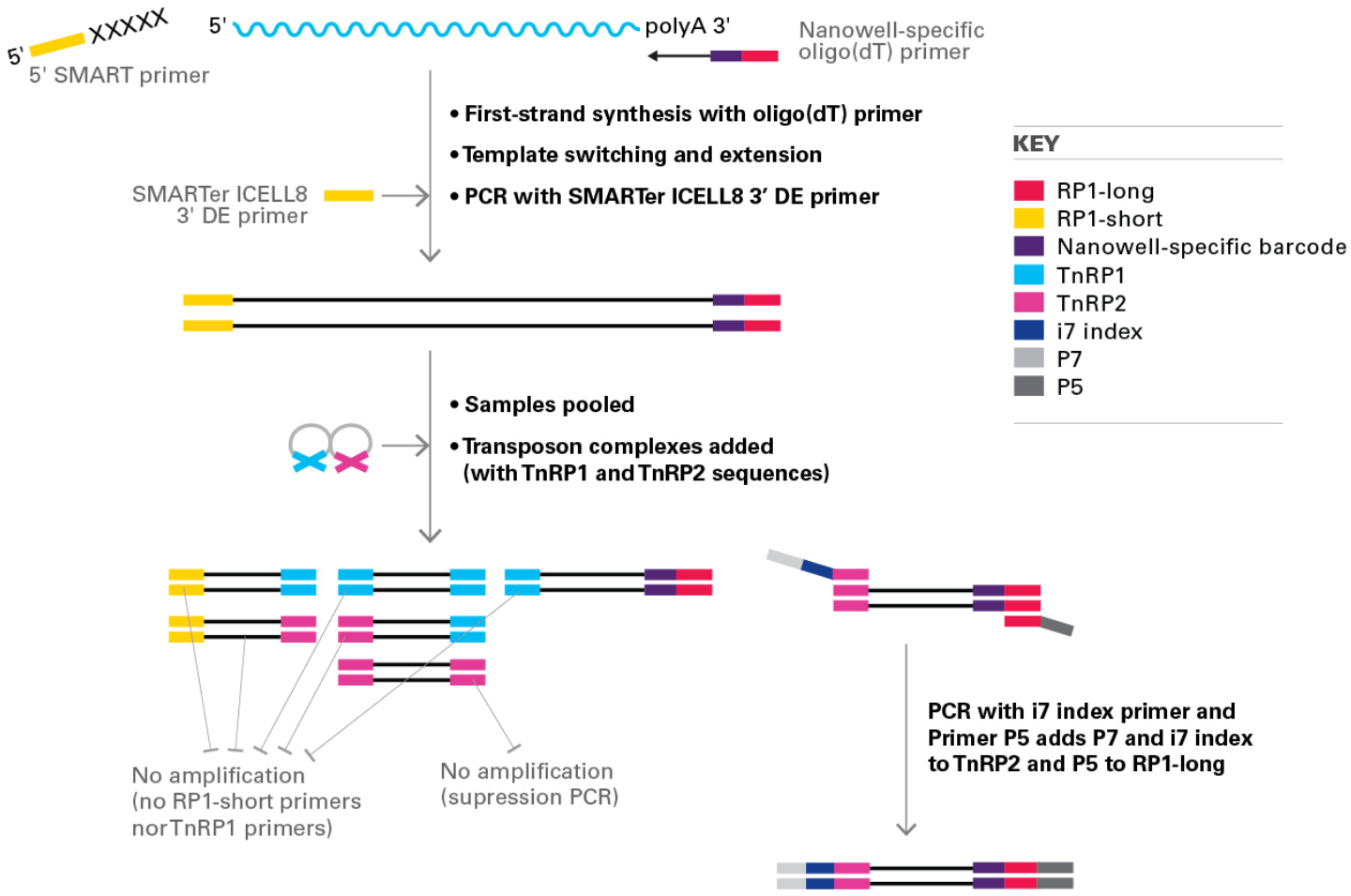


Figure 2. cDNA synthesis and sequencing library construction with the ICELL8 Chip and Reagent 3′ DE Kit. First-strand synthesis is primed by oligo(dT) primers, which include nanowell-specific barcode sequences, that are preprinted into each nanowell of the ICELL8 3′ DE Chip. The oligo(dT) primers also include random nucleotide sequences of fixed length (not shown in legend) 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 Chip and Reagent 3′ DE 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.

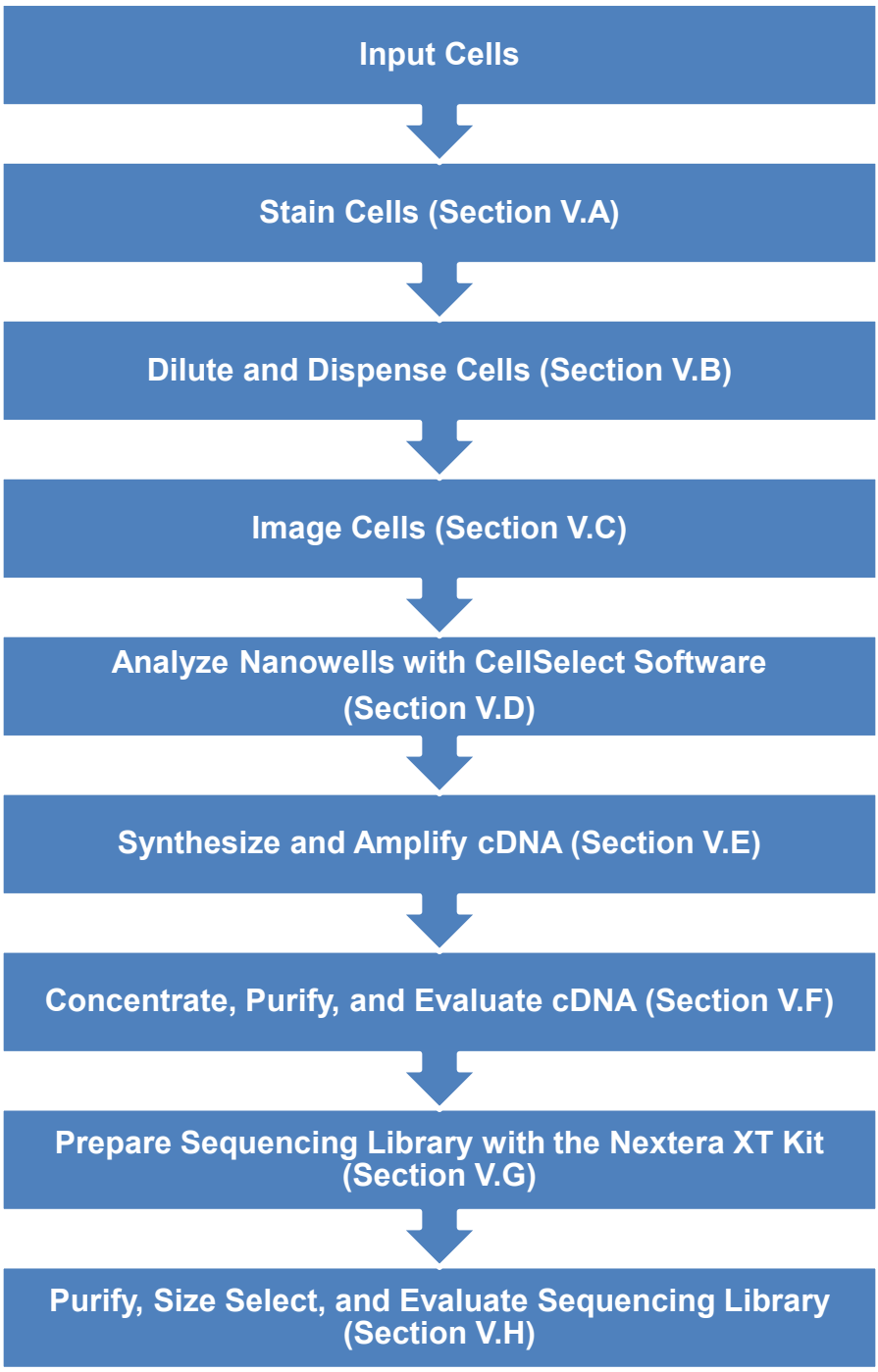


Figure 3. Protocols included in this user manual.

II. List of Components

The ICELL8 Chip and Reagent 3′ DE Kit consists of the ICELL8 3′ DE Reagent Kit, the ICELL8 3′ DE Chip, the ICELL8 Collection Kit, and the ICELL8 Loading Kit, which are provided in equal proportions for each kit size:

ICELL8 Chip and Reagent 3′ DE Kit

ICELL8 Chip and Reagent 3′ DE Kit (see below for storage conditions)			
	Quantity per kit		
Component	**640164**	**640165**	**640166**
ICELL8 3′ DE Reagent Kit (Cat. No. 640167)	1	3	5
ICELL8 3′ DE Chip (Cat. No. 640143)	1	3	5
ICELL8 Collection Kit (Cat. No. 640048)	1	3	5
ICELL8 Loading Kit (Cat. No. 640109)	1	3	5

Component Breakdown

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)		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 minutes.

ICELL8 3′ DE Chip (Cat. No. 640143, store at room temperature)

ICELL8 Loading Kit (Cat. No. 640109, store at room temperature)	
Component	**Quantity per kit**
Blotting Paper	3
Optical Imaging Film	1
Sealing Film	1

ICELL8 Collection Kit (Cat. No. 640048, store at room temperature)	
Component	**Quantity per kit**
Collection Fixture	1
Collection Tube	2
Collection Film	1

ICELL8 Single-Cell System

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

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

*These components are also included as part of the ICELL8 and Reagent 3′ DE Kit.

III. Additional Materials Required

The following reagents and materials are required but not supplied with the ICELL8 Chip and Reagent 3′ DE Kit or the ICELL8 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
- Two pairs of tweezers for handling chips during imaging

For MSND general operation:

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

For staining and dispensing cells:

- 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)
- TrypLE Express (Life Technologies, Cat. No. 12604-021)

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)

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
- Agilent High Sensitivity DNA Kit (110 samples; Agilent, Cat. No. 5067-4626)

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
- Bioanalyzer instrument (or similar; Agilent)

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 10^6 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 on the ICELL8 system.
- 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 minutes or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
- Use deionized (e.g., Milli-Q) water for the MSND pressure bottle and humidifier; clean these reservoirs routinely. Use 0.2% bleach solution for the MSND wash bottle.
- 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 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 MSND Application Notes

Refer to the ICELL8 MSND User Manual for full details. Included below are general reminders.

- Before operating the instrument, check the water level in the pressure reservoir. Refill the pressure reservoir if the weighing scale below the pressure reservoir displays a value ≤4 kg (≤8 lbs).
- Add deionized water (e.g., Milli-Q water) to the reservoir as indicated in the following steps, and in Figure 4 (below):
    a. Close the helium in-line stopcock and open the vent stopcock.
    b. Fill the pressure reservoir with deionized water to the fill line (top of the protective cover rim). The weighing scale below the pressure reservoir should display a value of ~4 kg.
    c. Reattach the cap and let sit for ~20 minutes to degas. Make sure that the cap is properly threaded and securely fastened to prevent the helium from leaking out.
    d. Close the system by opening the helium in-line stopcock and closing the vent stopcock.

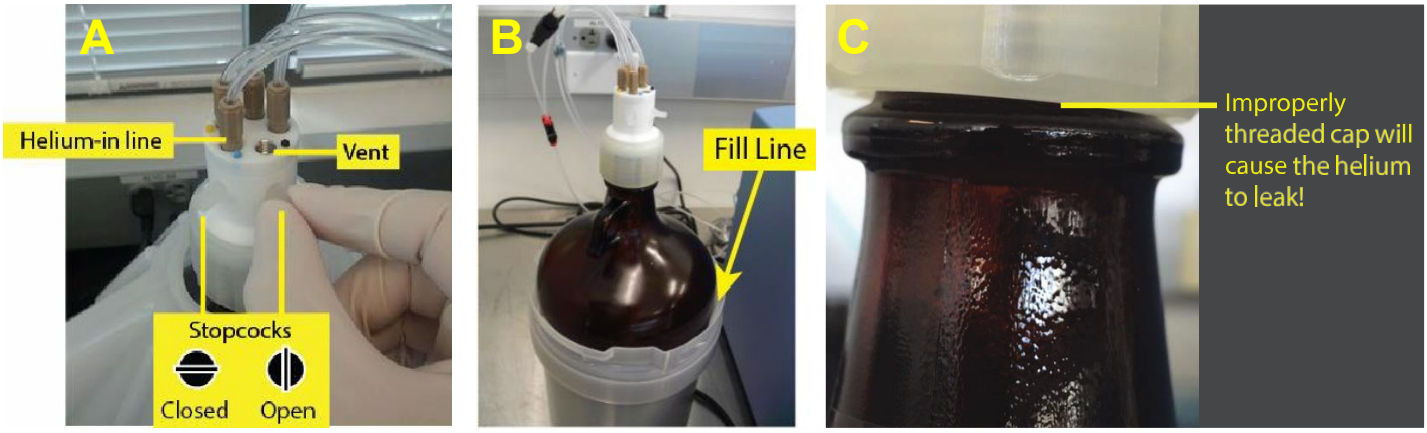


Figure 4. Filling the pressure reservoir. **Panel A.** Close the helium in-line stopcock and open the vent stopcock. **Panel B.** Add Milli-Q water to the fill line of the pressure reservoir. **Panel C.** Make sure that the cap on the pressure reservoir is properly attached.

- Check the water level in the humidifier and fill if needed. Clean the humidifier regularly.
- Check the pressure on the helium tank. The regulator should be set to a supply input of >500 psi (3.5 MPa) and an output of 30–40 psi. If the tank pressure drops to <500 psi, replace it with a full tank.
- Check the bleach level in the wash bottle. If it is <25% full or ≥3–4 days old, replace it with 500 ml of freshly made 0.2% sodium hypochlorite solution prepared from reagent-grade sodium hypochlorite in deionized, filtered water (e.g., Milli-Q, ELGA system, or equivalent with 0.2-µm filtration).
- Check the waste container. If full, dispose the liquid waste appropriately and replace with an empty container.

- All dispensing steps in the MSND Stage Module should be performed with the 384-well source plate oriented with the A1 well positioned at the top-right corner of the 384-well Plate Nest, and the chip oriented with the chamfered (notched) corner positioned towards the lower-right corner of the Dispensing Platform (Figures 5–7, below).

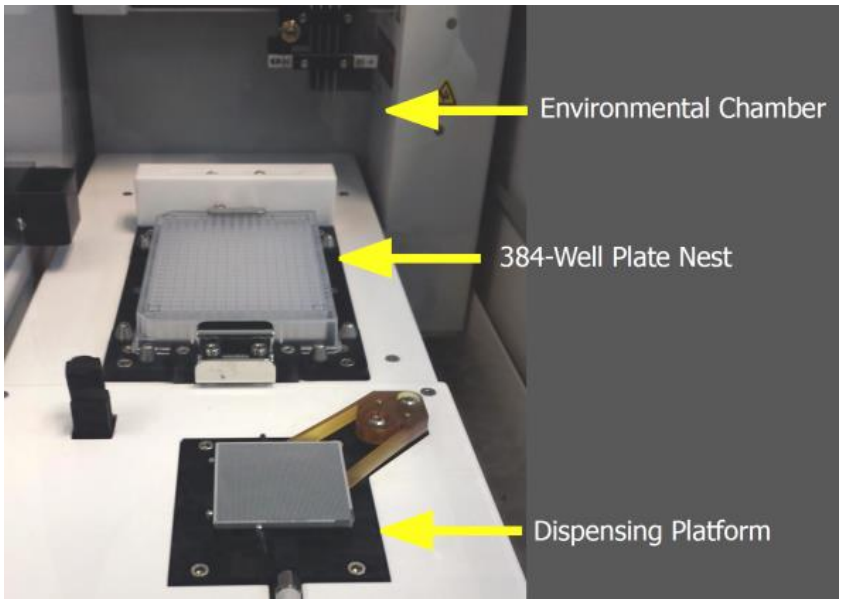


Figure 5. ICELL8 MSND Stage Module.

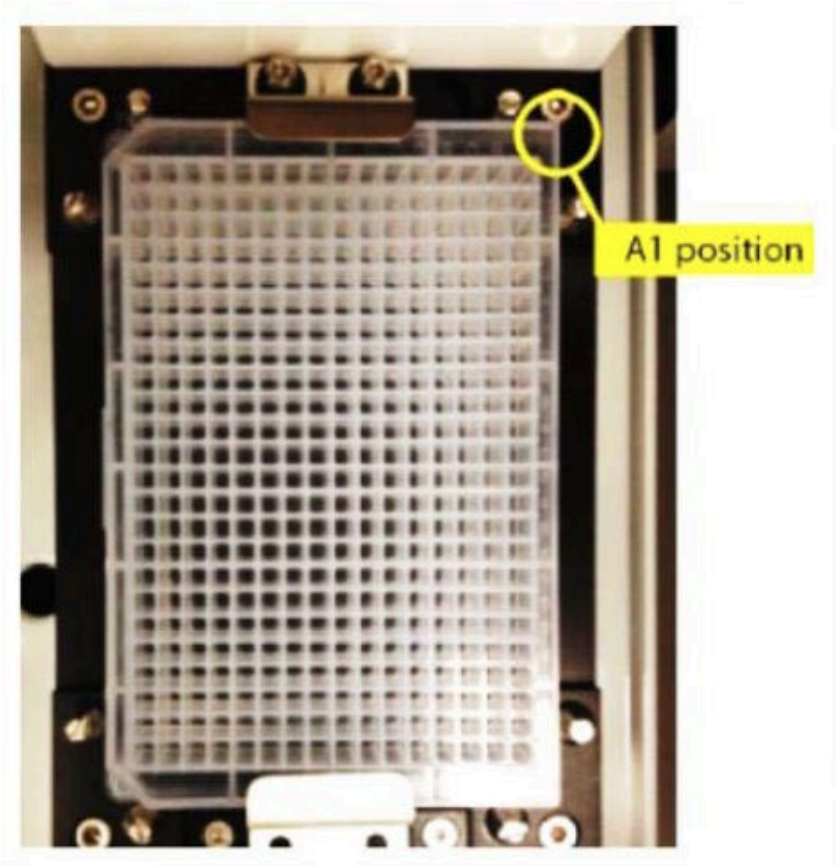


Figure 6. 384-well source plate in the Plate Nest.

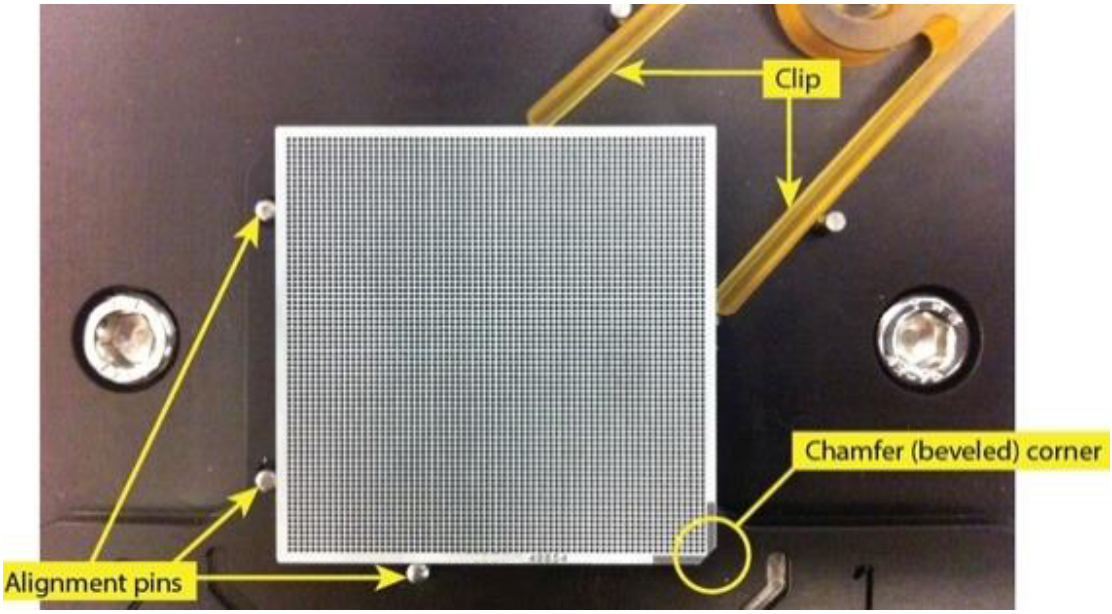


Figure 7. Chip seated in the Dispensing Platform. Stretch the arms of the clip apart and angle the chip onto the Dispensing Platform with the chamfered (notched) corner of the chip oriented towards the lower-right corner of the Dispensing Platform. The edges of the chip should be pressed against the three alignment pins.

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

E. ICELL8 Imaging System and CellSelect Software

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

F. ICELL8 3′ DE Chip

- Each ICELL8 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 3′ DE Chip; the printing lot number is stamped onto the chip (Figure 9, below). A total of 5,184 unique nanowell barcodes are available; each nanowell barcode was printed once into the chip.

Figure 8. ICELL8 3′ DE Chip packaging.

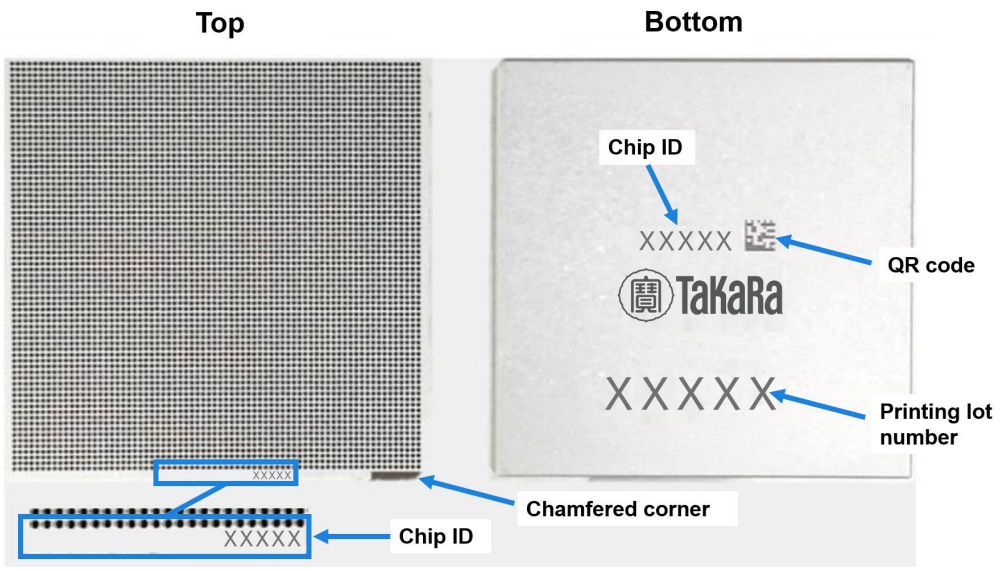


Figure 9. ICELL8 3′ DE Chip features. **Top.** In addition to the 5,184 nanowells, note the chamfered (notched) corner at the bottom right. "TaKaRa" and the chip ID (unique to each chip) are engraved on the chip border, near the chamfered corner. **Bottom.** The chip ID is also engraved on the other side of the chip with a corresponding QR code that can be scanned by a barcode reader, allowing for the chip ID to be easily entered into the software. The lot number corresponding to the printing of the barcode- and UMI-containing oligos is also engraved on this side of the chip.

V. Procedure

A. Protocol: Stain Cells

In this protocol, sample cells are stained with Hoechst 33342 and propidium iodide dyes that enable imaging, analysis, and selection of candidates suitable for downstream analysis following dispensation into the ICELL8 3′ DE Chip. Start from Section V.A.1 if working with cells in suspension. Start from Section V.A.2 and then proceed to Section V.A.1 if working with adherent cells.

Prerequisite:

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

Before you start:

- Initialize the ICELL8 MSND; perform daily warm-up via the *Startup* window, then pull up the *Advanced* window and execute the **Wash Prime** function followed by **Tip Clean**. Refer to the ICELL8 MSND User Manual for more details.
- Prefreeze Chip Holder(s) at –80°C.
- Set the chip centrifuge to 22°C.
- Start the ICELL8 Imaging System by turning on system components in the following order: the fluorescence light source, the camera, the controllers for the microscope imaging filters and stage, and then the computer. Please note that the fluorescence light source requires a warmup period of ~5 minutes. Refer to the ICELL8 Imaging System User Manual for detailed setup instructions before first use.
- Thaw Second Diluent (100X), ICELL8 Fiducial Mix (1X), and Nuclease-Free Water (all provided with the ICELL8 Chip and Reagent 3′ DE Kit) on ice. Once thawed, keep on ice for the remainder of the protocol.

- Prewarm 1X PBS (Ca^{2+} and Mg^{2+} free, pH 7.4), TrypLE Express*, and cell culture medium* at 37°C. *Required only if performing dissociation of adherent cells.
- Dilute Control K-562 RNA (1 µg/µl) 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:
    - Dilute Control K-562 RNA 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.
    - Dilute Control K-562 RNA 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.

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 1:1 mixture of Hoechst 33342 and propidium iodide. Combine 80 µl of each dye per ml of cells to be stained. An example using 2 ml of cells is described below (e.g., prepare 320 µl of premixed dye solution).

   NOTE: Protect this mixture from light until ready for use.
2. Transfer ~2.1 ml of suspension cells to a fresh 5-ml tube.
3. Determine cell concentration using a Moxi automated cell counter and Moxi Z cassette or your preferred method (e.g., use Moxi Z cassette MF-M for K-562 cells; refer to a Moxi Z user manual for guidance in selecting an appropriate cassette size for the cells being analyzed). Use a pipette to transfer 75 µl of cell suspension to one end of the loading platform. Take a reading and record the cell concentration.
4. Add 320 µl of the premixed Hoechst 33342 and propidium iodide dye mix to the cells. Mix gently by inverting the tube 5 times. Do not vortex or overagitate the cells.
5. Incubate cells at 37°C for 20 minutes.
6. Add an equal volume of 1X PBS (prewarmed to 37°C) to stained cells. For the example described here, 2 ml of prewarmed 1X PBS is added to the 5-ml tube containing the stained cell suspension.
7. Mix the stained cell suspension and PBS by gently inverting the tube 5 times. Do not vortex or overagitate the cells.
8. Pellet the cells by centrifugation at 100*g* for 3 minutes at room temperature. Avoid over-centrifugation or pelleting into a firm pellet or clump.

NOTE: Optimal centrifugation speed and time may vary depending on the cell type being analyzed. Examples:
- K-562 or 3T3 cells: 100*g* for 3 minutes.
- PBMCs or similarly sized cells: 500*g* for 3 minutes.

9. Gently remove tube from the centrifuge without disturbing the cell pellet.
10. Carefully decant the supernatant without disturbing the cell pellet.
11. Wipe remaining fluid from the tube top using a fresh Kimwipe such that the last remaining drop on the tilted tube top is gently removed.
12. Gently add 1 ml of 1X PBS (prewarmed to 37°C) to the tube side wall.
13. Use a wide-bore 1-ml pipette tip to gently mix the cell suspension by slowly pipetting up and down ~5 times. Do not vortex or overagitate the cells.
14. Count the cells using a Moxi automated cell counter and an appropriate cassette (or your preferred method). Take two readings for each stained cell sample and average the results. The recommended concentration range of stained cell suspension is 1.2 x 10^5–5.0 x 10^5 cells/ml. If you end up with a concentration lower than 1.2 x 10^5 cells/ml, repellet the stained cell suspension and resuspend in a lower volume of 1X PBS to achieve a concentration in the recommended range.
15. Proceed to the next protocol (Section V.B).

2. 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 TrypLE Express prewarmed to 37°C to the flask to dissociate the cells.
5. The efficiency of cell dissociation from the flask surface may vary with cell type. Monitor the process visually using a microscope.
6. Once cell dissociation has occurred, neutralize the trypsinization reaction by gently adding 7 ml of complete media (containing 10% serum), prewarmed to 37°C. Do not vortex or overagitate cells.
7. Follow the procedure for Preparation of Stained Suspension Cells (Section V.A.1, above) starting from Step 1.

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 3′ DE Chip using the ICELL8 MSND. Refer to the ICELL8 MSND User Manual for detailed information about instrument setup and operation.

Before you start

- Confirm that initialization and setup procedures for the ICELL8 MSND and ICELL8 Imaging System have been completed (see "Before you start" in Section V.A, above).
- Prefreeze the empty Chip Holder (Figure 10, below) at –80°C.
- Aliquot 300–500 µl of 1X PBS (Ca^{2+} and Mg^{2+} free, pH 7.4) on ice for positive and negative control.
- This protocol requires an MSND 384-Well Source Plate (with seal) and the following components from the ICELL8 Chip and Reagent 3′ DE Kit: Control K-562 RNA (diluted to 10 ng/µl in the previous protocol), Second Diluent (100X), ICELL8 Fiducial Mix, RNase Inhibitor (40 U/µl), Blotting Paper, and Optical Imaging 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 cell/50 nl in a total volume of 1 ml.

Figure 10. Chip Holder. The engraved chip number can be seen through the center opening.

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 (below). Mix the combined reagents by vortexing, then spin the tubes briefly to collect contents at the bottom.

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

3. To the 1.5-ml microcentrifuge tube from the previous step (containing 1X PBS, RNase Inhibitor, and Second Diluent), add the calculated volume of stained cell suspension that will yield a final concentration of 1 cell/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 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 (below).

NOTES:
- Keep the positive control sample on ice.
- Mix well, but do not vortex the positive and negative control samples.
- The final concentration of Control K-562 RNA should be 10 pg/50 nl (equivalent to the total RNA content from 1 cell).

Table I. 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 cell/50 nl*	Dilute to 1 cell/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 cell/50 nl (i.e., 20,000 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 11, below).

Prepare cell dispense source plate

Refer to the ICELL8 MSND 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 an MSND 384-Well Source Plate as indicated in Figure 11 (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 Control, Negative Control, and ICELL8 Fiducial Mix (1X) to the 384-well source plate as indicated in the following steps and in Figure 11 (below):
    - Add 25 µl of prepared Positive Control to well P24.
    - Add 25 µl of prepared Negative Control to well A24.
    - Add 25 µl of ICELL8 Fiducial Mix (1X) to well P1.

NOTES:
- Be careful not to splash liquid into neighboring wells.
- 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.

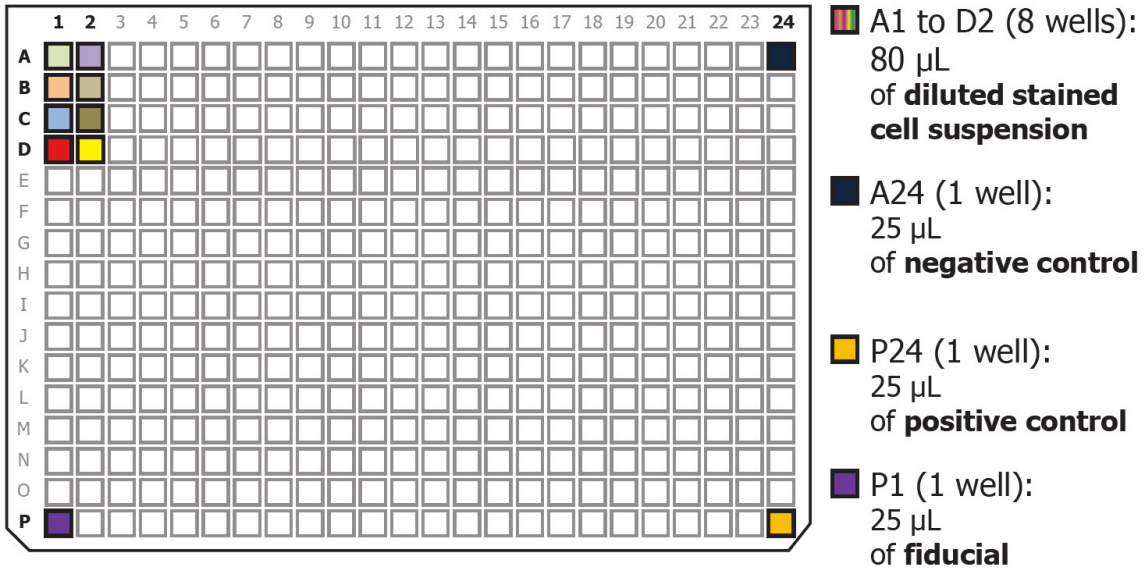


Figure 11. Setting up the 384-well source plate for dispensing cell samples and controls.

8. Seal the 384-well source plate with the provided Plate Seal. Do not centrifuge the plate.
9. Open the packet containing the sealed ICELL8 3′ DE Chip and remove the seal from the preprinted chip.
10. In the ICELL8 MSND software GUI, click the *Single Cell* tab and then scan the barcode on the back of the chip or enter the chip ID into the text field.

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

11. Place the chip on the chuck of the Dispensing Platform in the MSND with the chamfered (notched) corner of the chip at the lower right corner of the Dispensing Platform, aligned with the chamfered corner of the chuck (see Figure 7, above).

    IMPORTANT: The edges of the chip should be pressed against the three alignment pins; **make sure that the chip is flat on the chuck from all sides and is in the correct orientation** (see Figure 7, above).

12. Place the 384-well source plate in the MSND with the A1 corner positioned at the top-right corner of the Plate Nest (see Figure 6, above). The beveled corners of the 384-well source plate should be on the left side.
13. Gently remove the seal from the 384-well source plate and lock the plate into position then close the MSND environmental chamber doors.
14. In the ICELL8 MSND Software GUI, click [Dispense cells] button (Figure 12, below) then click **OK** after carefully going through all questions prompted. The ICELL8 MSND will start dispensing 50 nl of cell suspension, positive/negative control, or fiducial to the appropriate nanowells.

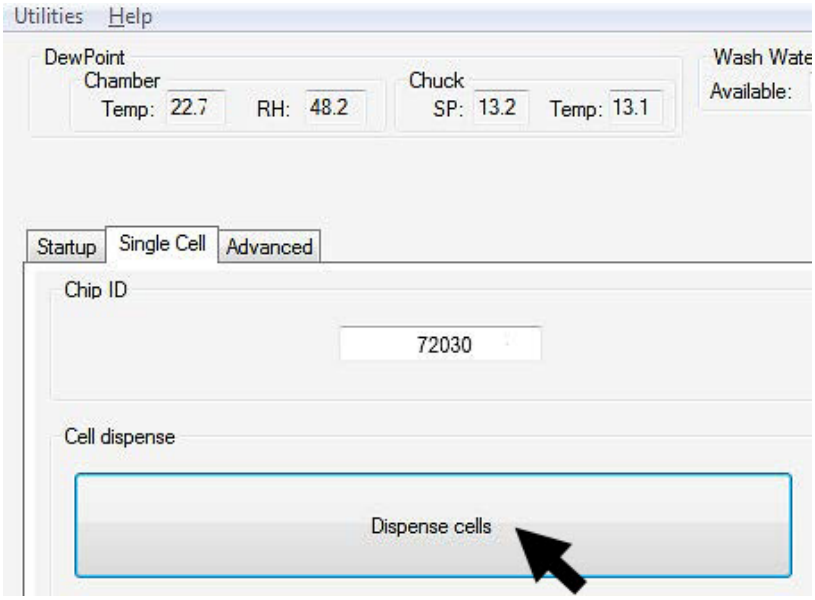


Figure 12. Using the MSND software to dispense cells into the ICELL8 3′ DE Chip.

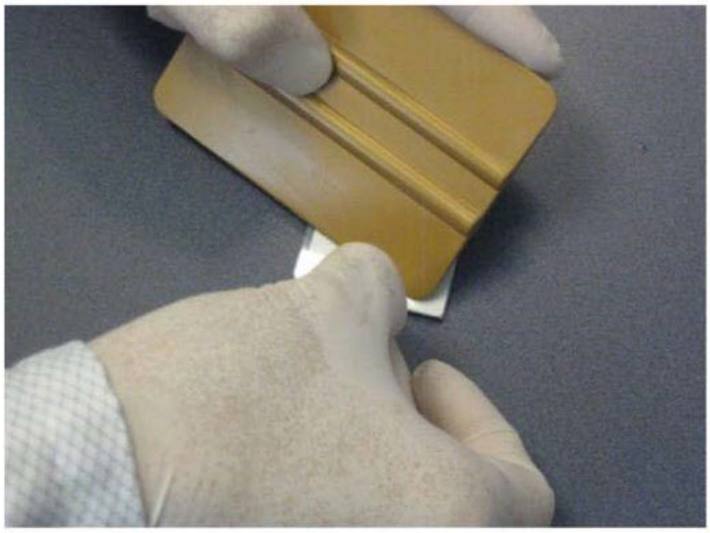
15. After dispensation is completed, remove the chip from the Dispensing Platform and gently blot for two seconds with a fresh piece Blotting Paper (provided in the ICELL8 Loading Kit) and Blotter.

Figure 13. Blot the chip with blotting paper and blotter.

16. Remove the liner from **only one side** of an imaging film and apply the exposed sticky side of the Imaging Film to the blotted chip (Figures 14 and 15, below).

Figure 14. Sealing film and imaging film. The sealing film (left) is provided with a white backing. The imaging film (right) is double sided and is provided between two layers of clear backing.

17. Use a Film Applicator to make sure chip is securely sealed to avoid well-to-well contamination (left panel of Figure 15). **Note the orientations of the chamfered corner and the imaging film tab** (right panel of Figure 15).



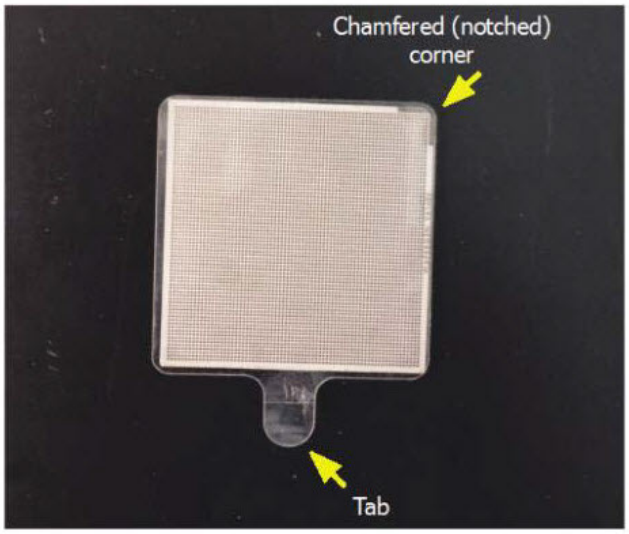


Figure 15. Sealing the blotted chip. Seal the blotted chip using a Film Applicator (left). The tab of the imaging film should be oriented downward relative to the chamfered corner of the chip (right).

18. Place the sealed chip on a centrifuge spin plate and centrifuge the sealed chip at 300*g* for 5 minutes at 4°C or 22°C, as required for your cells, with full acceleration and full brake (Figure 16, 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.

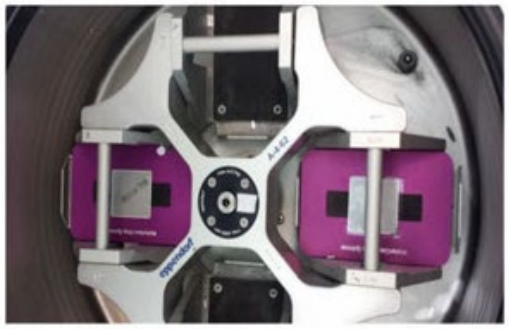


Figure 16. Centrifuge Chip Spinner.

C. Protocol: Image Cells

In this protocol, images of all 5,184 nanowells of the ICELL8 3′ DE Chip are acquired. Refer to the ICELL8 Imaging System User Manual for detailed information about instrument setup and operation.

ICELL8 Imaging System notes

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

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

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

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

Procedure

Start software and input chip information

1. Start the Micro-Manager software. Ensure that the manual shutter is in the Open position.
2. Click **Live** in the *Main* window to open a window showing a live image from the camera.
3. Click **Multi-D Acq.** to open the *Multi-Dimensional Acquisition* window.
4. Make sure that the **Multiple positions (XY)** box is checked.
5. Click **Load Chip type** and load the configuration file `72.72.pos` (if it is not preloaded).
6. Position the cursor so that it resides in the "Chip Number" field (indicated by the arrow in Figure 17A, below). Input the chip ID or use the barcode scanner to scan the two-dimensional barcode on the back of the chip WITHOUT completely inverting the chip (Figure 17, Panel B, below).

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

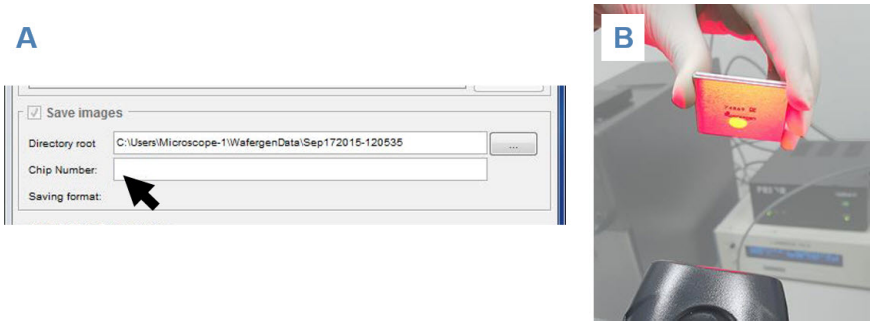


Figure 17. Inputting the chip ID. Place the cursor in the Chip Number field (**Panel A**), and either input the Chip ID manually, or scan the QR code on the bottom of the chip (**Panel B**).

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

Position chip on microscope

8. Place the chip on the microscope Holding Platform with the chamfered (notched) corner facing the upper right corner (Figure 18, below). **Ensure that the chip is sitting flat on the chuck from all sides and is in the correct orientation.**

   NOTE: The chip should be centrifuged prior to imaging.

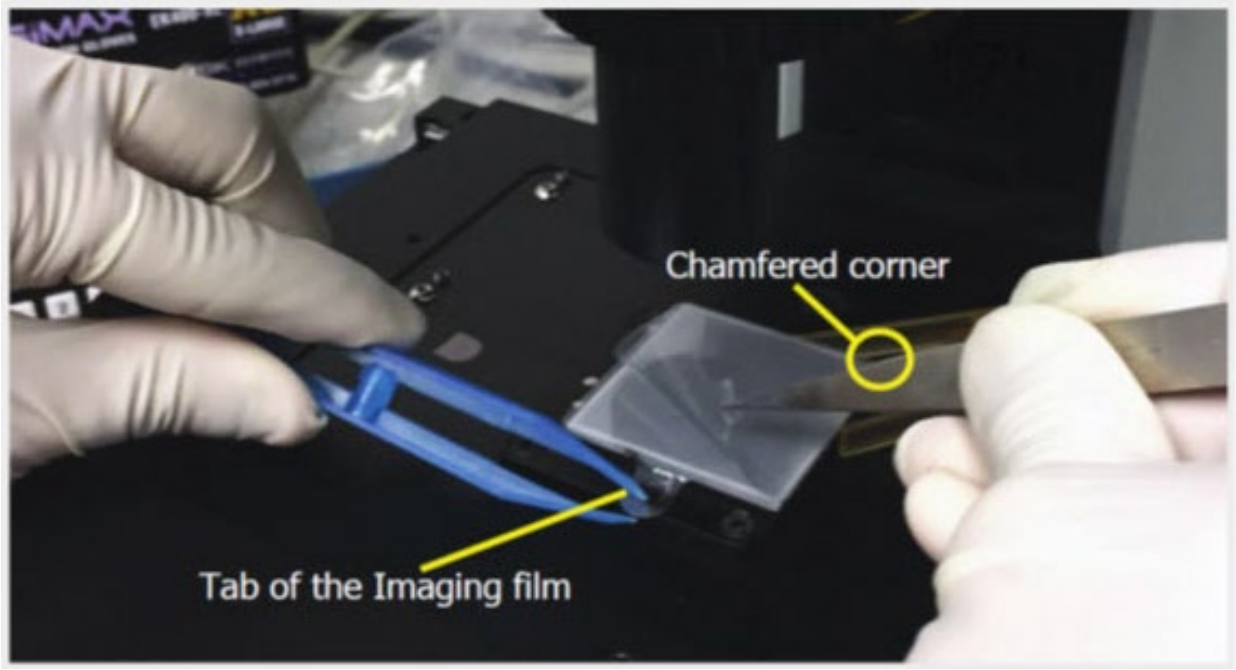


Figure 18. Seating the chip on the microscope holding platform.

9. Ensure the chip is perfectly flat and seated square relative to the pins before removing the second release liner. Peel off the top layer release liner of the imaging film using tweezers (Figure 18, above). **Save the release liner**. The chip will remain sealed by the double-sided intermediate film layer.
10. Move the objective over the chip. Ensure that the objective is vertical and centered (you should hear/feel a slight click).
11. Select the row "Pos0" in the *Stage Position List* window (Figure 19), and then click [Go to] button.

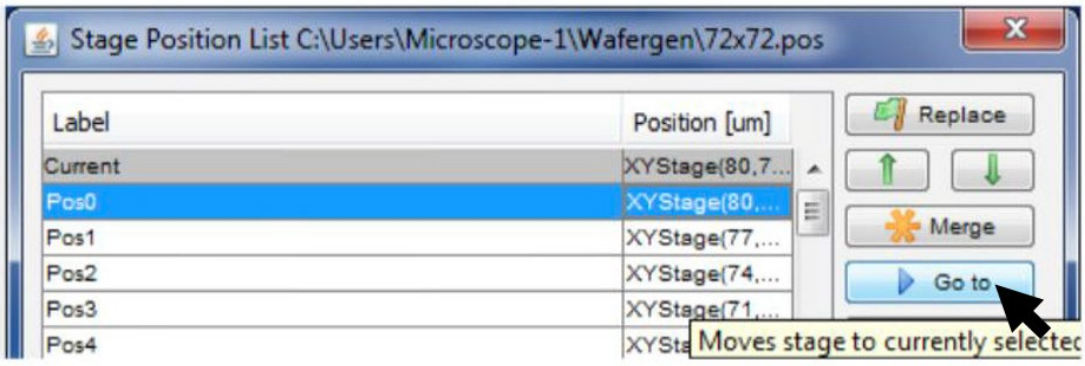


Figure 19. Moving the motorized stage to Position 0.

12. In the *Main* window, under "Configuration settings", select **Texas Red** as the illumination channel for examination of fiducial-containing nanowells (Figure 20).

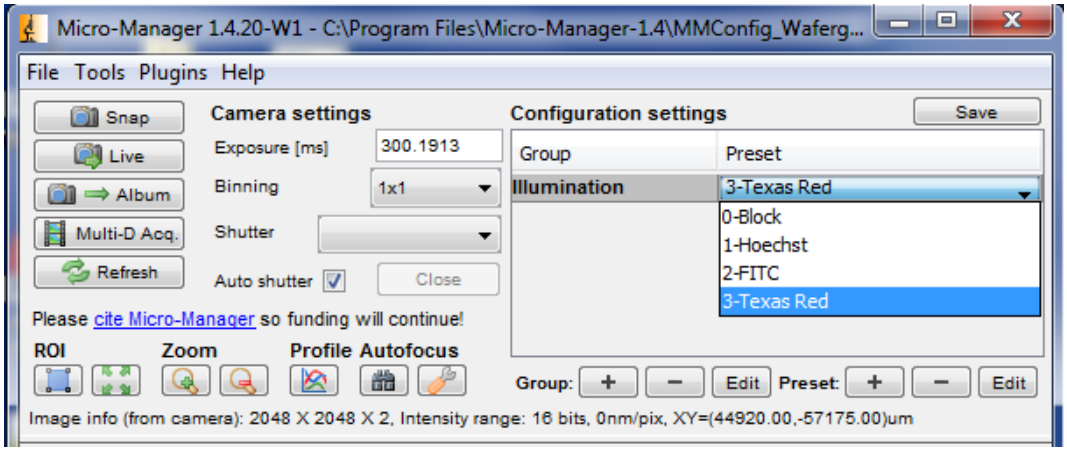


Figure 20. Setting the illumination channel to Texas Red.

13. Confirm that five fiducial-containing wells are present, forming an "X" shape at Pos0 (Figure 21). There are four fields of view across an entire chip that include fiducial-containing nanowells (Pos0, Pos11, Pos132, and Pos143). Each field of view includes 36 nanowells (a 6 x 6 grid).

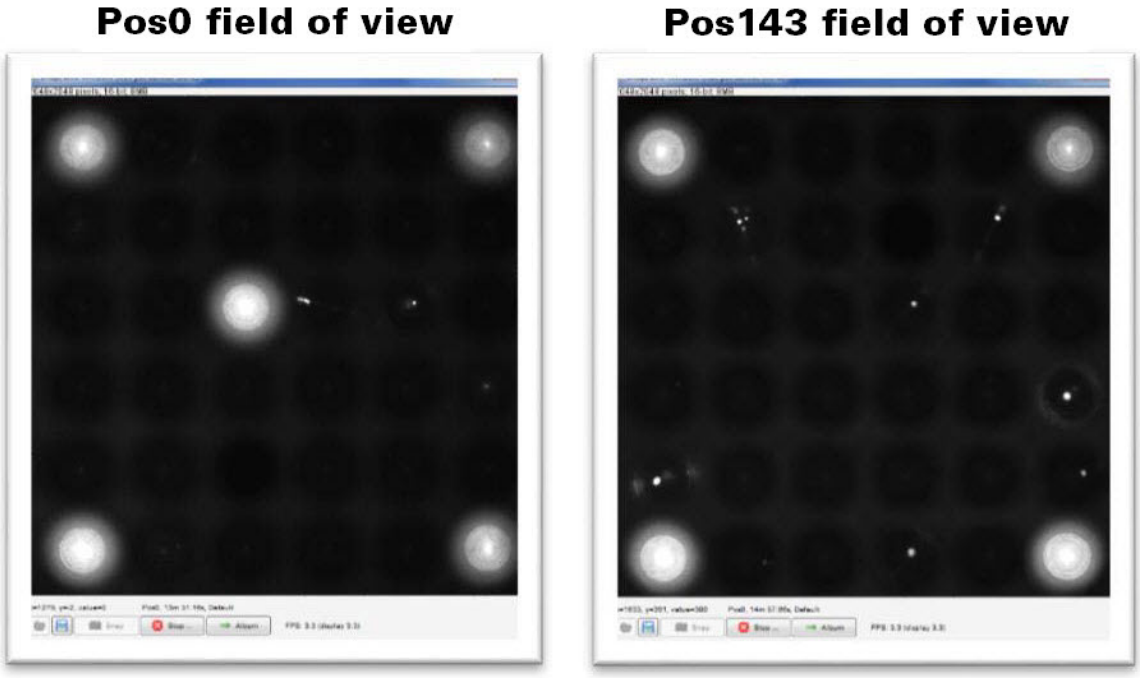


Figure 21. Multiwell images including fiducial-containing nanowells.

14. Select "Pos143" in the *Stage Position List* window and confirm that all 36 nanowells are properly captured in the image. There will be four fiducial-containing nanowells in the shape of a square, as shown in Figure 21 (above). Pos143 is located at the lower right corner of the stage fixture.

Optimize image focus

15. Adjust image contrast and focus settings for optimal visualization of single cells (refer to the ICELL8 Imaging System User Manual for more details):
    a. In the *Main* window, under "Configuration settings", select **Hoechst** as the illumination channel for examination of Hoechst-stained cells.

b. Use the microscope's manual focus to achieve the optimal Z-plane focal depth to visualize a field of single cells.

c. Use the zoom function to zoom in on a well containing one or a few cells that are off center (i.e., not in the center of a well).

d. Adjust the fine contrast knob back and forth to find the optimal focal point for a cell (smallest, sharpest view) or the optimal focal point between two cells on the Z-plane (blue box in Figure 22, below).

e. Zoom out to view all 36 nanowells at Pos143 and confirm that the focus is optimal.

16. **NOTE:** If the cells are truly out of focus, ensure that the chip is perfectly flat on the Dispense Platform. Close the *Stage Position List* window.

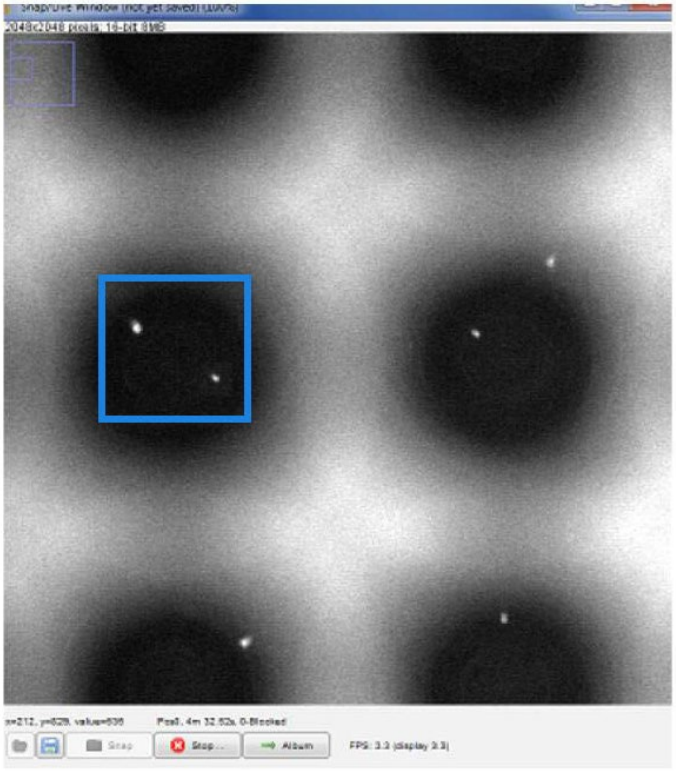


Figure 22. Adjusting the focus on single cells.

Acquire images

17. In the "Channels" table of the *Multi-Dimensional Acquisition* window, the following two preconfigured channels should be preselected: **Hoechst** (exposure: 200) and **Texas Red** (exposure: 300). The Hoechst channel enables visualization of Hoechst staining, while the Texas Red channel is used for visualization of propidium iodide staining.

18. Click **Acquire!** and accept the prompt to create a new folder based on the current date. The camera will acquire TIFF images from 144 fields of view (Pos0–Pos143) using Hoechst and Texas Red filters, respectively, with each field of view including 36 nanowells in a 6 x 6 grid. Following acquisition, the entire image folder should contain a total of 288 TIFF files comprising the 144 fields of view in both the Hoechst and Texas Red channels.

    a. In the *Main* window, select the *Contrast* tab and ensure that "Camera Depth" menu is set to **12 bit (0-4095)** for Hoechst and **10 bit (0-1023)** bit for Texas Red in the (purple box in Figure 23, below).

b. Next, confirm that the [Autostretch] and [Ignore] boxes are selected, and that the percentage value is in the range of **1–3%** for Ignore option (blue box in Figure 23). If this does not provide optimal contrast, drag the ends of the diagonal line (see arrows in Figure 23) closer to the range of the histogram peaks and zoom out when satisfied with the focus in the Z-plane.

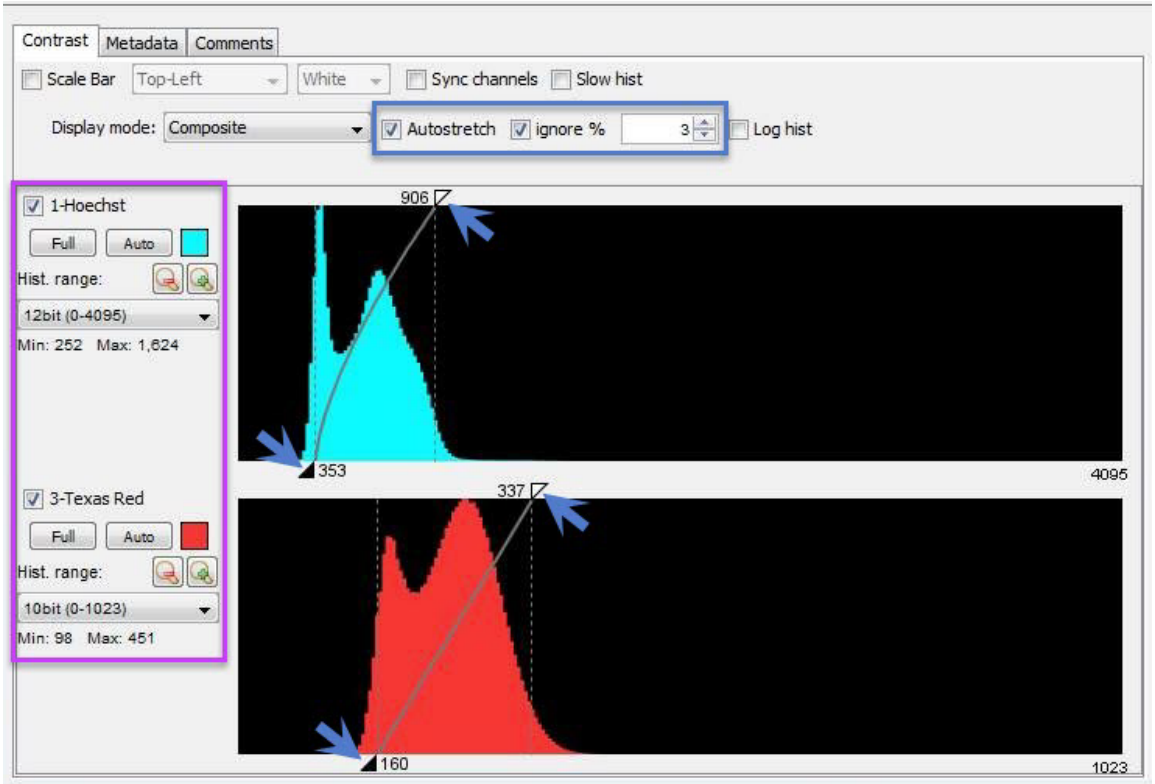


Figure 23. Adjusting camera depth and image contrast.

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

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

20. Reapply the peeled release liner onto the top side of the double-sided intermediate film on the chip.
21. Place the imaged chip into an empty Chip Holder that has been prechilled at –80°C (Figure 10, above). Make sure that the imaging film is well sealed over the chip. The Chip Holder should click closed and should close evenly, indicating a proper magnetic seal.
22. Freeze cells at –80°C for a minimum of 30 minutes before proceeding to RT-PCR (Section V.E, below).
23. Turn off the controllers for the microscope imaging filters and stage, the camera, and the fluorescence light source when the imaging is completed.

SAFE STOPPING POINT: The chip can be frozen in the Chip Holder at –80°C overnight.

D. Protocol: Analyze Nanowells with CellSelect Software

In this protocol, CellSelect software is used to analyze the images of the ICELL8 3′ DE Chip in order to identify nanowells containing viable single cells that are suitable for further processing and analysis via RT-PCR. Refer to the CellSelect Software User Manual for detailed information about using the software.

Load images

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

2. Click **YES** when prompted to convert the detected Micro-Manager image stack.

Figure 24. Converting image stack.

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

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

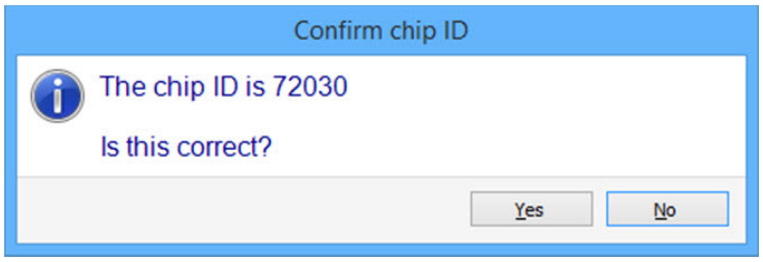


Figure 25. Confirming the chip ID.

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

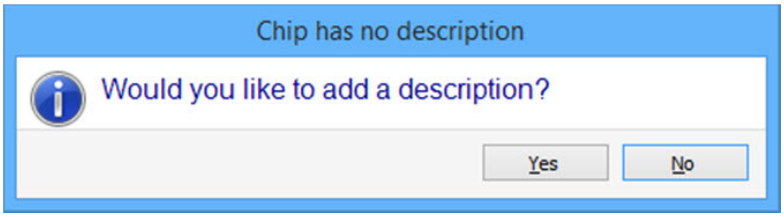


Figure 26. Adding a description for the chip.

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

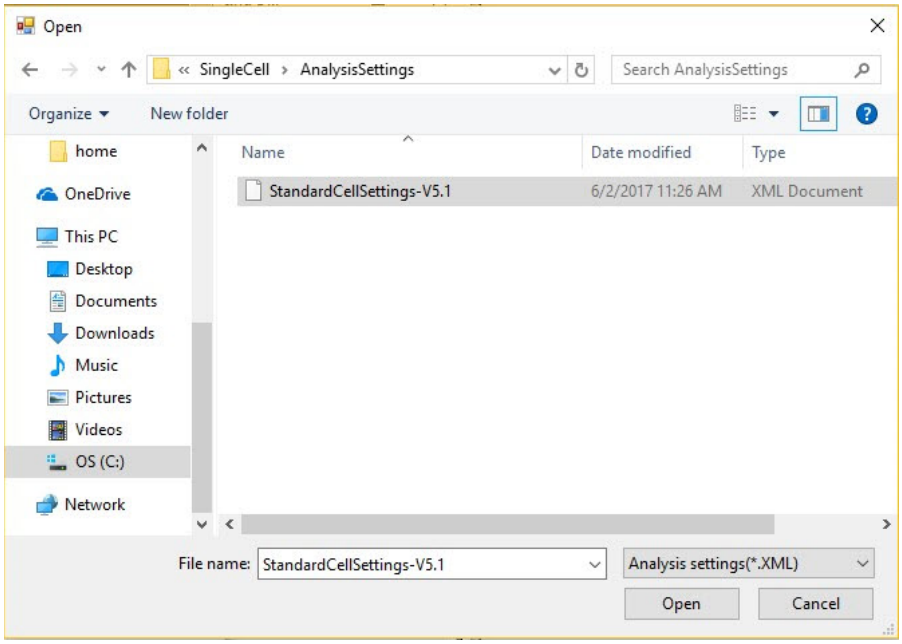


Figure 27. Loading the settings file.

Define fiducials

6. The software will automatically detect fiducials and prompts you with four consecutive fiducial images. The [Define fiducials] box in the top-left portion in the *Main* window will be highlighted as this process occurs (see Figure 28). The first fiducial image (Pos0) shows five green circles, while the other three fiducial images (Pos11, Pos132, Pos143) show four green circles. The green circles highlight the centers of the nanowells (Figure 28).

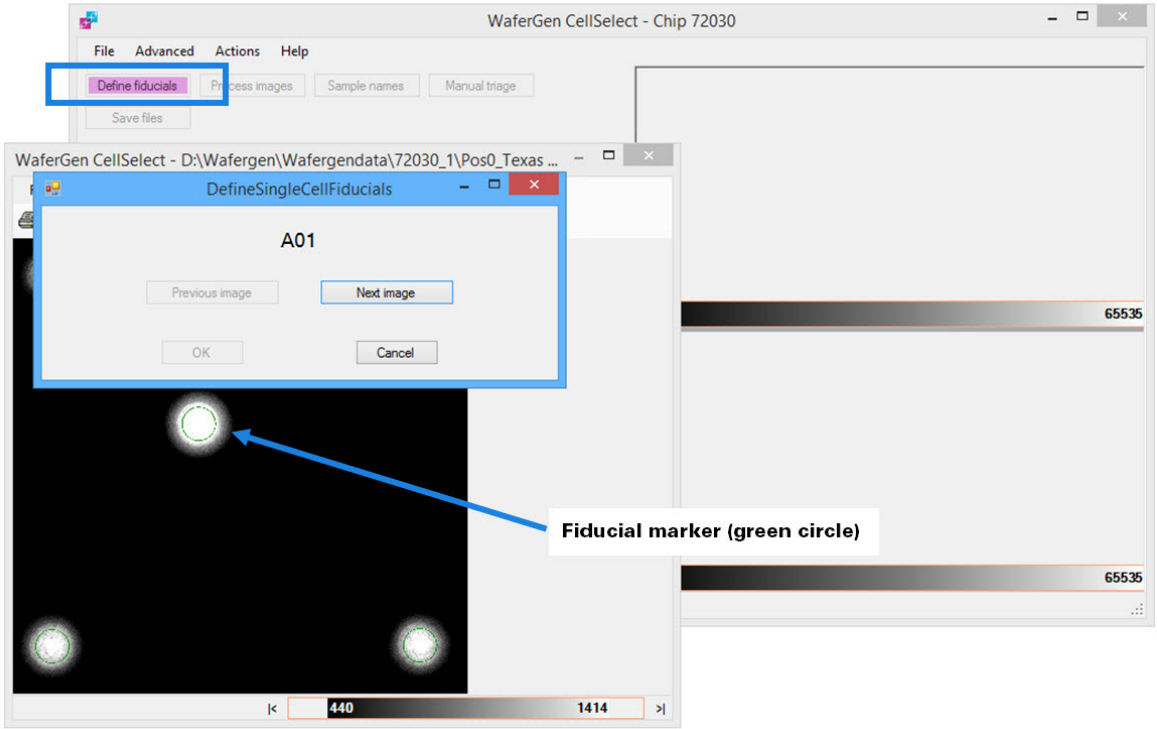


Figure 28. Defining fiducials.

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

    a. Click and drag the *DefineSingleCellFiducials* prompt window to the side to view the fiducials image (see Figure 29, below).

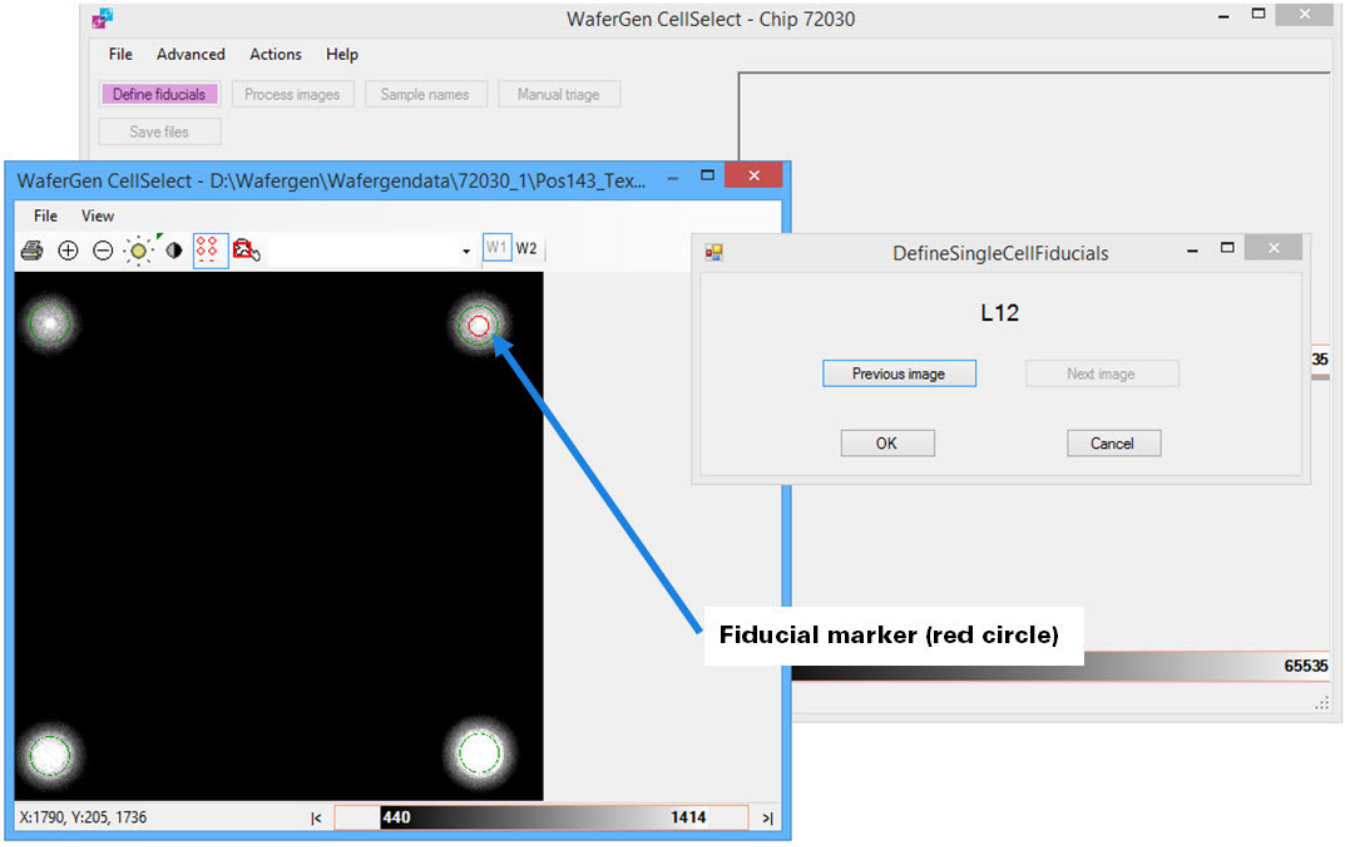


Figure 29. Inspecting and adjusting fiducials.

    b. If the green circles are not centered properly, click on the nanowell centers with the mouse pointer (changes to red circle; see Figure 29, above).

    c. If necessary, resize the fiducial image to view a 6 x 6 grid (36 nanowells). Use the following tools in the *Image Viewer* toolbar to check and correct fiducials: **Zoom in**/**Zoom out**, **Image contrast**, **Auto contrast**, **Well overlay**, **Wavelength 1 or 2** (W1, W2). Figure 30 provides a legend for the *Image Viewer* toolbar icons.

Figure 30. *Image Viewer* toolbar icons.

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

Load barcode file

8. In the *Main* window, click the **File** tab and select **Load barcode file**.

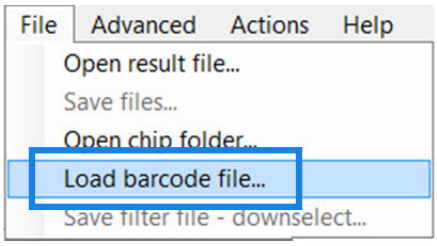


Figure 31. File menu.

9. Select the preloaded file `3'DE.xml`. This eXtensible Markup Language (XML) file contains nanowell barcode sequences for each preprinted nanowell location.

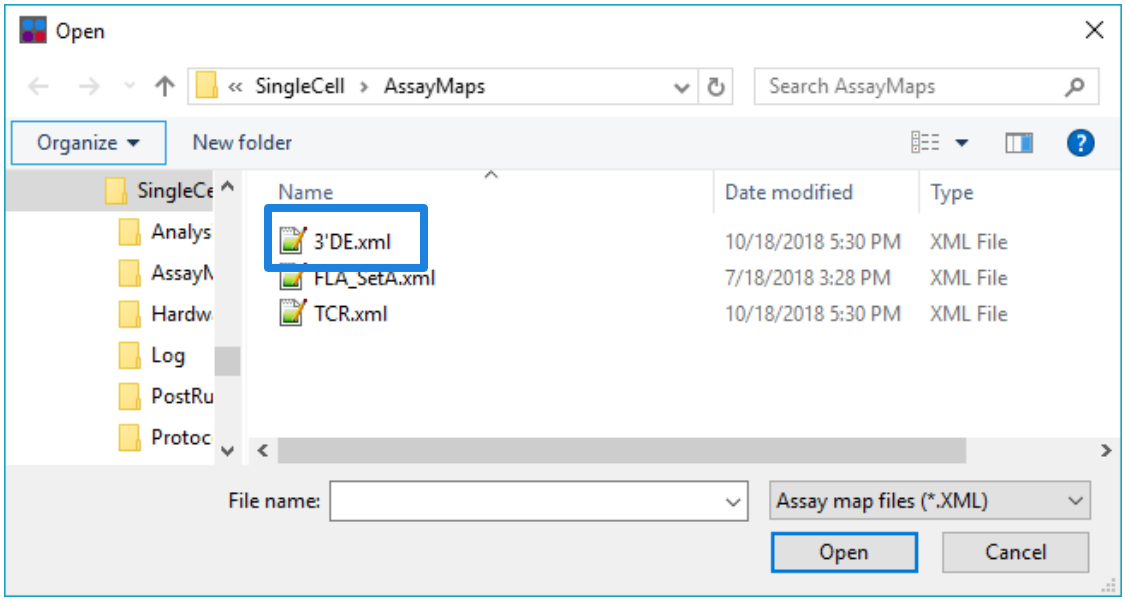


Figure 32. Loading the barcode file for the preprinted chip.

Process images

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

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

- The cell must appear in the Hoechst channel and NOT in the Texas Red channel.

- No other cells are visible in the well.
- The cell must satisfy the size/shape parameters specified in *Settings*.

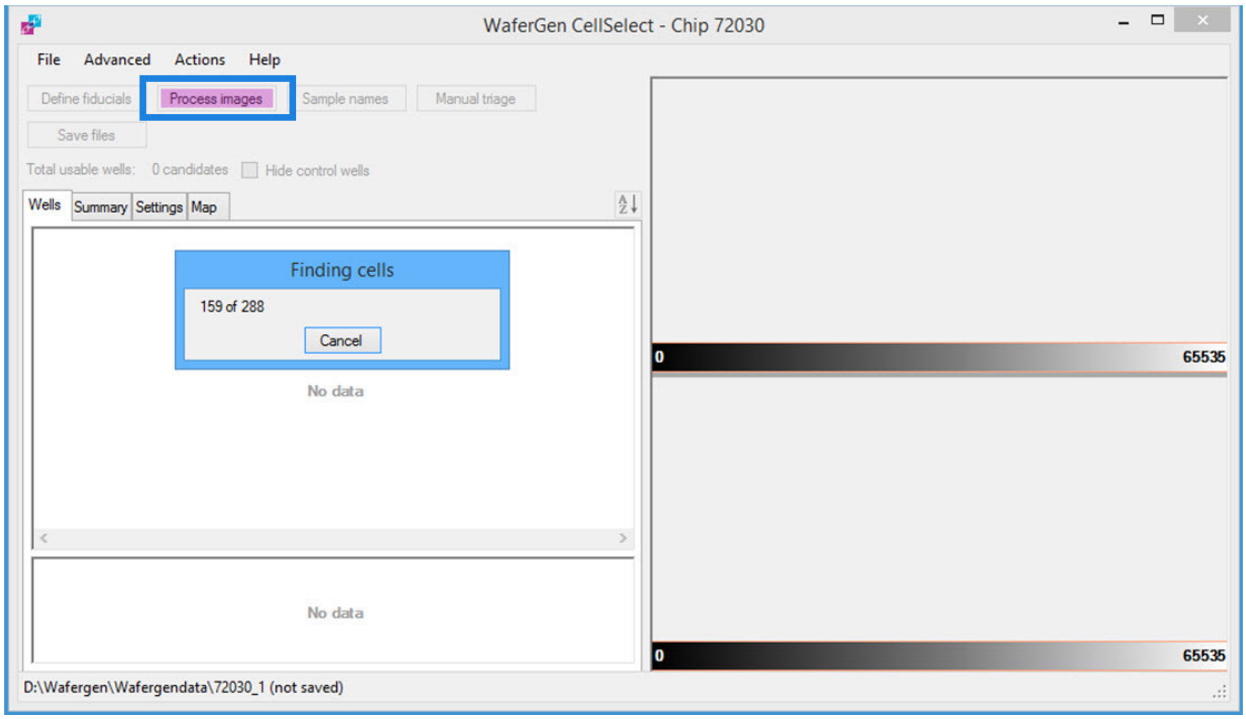
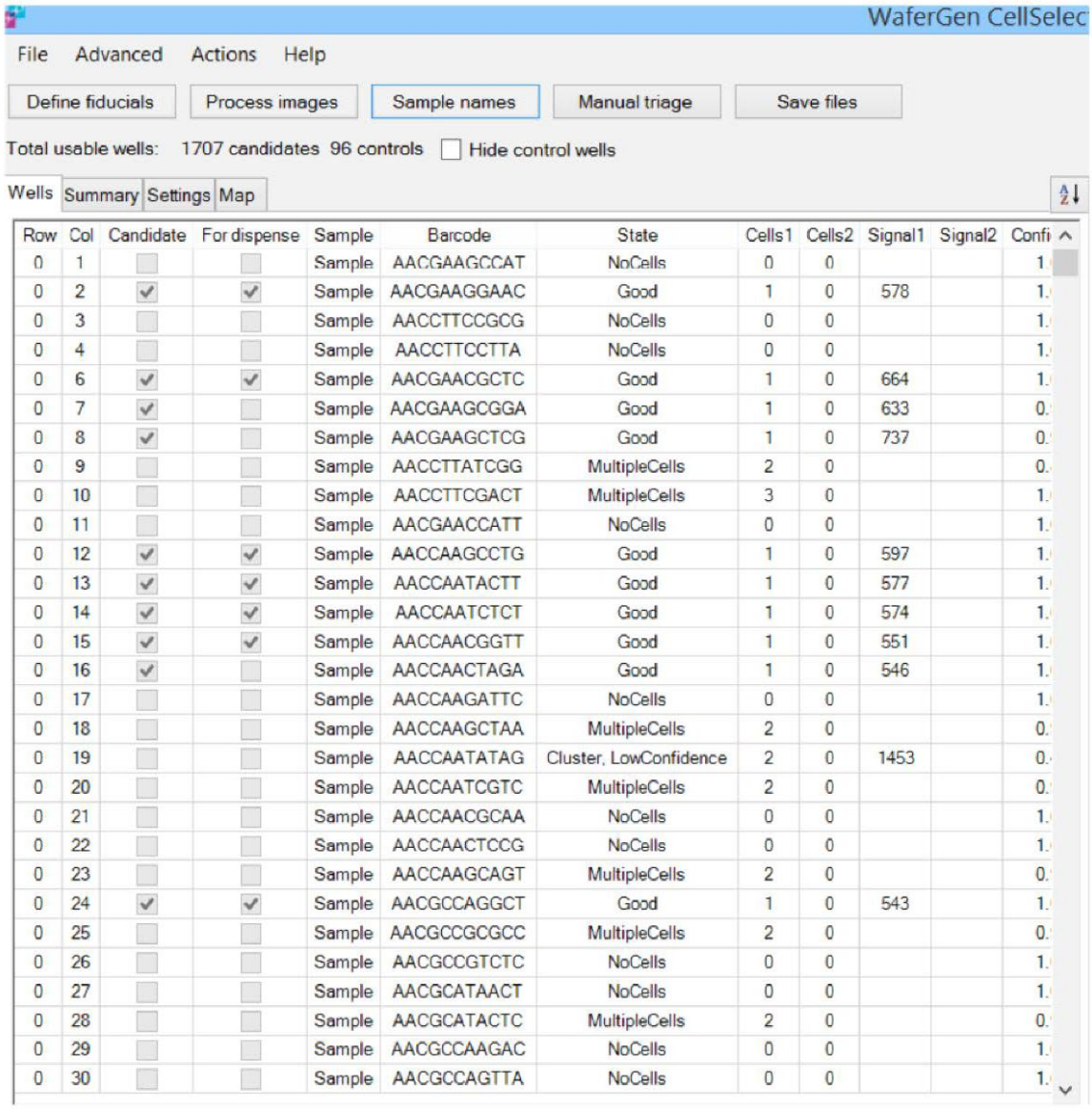


Figure 33. Having the software find cells based on parameters defined in *Settings*.

11. After processing is complete, the software will provide a prompt for saving results. Click **Yes** and input a file name prefixed with the chip number (`<Chip ID>_<Date>.wcd`). The software will then do the following:
    - Select wells among all those that are checked under Candidate according to the best parameters defined in the *Settings* tab.
    - Save the result file under the specified name (`<Chip ID>_<Date>.wcd`). The result file will contain the selected nanowells and setting parameters.
    - Automatically generate the filter file for dispensing RT reagents (`<Chip ID>_FilterFile.csv`). The selected wells in the filter file will be displayed in the *For Dispense* tab.
12. Each preprinted chip contains a total of 5,184 unique nanowell barcodes. You can load the results along with the settings from the saved WCD file (Figure 34, below). The software uses the following legend when examining nanowells:
    - **Green** = a cell
    - **Yellow** = ignored
    - **Blue** = a reflection

    Refer to the CellSelect Software User Manual for more information.



Row	Col	Candidate	For dispense	Sample	Barcode	State	Cells1	Cells2	Signal1	Signal2	Confi
0	1	☐	☐	Sample	AACGAAGCCAT	NoCells	0	0			1
0	2	☑	☑	Sample	AACGAAGGAAC	Good	1	0	578		1
0	3	☐	☐	Sample	AACCTTCCGCG	NoCells	0	0			1
0	4	☐	☐	Sample	AACCTTCCTTA	NoCells	0	0			1
0	6	☑	☑	Sample	AACGAACGCTC	Good	1	0	664		1
0	7	☑	☐	Sample	AACGAAGCGGA	Good	1	0	633		0
0	8	☑	☐	Sample	AACGAAGCTCG	Good	1	0	737		0
0	9	☐	☐	Sample	AACCTTATCGG	MultipleCells	2	0			0
0	10	☐	☐	Sample	AACCTTCGACT	MultipleCells	3	0			1
0	11	☐	☐	Sample	AACGAACCATT	NoCells	0	0			1
0	12	☑	☑	Sample	AACCAAGCCTG	Good	1	0	597		1
0	13	☑	☑	Sample	AACCAATACTT	Good	1	0	577		1
0	14	☑	☑	Sample	AACCAATCTCT	Good	1	0	574		1
0	15	☑	☑	Sample	AACCAACGGTT	Good	1	0	551		1
0	16	☑	☐	Sample	AACCAACTAGA	Good	1	0	546		1
0	17	☐	☐	Sample	AACCAAGATTC	NoCells	0	0			1
0	18	☐	☐	Sample	AACCAAGCTAA	MultipleCells	2	0			0
0	19	☐	☐	Sample	AACCAATATAG	Cluster, LowConfidence	2	0	1453		0
0	20	☐	☐	Sample	AACCAATCGTC	MultipleCells	2	0			0
0	21	☐	☐	Sample	AACCAACGCAA	NoCells	0	0			1
0	22	☐	☐	Sample	AACCAACTCCG	NoCells	0	0			1
0	23	☐	☐	Sample	AACCAAGCAGT	MultipleCells	2	0			0
0	24	☑	☑	Sample	AACGCCAGGCT	Good	1	0	543		1
0	25	☐	☐	Sample	AACGCCGCGCC	MultipleCells	2	0			0
0	26	☐	☐	Sample	AACGCCGTCTC	NoCells	0	0			1
0	27	☐	☐	Sample	AACGCATAACT	NoCells	0	0			1
0	28	☐	☐	Sample	AACGCATACTC	MultipleCells	2	0			0
0	29	☐	☐	Sample	AACGCCAAGAC	NoCells	0	0			1
0	30	☐	☐	Sample	AACGCCAGTTA	NoCells	0	0			1

Figure 34. Viewing the results list after reading the image stack.

Specify sample names

13. Click the [Sample names] box and enter the sample description; enter one sample name for all samples
    (Figure 35, below), or multiple sample names based on the source plate configuration layout in Figure 11, Section V.B (above).

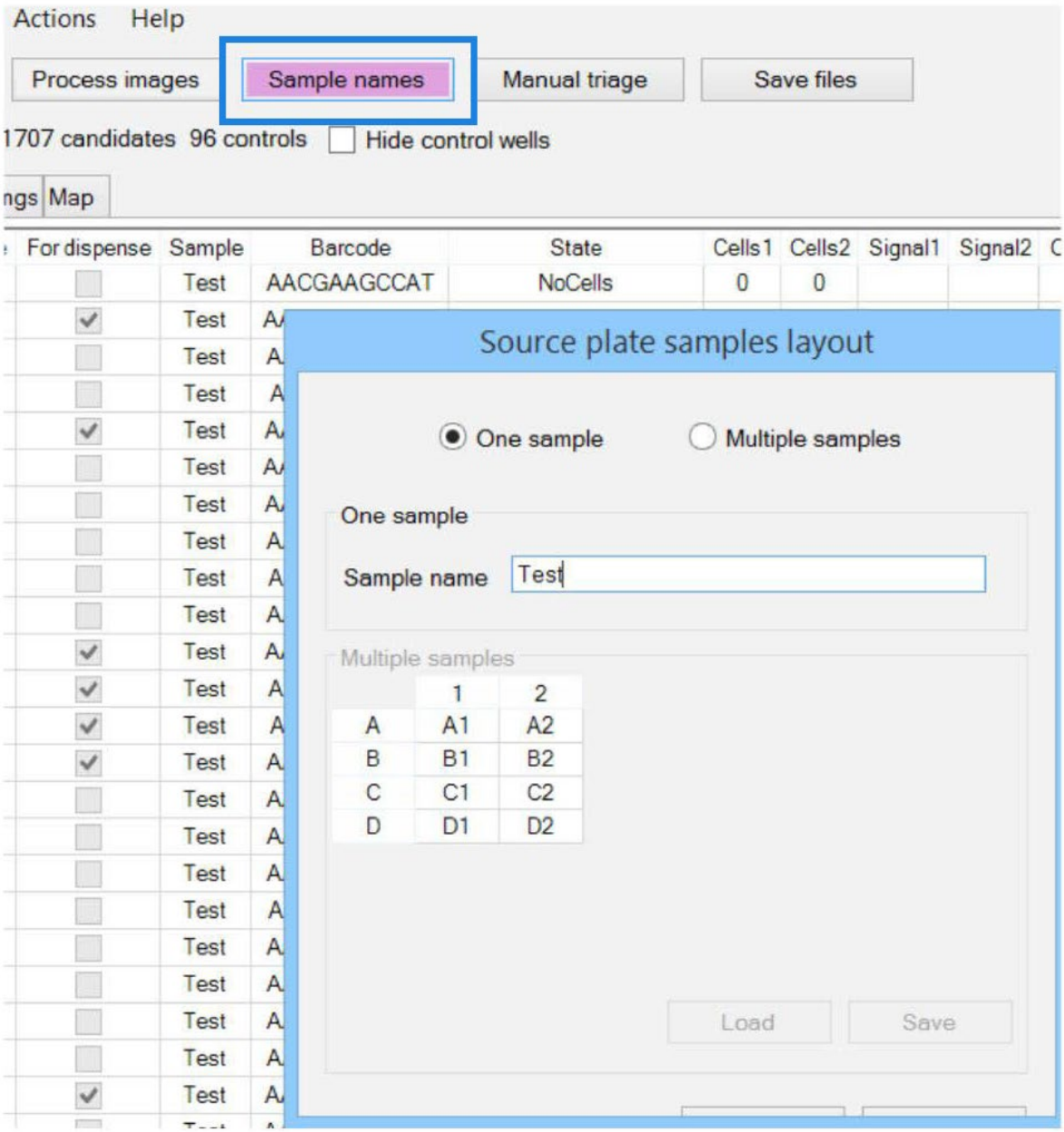


Figure 35. Specifying sample names. For the "One sample" option, "Test is repeated down the Sample column. Control and fiducial wells are identified automatically.

Review images

14. Click the *Wells* tab and sort the nanowell statistics in the "Candidate" column by clicking the row heading (see Figure 36, below). The software will sort and consolidate all selected nanowells (i.e., "candidates") to the top of the table.

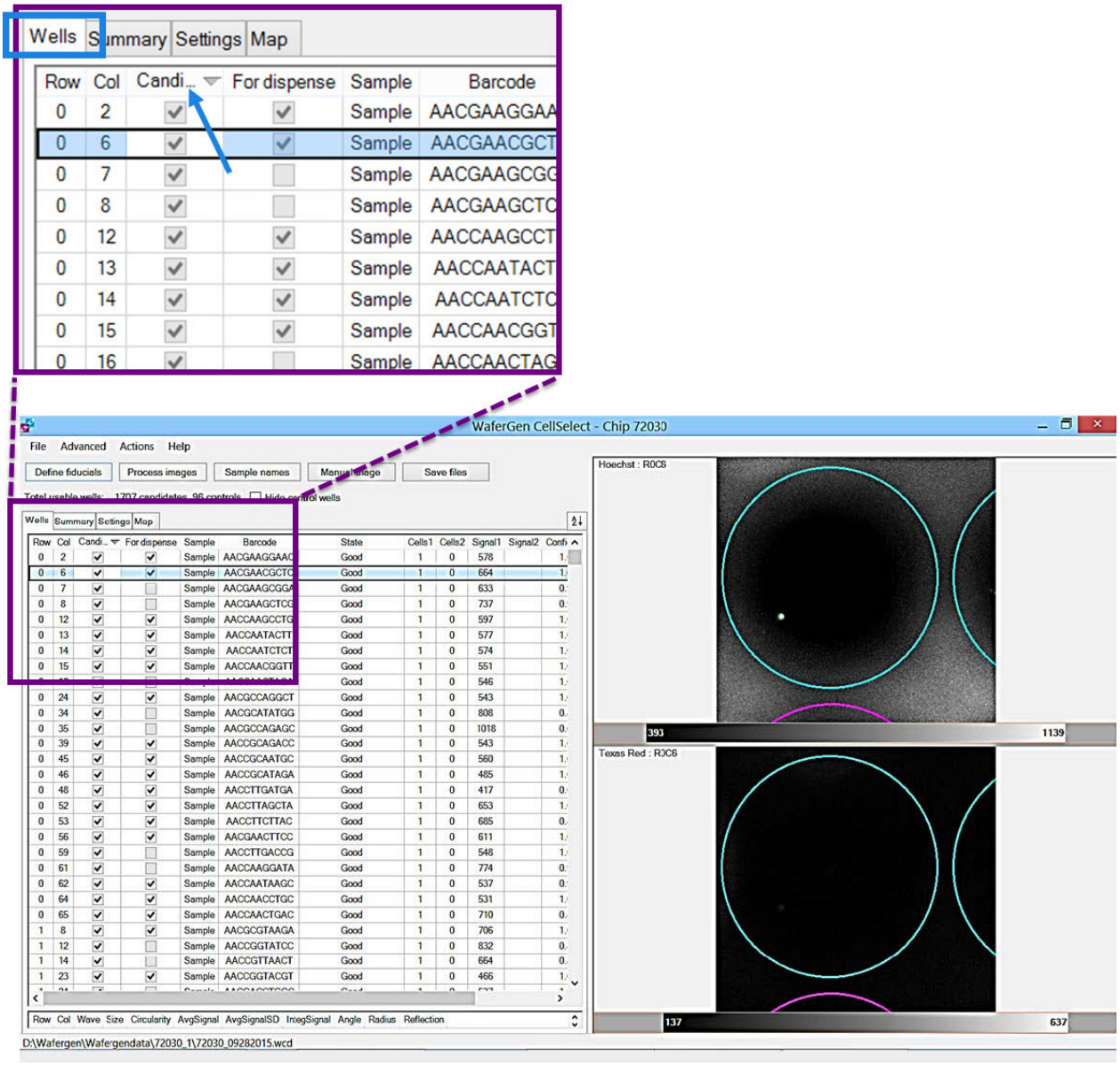


Figure 36. Sorting the candidate column to consolidate all selected nanowells to the top of the table.

15. If desired, manually inspect the selected nanowells. Click any row for closeup views of the selected nanowell in both the Hoechst and Texas Red channels (top and bottom single-well images, respectively, in Figure 36, above).
16. Selected nanowells can also be viewed in the context of the corresponding multi-well image via the *Image Viewer* window: click the **Advanced > Image viewer** menu item (Figure 37, below). A multi-well image highlighting the nanowell selected in the results table (on the *Wells* tab) will appear.

17. Arrange the windows as demonstrated in Figure 37 (below). When you click on any of the Candidate rows in the results table (on the *Wells* tab), the software will present corresponding single-well images, and display the corresponding multi-well image in the *Image Viewer* window.
18. To manually exclude one or more candidate wells, right-click the highlighted rows on the *Wells* tab and select **Exclude selected wells**. To exclude several candidate wells, consider using the **Manual triage** function. Refer to the corresponding section of the CellSelect Software User Manual for more information.

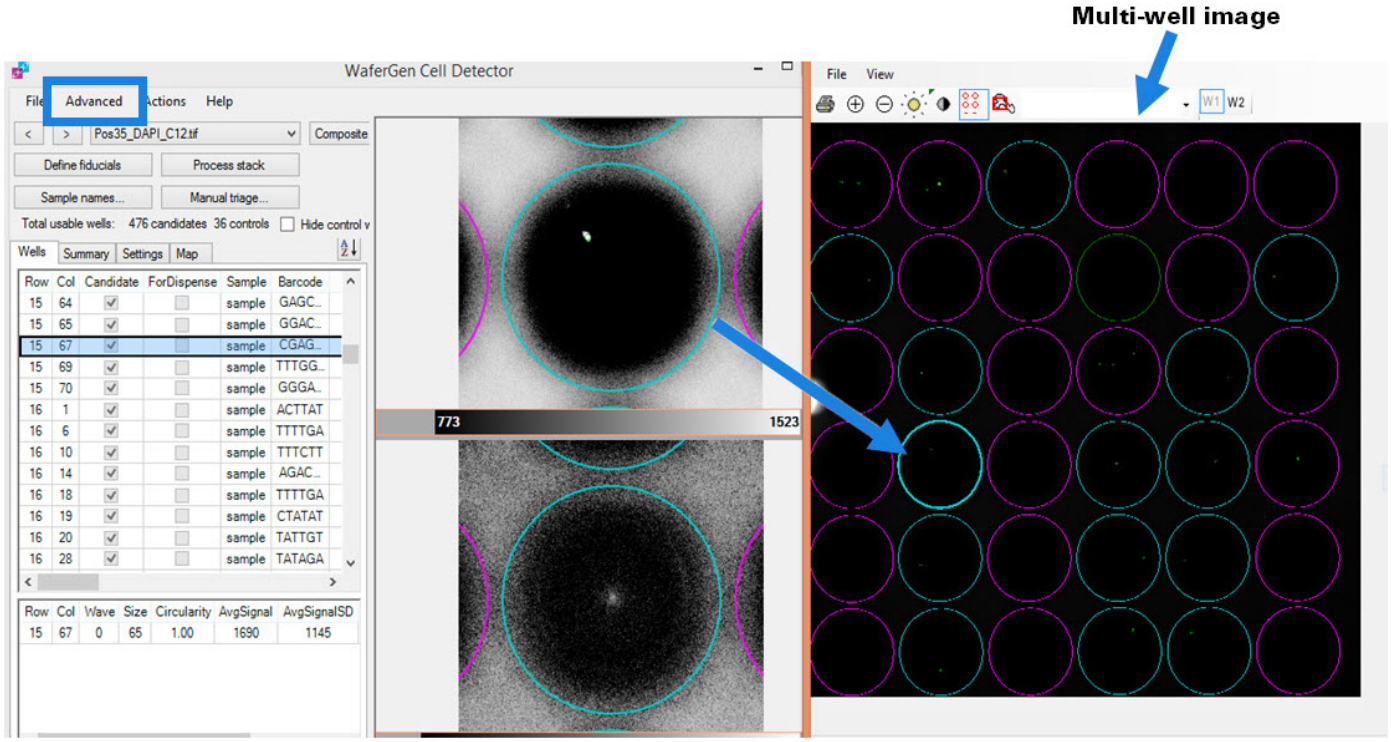


Figure 37. Arrangement of CellSelect windows. The image on the far right is the multi-well image. The horizontally split images in the center show a single cell in a nanowell stained by Hoechst 33342 (top) and the absence of propidium iodide stain in the corresponding position (dead-cell stain; bottom). In the bottom image, the green dot in the center (if seen) marks the bottom of the nanowell and is probably not from a dead cell because it does not match the location of the Hoechst-stained cell.

Save files

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

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

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

21. If you would like to downselect nanowells, click the **Actions > Downselect** menu item.

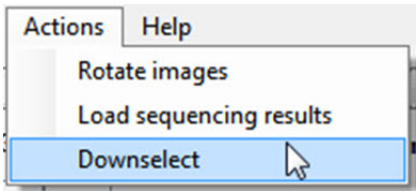


Figure 38. Choosing Downselect from the Actions menu.

22. Enter the desired number of control and sample wells to be included (Figure 39, below).

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

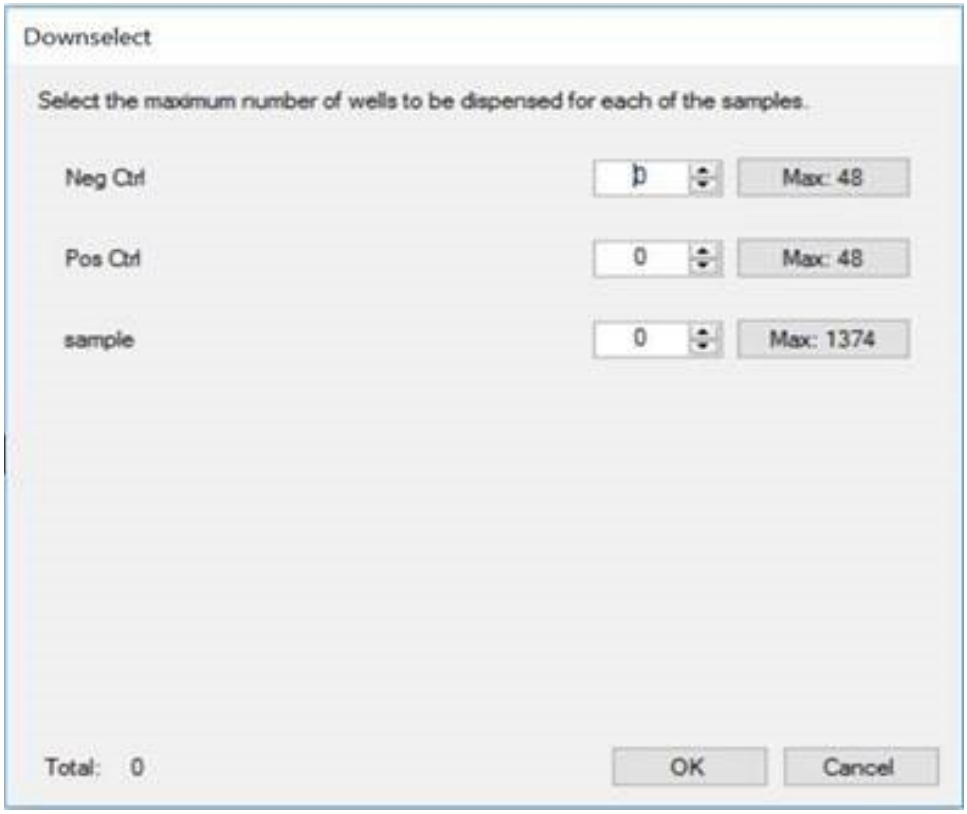


Figure 39. Downselecting nanowells for the filter file.

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

E. Protocol: Synthesize and Amplify cDNA

In this protocol, reagents for cDNA synthesis and amplification are dispensed into selected wells of the ICELL8 3′ DE Chip using the ICELL8 MSND, 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.

Before you start:

- Initialize the ICELL8 MSND; perform daily warm-up via the *Startup* window, then pull up the *Advanced* window and execute the **Wash Prime** function followed by **Tip Clean**, then repeat the **Tip Clean** function 2–3 more times.
- Set the centrifuge(s) used for spinning the chip and 384-well source plate to 4°C.
- This protocol requires an MSND 384-Well Source Plate (with seal) and the following components from the ICELL8 Chip and Reagent 3′ DE 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 DNA Polymerase, Blotting Paper, PCR Film, Collection Fixture, Collection Tube, and Collection Film.
- Thaw all reagents on ice except for 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.
- Remove the chip holder containing the ICELL8 3′ DE Chip from the –80°C freezer. Take the chip out of chip holder and thaw the chip at room temperature for 10 minutes to lyse cells. 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 minutes at 4°C. Keep the chip on ice until ready for dispensation of RT-PCR mix.

Procedure:

1. 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.
2. Add ICELL8 3′ DE Oligo Mix. Vortex gently and spin down. Keep the mixture on ice.
3. 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

4. Pipette 50 µl of the RT-PCR reaction mix into wells A1, B1, C1, and D1 of a MSND 384-Well Source Plate, as shown in Figure 40.

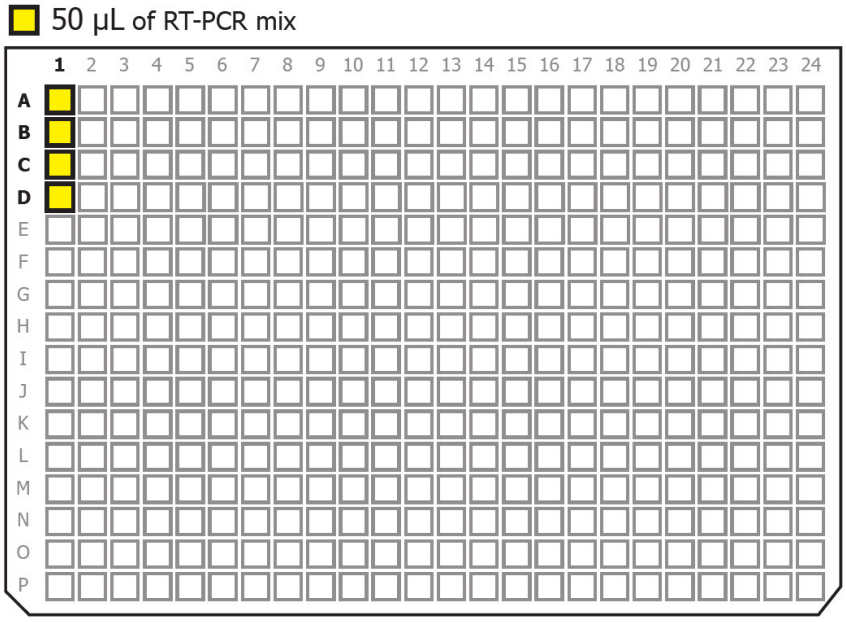


Figure 40. Setting up the source plate for dispensing RT-PCR mix.

5. Seal the 384-well source plate with the provided Plate Seal.
6. Centrifuge the source plate at 3,220*g* (minimum 2,600*g*) for 3 minutes at 4°C.
7. In the ICELL8 MSND GUI, load the filter file generated in the previous protocol (Section V.D, above); click the **Single Cell** tab followed by the **Browse** button next to the *Filter file* field ("…"; Figure 41, below) then select the appropriate filter file (`.csv`). Click within the Chip ID field and enter or scan the corresponding chip ID (refer to the ICELL8 MSND User Manual for more details):
8. Place the 384-well source plate in the MSND with the A1 corner positioned at the top-right corner of the Plate Nest (see Figure 6, above). The beveled corners of the 384-well source plate should be on the left side.
9. Gently remove the seal from the 384-well source plate and lock the plate into position.
10. Peel off the imaging film from the chip. Place the chip on the chuck of the Dispensing Platform in the MSND with the chamfered (notched) corner of the chip at the lower-right corner of the Dispensing Platform, aligned with the chamfered corner of the chuck (see Figure 7 in Section IV.D, above).

    IMPORTANT: The edges of the chip should be pressed against the three alignment pins; **make sure that the chip is flat on the chuck from all sides and is in the correct orientation** (see Figure 7, above).

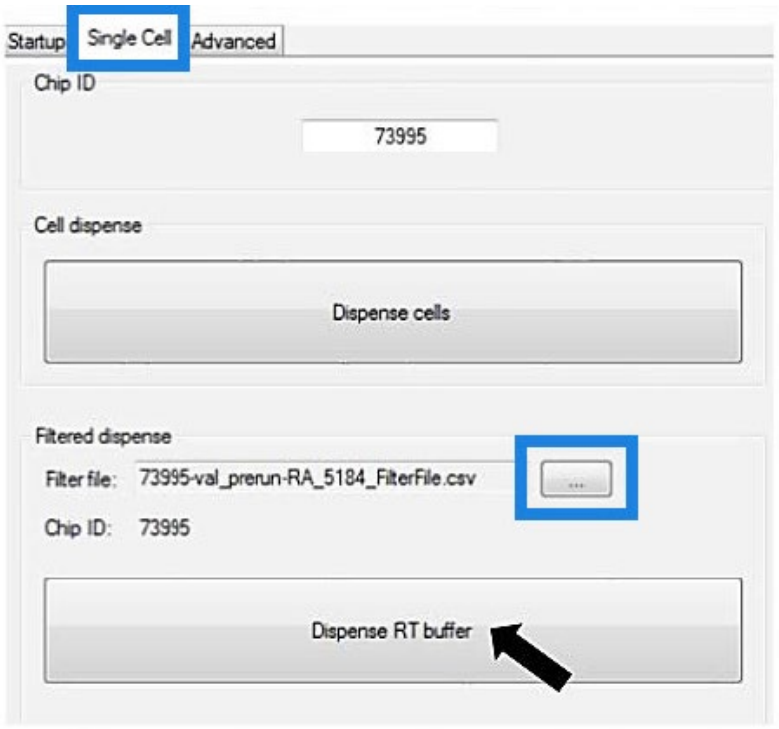


Figure 41. Loading the filter file and dispensing RT-PCR mix with the MSND software.

11. Click **Dispense RT buffer**, carefully go through all questions prompted, and then click **OK**. The ICELL8 MSND will dispense 50 nl of RT-PCR reaction mix into each chip nanowell.
12. After dispensation is completed, remove the chip from the Dispensing Platform and gently blot for two seconds with a fresh Blotting Paper and Blotter (Figure 13, above).
13. Seal the chip with a PCR Film using the Film Applicator. Make sure that the chip is securely sealed to avoid well-to-well contamination.
14. Centrifuge the ICELL8 3′ DE Chip at 3,220g for 3 minutes at 4°C.
15. Using the ICELL8 MSND GUI, repeat the Tip Clean procedure 3–4 more times.

16. Place the ICELL8 3′ DE Chip into the Chip Cycler to perform the RT-PCR reaction using the following preinstalled program.

NOTE: The RT-PCR program described below is preinstalled on the Chip Cycler provided with the ICELL8 system, 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.

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 Chip Cycler at 4°C overnight.

17. Remove the chip from the Chip Cycler. Centrifuge the chip at 3,220*g* for 3 minutes at 4°C.
18. Open the supplied ICELL8 Collection Kit and label the Collection Tube with the engraved chip number. Assemble the Collection Module by attaching the Collection Tube to the Collection Fixture.

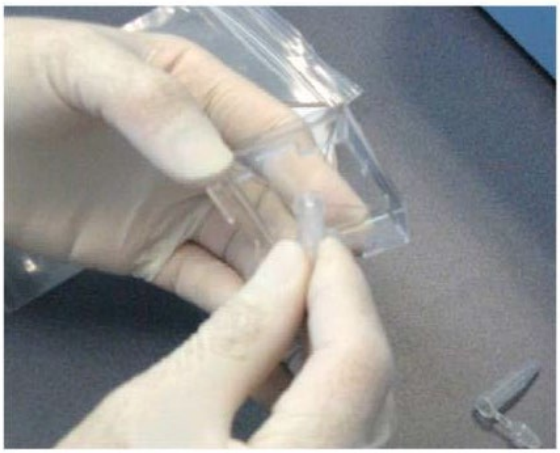


Figure 42. Assembling the Collection Module.

19. Carefully peel off the PCR Film from the chip (Figure 43, below).

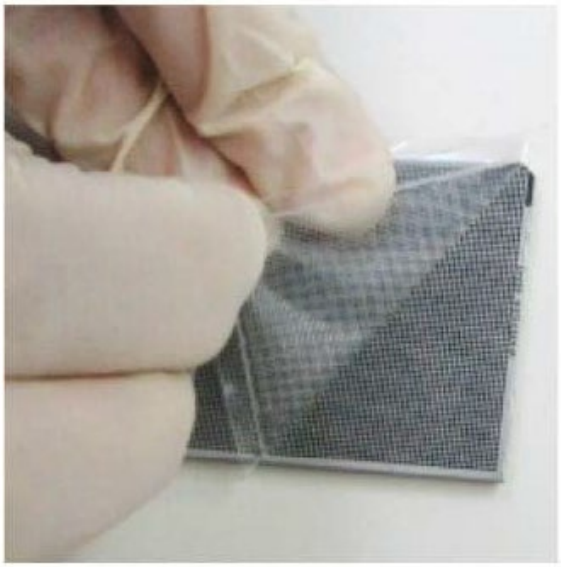


Figure 43. Removing the PCR sealing film from the chip. Start from one corner and apply even pressure to pull.

20. With the nanowells facing down, place the chip into the assembled Collection Module (Collection Tube plus Collection Fixture; Figure 44, below). Surface tension will hold the liquid in the nanowells.

Figure 44. Placing the chip into the Collection Module.

21. Seal the chip and the top of the Collection Module with a supplied Collection Film (Figure 45, below).

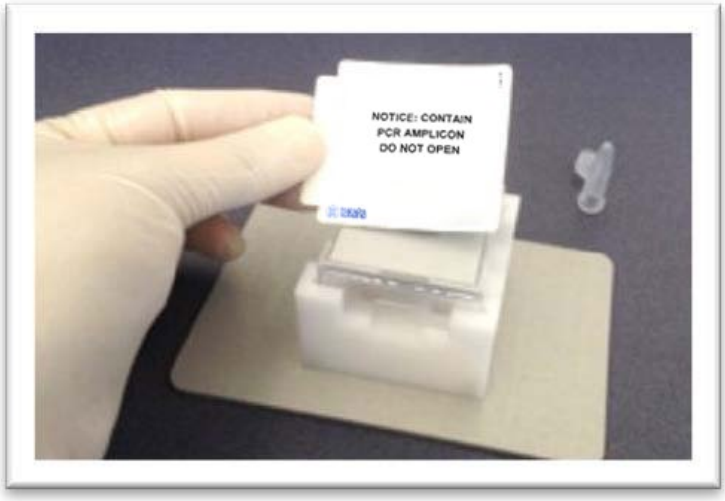


Figure 45. Securing the Collection Module with Collection Film.

22. Using a balance or blank chip, assemble another Collection Module. Centrifuge both Collection Modules at 3,220*g* (minimum 2,600*g*) for 10 minutes at 4°C.
23. Carefully remove the Collection Tube containing the extracted cDNA (Figure 46, below).

IMPORTANT: Measure the volume of the collected eluent. The volume should be no less than 15% of the expected value. For example, if 500 nanowells were selected for processing, you should collect 260–284 µl of eluent.

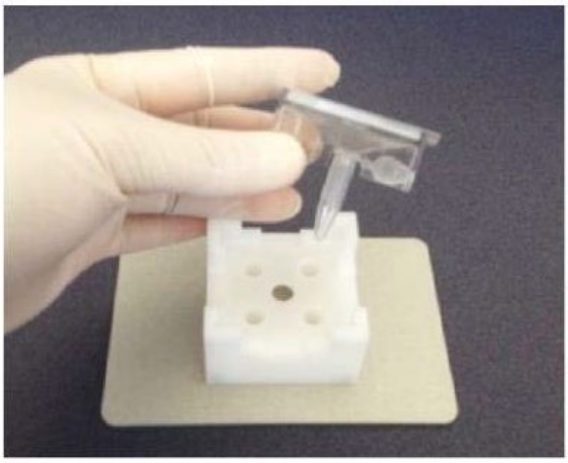


Figure 46. Removing the Collection Tube containing the cDNA eluent.

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

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

F. 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 minutes 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 Chip and Reagent 3′ DE 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 minutes and then on a magnetic stand for ≥2 minutes 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 minutes or until the ethanol has just evaporated and the pellet is no longer glossy. Do not overdry 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 minutes 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 47, 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).

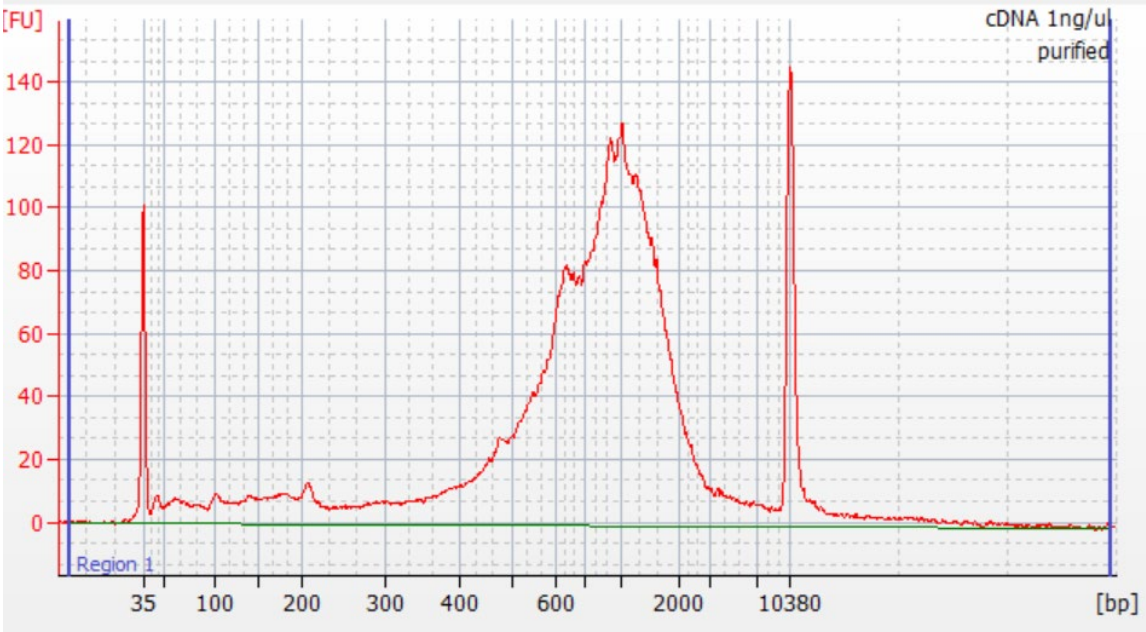


Figure 47. Typical Bioanalyzer trace for purified full-length cDNA that has been normalized to 1 ng/µl.

G. 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). Using the Qubit quantification, 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 Chip and Reagent 3′ DE 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
10°C	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 minutes.
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 Chip and Reagent 3′ DE 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

H. 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 minutes 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 Chip and Reagent 3′ DE Kit.

Procedure:

Purify and size select sequencing library

1. To each 50-µl volume of PCR product from the previous protocol (Section V.G, 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 minutes and then on a magnetic stand for ≥2 minutes 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 minutes 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 minutes and then incubate on a magnetic stand for 2 minutes 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 minutes and then on a magnetic stand for ≥2 minutes 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 minutes and then on a magnetic stand for ≥2 minutes 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 minutes 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 minutes 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 48, 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.

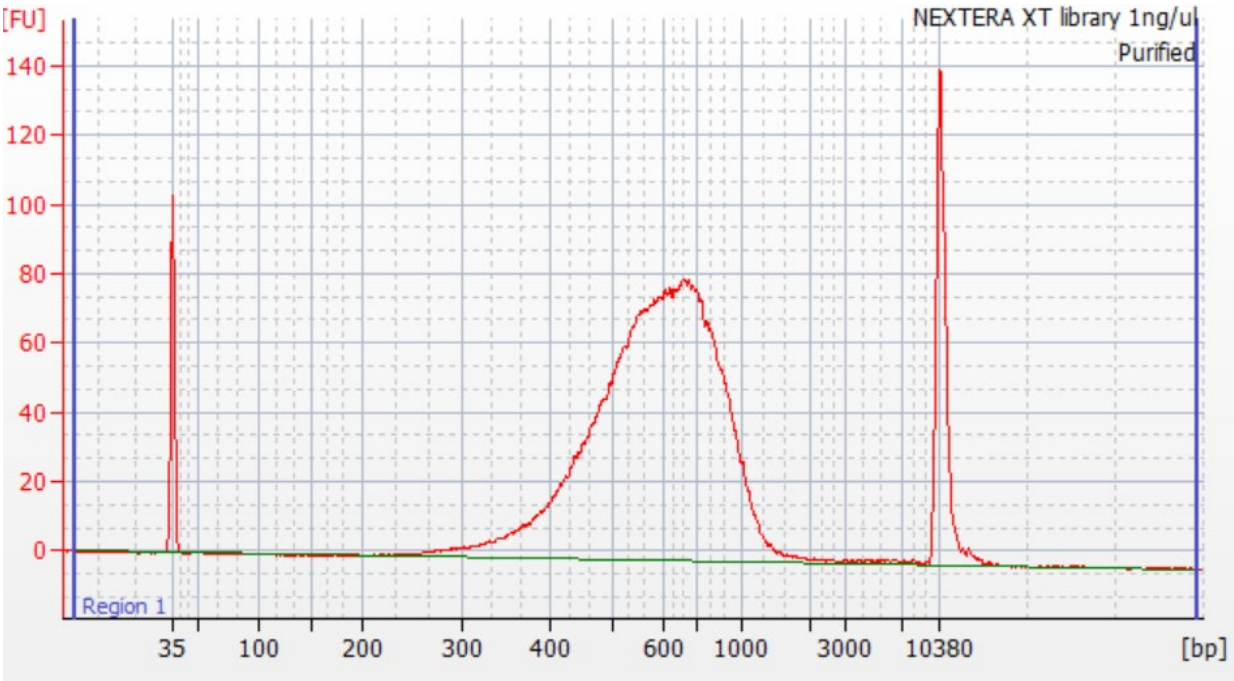


Figure 48. Typical Bioanalyzer trace for purified Nextera XT NGS library that has been normalized to 1 ng/µl.

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