Illumina Bio-Rad SureCell WTA 3' Library Prep for Nuclei Samples

Demonstrated Protocol

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Chapter 1 Overview

Introduction

This protocol describes how to prepare 3′-tagged RNA-Seq libraries from single nuclei for whole transcriptome gene profiling analysis on Illumina sequencing systems. The protocol requires a Bio-Rad ddSEQ Single-Cell Isolator and reagents provided in the Illumina® Bio-Rad® SureCell™ WTA 3′ Library Prep Kit for the ddSEQ™ System to isolate single nuclei and barcode individual transcriptomes. This protocol has been demonstrated with nuclei isolated from cell lines. Preparation of nuclei from other sources, such as tissue, may require further optimization beyond the guidelines included here.

The SureCell WTA 3' Library Prep Kit for the ddSEQ System is optimized for 1200 single nuclei as output for each ddSEQ Single-Cell Isolator cartridge. Each cartridge has four chambers that can be loaded with up to four unique samples, for an average output of 300 single nuclei per chamber.

The SureCell WTA 3' Library Prep Kit for the ddSEQ System protocol includes the following features:

- Comprehensive workflow for single-nuclei analysis of 3′ RNA transcripts
- Significant reduction in the time from cell culture to nuclei isolation using the ddSEQ Single-Cell Isolator
- Individual droplets have nuclear lysis, nuclear barcoding, and unique molecule tagging
- Fifteen-minute tagmentation process to fragment cDNA and add adapter sequences
- Benefits of using master mixed reagents, saving reagent containers, pipetting, and hands-on time

Data analysis is conducted using the Illumina SureCell RNA Single-Cell app in BaseSpace Sequence Hub. The SureCell RNA Single-Cell app performs sample demultiplexing, single-cell identification, genome alignment, 3′ gene counting, and cell clustering. The SureCell RNA Single-Cell app supports multiple species, including, but not limited to, human, mouse, and rat genomes.

DISCLAIMER

The information in this Illumina Demonstrated Protocol is being provided as a courtesy. In some cases, reagents are required to be purchased from non-authorized third-party suppliers. Illumina does not guarantee or promise technical support for the performance of our products used with any reagent purchased from a non-authorized third-party supplier.

Additional Resources

Visit the SureCell WTA 3' Library Prep Kit for the ddSEQ System support page on the Illumina website or ddSEQ Single-Cell Isolator page on the Bio-Rad website for documentation, software downloads, training resources, and information about compatible Illumina and Bio-Rad products.

The following documentation is available for download from the Illumina website.

Resource	Description
Custom Protocol Selector	A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
Consumables and Equipment List (document # 1000000021455)	Provides an interactive checklist of user-provided consumables and equipment.

The following documentation is available for download from the Bio-Rad website.

Resource	Description
ddSEQ Single-Cell Isolator Instruction Manual (Document # 10000069430)	Provides instructions for installing and operating the Bio-Rad ddSEQ Single-Cell Isolator.

Chapter 2 Protocol

Introduction

This chapter describes a demonstrated protocol for processing single nuclei using the SureCell WTA 3' Library Prep Kit for the ddSEQ System, from nuclei isolation through qualification and quantification of final libraries for sequencing.

Before you begin, do the following.

- Review SureCell Best Practices on the Illumina website.
- Confirm that Illumina Experiment Manager v1.13 or later is used to set up the sequencing sample sheet if BaseSpace Prep Tab is not used. Version 1.13 or later has the appropriate UMI settings and index sequences for sample demultiplexing.
- When using BaseSpace Sequence Hub for sequencing analysis, confirm that First Analysis App App v1.2 or later is used.
- Confirm that bcl2fastq v2.18 or later is used for FASTQ generation.
- This protocol is verified to process up to four cartridges in one experiment. If this is your first experiment, process 1–2 cartridges. If you are processing more than four cartridges, contact Illumina Tech Support for a modified protocol.
- Confirm that the ddSEQ Single-Cell Isolator is installed and operating properly.
- Review pipetting techniques in the ddSEQ Single-Cell Isolator Instruction Manual.
- Confirm kit contents and make sure that you have the required equipment and consumables. This protocol requires two different magnetic stands during library clean-up procedures. See *Supporting Information* on page 39.
- Review the color-coded caps that identify the associated suspension mix of the reagents in this protocol.
  - Red caps identify reagents used to create nuclei enzyme mix
  - Blue caps identify reagents used to create barcode suspension mix
- Use a Lab Tracking Chart to record sample observations throughout the protocol. See *Lab Tracking Chart* on page 42.

Critical Workflow Steps

Several steps within the workflow require additional attention and are key to single-nuclei library success.

1. Confirm that cells are completely lysed using a manual count or an automated count.
2. Confirm that isolated nuclei are in a single-nuclei suspension.
3. Keep your single-nuclei suspension and all buffers on ice at all times during nuclei prep to prevent nuclei from clumping.
4. A bead purification step purifies single-nuclei DNA from sample wells containing separate oil and aqueous layers. Visually confirm that magnetic beads are well-mixed.
5. A custom sequencing primer is provided for Read 1. Dilute the provided primer to the concentration specified for the sequencer you are using.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Designating Separate Areas

- Conduct all tissue and cell activities in a designated aseptic area that is restricted to cell culture work.
- Conduct all pre-PCR activities (cell lysis, tagmentation, and amplification preparation procedures) in a dedicated environment physically separated from amplified genetic material (post-PCR).
- Do not pass material or equipment from the post-PCR area to the pre-PCR area.
- Consult your local regulations for universal precautions regarding amplicon control practices and biohazardous material handling.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- Use aerosol-resistant pipette tips to reduce the risks of reagent carry-over and sample-to-sample cross-contamination.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Centrifuge steps
  - Thermal cycling steps
- Use 8-strip tube caps to seal plates.

Handling Purification (Magnetic) Beads

This protocol does not include excess Purification Beads (SPB) reagent volume for dispensing from a reservoir and discarding excess volume. Use a single-channel pipette to transfer SPB from the reagent tube to individual sample wells.

- Use beads at room temperature.
- Vortex immediately before use. Confirm that the beads are well-dispersed and the color appears homogeneous.
- Pipette accurate bead volume as this is essential to protocol success:
  - Beads are viscous; pipette beads slowly from stock tube to obtain full volume.

- Remove any additional drops collected on the outside of the tip before dispensing to the sample plate.
- Dispense beads slowly into the sample plate, allowing time to ensure the entire volume has been dispensed from the pipette tip.

- When washing beads:
  - Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air, impacting your results.
  - Use the specified magnet for the plate.
  - Dispense liquid so that beads on the side of the wells are wetted.
  - Keep the plate on the magnet until the instructions specify to remove it.
  - Do not agitate the plate while on the magnetic stand. Do not disturb the bead pellet.
  - If beads are aspirated into the pipette tips, dispense them back to the plate on the magnetic stand and wait a few minutes until the liquid is clear.
  - Do not let the beads dry out.

Preliminary Cell Prep Optimization Guidelines

Before planning your first experiment using the SureCell WTA 3' assay, optimize cell preparation protocol for your sample by following these guidelines. These protocols are applicable to many adherent or suspension cell lines, though preparation from other sources, such as tissue, may require additional changes for success. Additionally, cell dissociation, washing, quantitation, or growth conditions may require further optimization and specific techniques appropriate for the individual cell type or cell source.

- Optimized cell preparation—When performing the SureCell WTA 3' assay for the first time or working with a new cell type, prepare additional cells that can be used to optimize cell dissociation conditions prior to starting a full experiment. Consider these methods when optimizing cell dissociation:
  - Types of trypsin or alternative enzymes used for adherent cell or tissue dissociation
  - Incubation time and temperature for trypsinization or enzyme digestion
  - Intensity of pipetting
  - Types (diameter) of serological pipettes or micropipette tips to be used

CAUTION

- Before starting the protocol, make sure you have the specified cell quantities, consumables, and equipment required to complete the protocol.
- After cells have been prepared, there are no safe stopping points until *Synthesize Second Strand cDNA* has begun. Proceed immediately to each step in the protocol.
- Cells and nuclei must be kept cold on ice at all times but should not be frozen. Do not remove the nuclei from ice until instructed to do so during *Isolate Single Nuclei* on page 15.

The following attributes are critical for the success of the SureCell WTA 3' assay.

- Consistent confluency—Cell confluency can impact the gene expression profile of cells. Start with cell samples at a consistent confluency (between 60–90%) to help ensure reproducible expression profiles from cells prepared on different days or compared between different conditions. Freshly split cell lines one day prior to starting the SureCell WTA 3' workflow and seed several flasks as different confluencies to increase the chances of achieving 60–90% confluency on the day of the experiment.
- High viability (>95%) and integrity of cells—Dead or damaged cells can release nucleic acids into the cell suspension buffer. This background signal from these cells remains through subsequent steps, and may impact the quality of the resulting analysis.
- Accurate nuclei count—Accurate nuclei count is critical to achieve target nuclei throughput and to avoid mutiplets. Size-based gating for automated counters or manual count may be required to avoid counting cell

debris. Nuclei are typically counted on automated cell counters as dead (non-viable) cells due to their small diameter, and should make up >95% of the final loaded sample (viability should be <5%). Both viable and non-viable cells should be included in total cell count.

Library Prep Workflow

Figure 1 SureCell WTA 3' Library Prep Kit for the ddSEQ System Workflow

Prepare, Count, and Assess Viability of Single-Cell Suspension

This section describes preparation of a single-cell suspension, counting of cells, and assessment of cell viability. The SureCell WTA 3' workflow has been successfully tested down to a minimum starting input of 500,000 whole cells, though you may proceed with as few as 100,000 whole cells per cartridge. Expected recovery of nuclei from whole cells is ≥ 50% (typically 70–90%).

After cells have been prepared, there are no safe stopping points until the Second Strand Synthesis has started. Proceed immediately to each step in the protocol.

CAUTION

Delays during cell preparation and handling can lead to sample failure. Make sure you have all required consumables (see *Consumables and Equipment* on page 46) before you begin and always keep samples on ice. Do not stop during or between steps.

Consumables

- BSA (Bovine Serum Albumin)
- PBS (Phosphate-Buffered Saline)
- Cell filter with appropriate pore size to exclude doublet cells
- Trypan blue
- Hemacytometer or slides for cell counting
- Rainin pipettes
  - P200 single channel or P1000 single channel
- [Optional] Trypsin, TrypLE Express, or other enzymatic disaggregation reagent

Guidelines

- Review *Preliminary Cell Prep Optimization Guidelines* on page 5before you begin.

Preparation

1. Prepare 1X PBS + 0.1% BSA and store on ice.
2. Chill a cell filter on ice for at least 5 minutes.

Procedure

1 Dissociate cells as directed in *Preliminary Cell Prep Optimization Guidelines* on page 5 for the cell or tissue type you are using in this assay.

2 If using adherent cells, neutralize the trypsin by adding 4x the volume of completely warmed medium.

3 Wash cells once in cold 1X PBS + 0.1% BSA at a volume sufficient to remove carryover components from the media or dissociation procedure.

4 Centrifuge and resuspend cells in 1 ml of cold 1X PBS + 0.1% BSA.

5 Pipette cell suspension through the chilled cell filter and transfer filtered cells from the cell filter tube to a 15 ml conical tube.
Keep filtered cells on ice until use.

6 Use a microscope or automated cell counter imaging feature to assess cell viability and number.

7 Typically 50-90% of input whole cells are recovered as nuclei after preparation for droplet generation. This translates to a starting cell input requirement of 100,000 cells per chip. The recommended minimum starting cell input for best results is 500,000 whole cells. If the required number of cells is not available after washing and filtering, return to step 1 with additional cells.

8 Keep cells on ice and proceed to *Prepare Cell Lysis and Storage Buffers* on page 9
Single-cell suspension can remain on ice for up to one hour before cell lysis.

Prepare Cell Lysis and Storage Buffers

This section describes preparation of the buffers that are required for preparation of nuclei from whole cells for droplet generation.

Cell lysis buffer breaks down the cell membrane to allow access to the nucleus.

Storage buffer is used to wash the nuclei suspension of any unnecessary debris and provides a proper buffer for droplet generation.

Buffers can be partially prepared up to one day before use. If prepared in advance, leave out IGEPAL CA-630 and SUPERase IN RNase Inhibitor and add to the buffer on the day of use. Store buffers prepared in advance at 4°C.

Consumables

- 1X PBS (Phosphate-Buffered Saline)
- DEPC
- Nuclease-Free Water
- 1M Tris-HCL, pH 7.4
- NaCl
- $MgCl_2$
- IGEPAL CA-630
- SUPERase IN RNase Inhibitor (20 µl) (AM2694)
- BSA (Bovine Serum Albumin)
- Rainin pipettes
  - P20 single channel and P200 single channel (use in *Procedure* on page 14)

Procedure

1 Prepare 1.5 ml of 1X PBS + 1% DEPC per sample type and store on ice.

2 Prepare the cell lysis buffer by combining the following components in a 15 ml conical on ice. Prepare 1.5 ml for each sample type.

Cell Lysis Buffer Component	Final Concentration
Nuclease-free water	n/a
Tris-HCl	10 mM
NaCl	10 mM
$MgCl_2$	3 mM
IGEPAL CA-630	0.1%
SUPERase In RNase Inhibitor	1.0% (0.2U/µl)
BSA	10 mg/ml

3 Pipette mix the cell lysis buffer 10–15 times while on ice, and then centrifuge briefly and store on ice.

NOTE

It is normal to see bubbles after mixing and centrifugation.

4 Prepare 4 ml per sample type of storage buffer by combining the following components in a 15 ml conical on ice. If starting with more than 5 million cells, more buffer may need to be prepared.

Nuclei Storage Buffer Component	Final Concentration
Nuclease-free water	n/a
Tris-HCl	10 mM
NaCl	10 mM
$MgCl_2$	3 mM
SUPERase In RNase Inhibitor	1.0%
BSA	10 mg/ml

5 Pipette mix the nuclei storage buffer 10–15 times while on ice, and then centrifuge briefly and store on ice.

NOTE

It is normal to see bubbles after mixing and centrifugation.

CAUTION

Keep prepared buffers on ice (or refrigerated) when not in use. Improper handling temperatures can lead to sample failure.

Isolate and Prepare Nuclei for Nuclei Suspension

This section describes isolation of nuclei from whole cells, and their preparation and counting. This workflow is optimized for a final suspension concentration of 2500 nuclei/µl. Each cartridge can be loaded with up to four unique samples.

NOTE

For FACS, sorting into into 1X PBS + 0.1% BSA is recommended. If using nuclei that have been FACS sorted, go directly to *Prepare Nuclei and Barcode Suspension Mixes* on page 13.

NOTE

Nuclei are smaller than whole cells, and are counted by automated cell counters as nonviable cells. If using an automatic cell counter, use the nonviable count to estimate the fraction of nuclei versus intact cells. Typical samples prepared with this protocol contain <5% live cells, as measured by Bio-Rad TC20, indicating >95% nuclei.

After nuclei have been prepared, there are no safe stopping points until the Second Strand Synthesis has started. Proceed immediately to each step in the protocol.

CAUTION

Delays during nuclei preparation and handling can lead to sample failure. Make sure that you have all required consumables (see *Consumables and Equipment* on page 46) before you begin and keep samples and buffers on ice. Do not stop during or between steps.

Consumables

- Trypan blue
- Hemocytometer or slides for cell counting
- Rainin pipettes
  - P20 single channel and P200 single channel

Guidelines

- Review *Preliminary Cell Prep Optimization Guidelines* on page 5 before you begin.

Preparation

- Set centrifuge temperature to 4°C.

Procedure

NOTE

This procedure includes multiple wash steps of cells and nuclei. Because nuclei are inherently sticky, this can cause an undesirable loss of nuclei when using pipette mixing to resuspend samples. To avoid this, pulse vortex samples for 1–3 seconds until the pellet is resuspended for the wash steps, especially if starting with a low cell count. Avoid overly vigorous vortexing (high speed, extended amount of time), as this can damage nuclei and can impact sample purity.

1 Centrifuge cells at 300 × g for 3 minutes, then remove supernatant.

2 Resuspend cells in 1 ml of cold lysis buffer by pipette mixing the entire volume 10–15 times, then incubate on ice for 10 minutes.
The cell membrane lyses, leaving intact nuclei behind.

3 Centrifuge at 300 × g for 3 minutes, then wash in 1 ml of cold 1X PBS + 1% DEPC by pulse vortexing samples for 1-3 seconds until the pellet is resuspended.

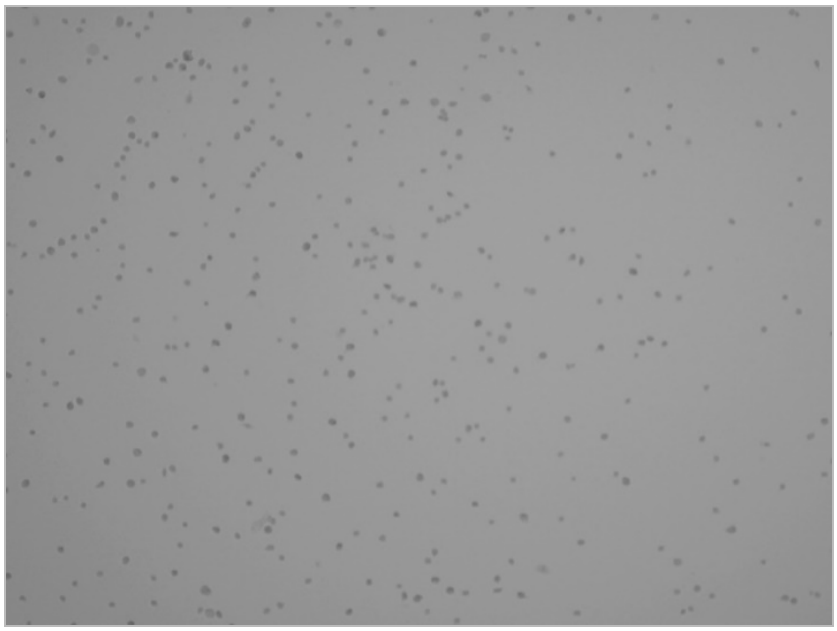
4 Centrifuge at 300 × g for 3 minutes, then wash in 1 ml of cold nuclei storage buffer by pulse vortexing samples for 1-3 seconds until the pellet is resuspended. Repeat step.

5 Centrifuge nuclei at 300 × g for 3 minutes and add an appropriate volume of nuclei storage buffer to achieve a final concentration of at least 2500 cells/µl. The following table provides recommendations for an initial resuspension volume.

Cell Input (cells)	Final Resuspension Volume (µl)
5 M+	1000
3–5 M	500
1–2 M	200
<1 M	100

6 Pipette mix the final nuclei suspension 20 times while on ice to achieve a single nuclei suspension, then use a microscope or automated cell counter imaging feature to assess nuclei isolation, dissociation, and concentration. If nuclei are not dissociated to single-nuclei suspension, pipette mix thoroughly and visualize nuclei again to confirm proper dissociation.

Figure 2 Nuclei with less than 10% doublets in single-nuclei suspension



7 When single nuclei suspension is achieved as shown in Figure 2, dilute the stock nuclei preparation to target 2500 nuclei/µl in nuclei storage buffer. Keep diluted nuclei on ice until use.

> **CAUTION**
>
> Nuclei concentration outside the range of 2250–2750 cells/µl can adversely affect assay performance.

8 Proceed to *Prepare Nuclei and Barcode Suspension Mixes* on page 13 immediately after preparing the single-nuclei suspension. Single-nuclei suspension can be stored on ice for up to 1 hour before loading on to the ddSEQ Single-Cell Isolator.

Prepare Nuclei and Barcode Suspension Mixes

This step prepares suspension mixes that add first strand synthesis components before loading on the ddSEQ Single-Cell Isolator.

Nuclei suspension mix includes all the reagents necessary to perform the first strand synthesis (RT) from the messenger RNA released from the encapsulated nuclei.

Barcode suspension mix contains the barcoded beads and UMI (unique molecular identifier) elements that allow specific tagging of messenger RNA.

> **CAUTION**
>
> Delays during cell preparation and handling can lead to sample failure. Do not stop during or between steps.

Consumables

- Cell Suspend Buffer [red cap]
- DTT (Dithiothreitol) [red cap]
- RNA Stabilizer [red cap]
- RT Enzyme [red cap]
- Enhancer Enzyme [red cap]

- Barcode Buffer [blue cap]
- 3′ Barcode Mix [blue cap]
- Rainin pipettes
  - P20 single channel and P200 single channel (use in *Procedure* on page 14)

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
Cell Suspend Buffer	-25°C to -15°C	Thaw on ice. Vortex vigorously to mix, and then centrifuge briefly.
DTT	-25°C to -15°C	Thaw at room temperature. Vortex to mix, then centrifuge briefly.
RNA Stabilizer	-25°C to -15°C	Gently invert the thawed tubes 3–5 times, and then centrifuge briefly.
RT Enzyme	-25°C to -15°C	Gently invert the thawed tubes 3–5 times, and then centrifuge briefly.
Enhancer Enzyme	-25°C to -15°C	Flick the thawed tubes 3–5 times, and then centrifuge briefly.
Barcode Buffer	-25°C to -15°C	Thaw on ice. Vortex vigorously to mix, and then centrifuge briefly.
3′ Barcode Mix	2°C to 8°C	Gently invert the tube 3–5 times. Keep on ice. Vortex to mix before use.
Encapsulation Oil	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Invert the tube five times to mix. This will be used in *Isolate Single Nuclei* on page 15.
ddSEQ Priming Solution	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex well to mix, then centrifuge briefly. This will be used in *Isolate Single Nuclei* on page 15.

Procedure

1 Create the nuclei enzyme mix by combining the following components (red caps) in a 1.7 ml tube on ice. Pipette mix the nuclei enzyme mix with a P200 single channel pipette 10–15 times while on ice, and then centrifuge briefly.

NOTE

It is normal to see bubbles after mixing and centrifugation.

Nuclei Enzyme Mix Component	Volume (µl) for 1 Cartridge (4 Samples)	Volume (µl) for 2 Cartridges (8 Samples)
Cell Suspend Buffer	60	120
DTT	8	16
RNA Stabilizer	6	12
RT Enzyme	13.2	26.4
Enhancer Enzyme	12	24
Total	99.2	198.4

2 Create the nuclei suspension mix for each cell type by combining the following components in a new 1.7 ml tube on ice. Before adding the single-nuclei suspension to the nuclei enzyme mix, pipette mix the entire volume of the single-nuclei suspension 20 times with an appropriately sized single channel pipette.

a To load four unique cell samples per chamber, make a single sample nuclei suspension mix for each cell type if loading 4 unique samples per chamber.

b To load the same cell sample across all four chambers, make a nuclei suspension mix using the volumes listed for 1 cartridge.

c All four sample chambers must be loaded with nuclei suspension mix. If you choose not to load any cells into a chamber, prepare and load the nuclei suspension mix, substituting an equivalent volume of nuclei storage buffer in place of nuclei.

Nuclei Suspension Mix Component	Volume (µl) per Sample	Volume (µl) for 1 Cartridge (4 Samples)	Volume (µl) for 2 Cartridges (8 Samples)
Nuclei Enzyme Mix	21.5	86	172
Single-nuclei suspension (2500 cells/µl)	4.5	18	36

NOTE

Proceed immediately to the next step. Do not mix the combined components until *Load Cartridge* on page 18.

3 Create the barcode suspension mix by combining the following components (blue caps) in a new 1.7 ml tube on ice. Before combining, resuspend the 3′ Barcode Mix by vortexing for 1 second, repeat 3 times, and immediately add to the Barcode Buffer.

Barcode Suspension Mix Component	Volume (µl) for 1 Cartridge (4 Samples)	Volume (µl) for 2 Cartridges (8 Samples)
Barcode Buffer	60	120
3′ Barcode Mix	60	120

NOTE

Proceed immediately to the next step. Do not mix the combined components until *Load Cartridge* on page 18.

Isolate Single Nuclei

This step uses the ddSEQ Single-Cell Isolator to coencapsulate nuclei (samples) and barcodes in droplets that create a highly parallelized library prep for single-cell analysis.

CAUTION

Delays during nuclei preparation and handling can lead to sample failure. Do not stop during or between steps.

Consumables

- 96-well cooling block
- ddSEQ cartridge
- ddSEQ cartridge holder
- Rainin pipettes
  - P20 single channel and multichannel, P50 multichannel, P200 single channel and multichannel (use in *Prime Cartridge* on page 17, *Load Cartridge* on page 18, and *Transfer Samples* on page 21)
- Encapsulation Oil
- ddSEQ Priming Solution
- Bio-Rad ddPCR plate (Bio-Rad catalog # 12001925)
- 8-tube strip (General Lab Supplier)
- 8-tube strip caps (Bio-Rad, catalog # TCS-0803)

- Multichannel Pipette Reservoir

Guidelines

- Make sure the ddSEQ Single-Cell Isolator is installed according to manufacturer instructions and the power indicator is lit.
- Review pipetting guidelines in the ddSEQ Single-Cell Isolator Instruction Manual.
- Use Rainin pipettes and corresponding tips to load the cartridge. Use of other tips can negatively impact ddSEQ cartridge performance.
- Make sure that the ddSEQ cartridge is in the cartridge holder when loading reagents.
- Avoid static generation while handling encapsulated samples.
  - Work in a clear, static-free area.
  - Do not use latex gloves when making or handling droplets.

About Reagents

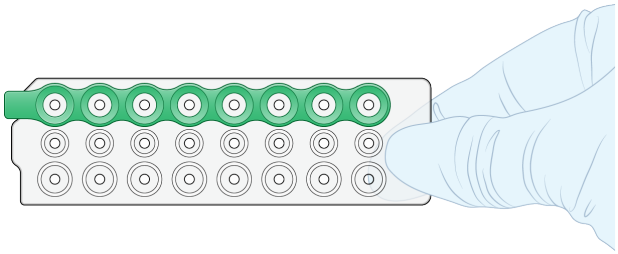
- To avoid bubbles, depress the pipette plunger only to the first stop when loading the cartridge.
- Aspirate and dispense Encapsulation Oil slowly due to the viscosity of the solution.

Preparation

Prepare Cartridge

1 Grip the cartridge by the tab and remove it from the package. Do not touch the wells or gaskets.

Figure 3 ddSEQ Cartridge



2 Insert the cartridge into the cartridge holder.

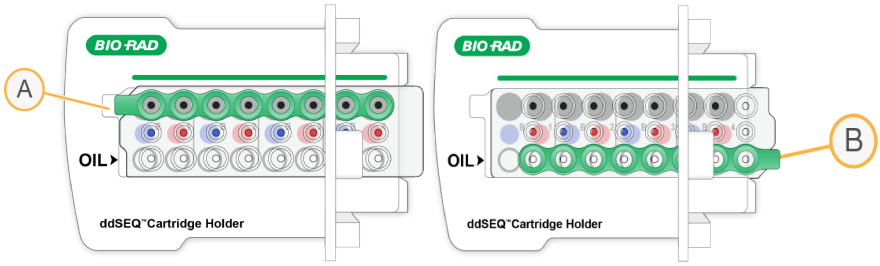
a Lift the cartridge holder lever.

b Orient the green gasket on the cartridge with the green stripe on the cartridge holder, insert the tab under the rails, then slide the cartridge into the holder.

c Check that the cartridge is fully inserted and lying flat against the bottom of the holder, then close the lever. If the lever does not close completely, reinsert the cartridge.

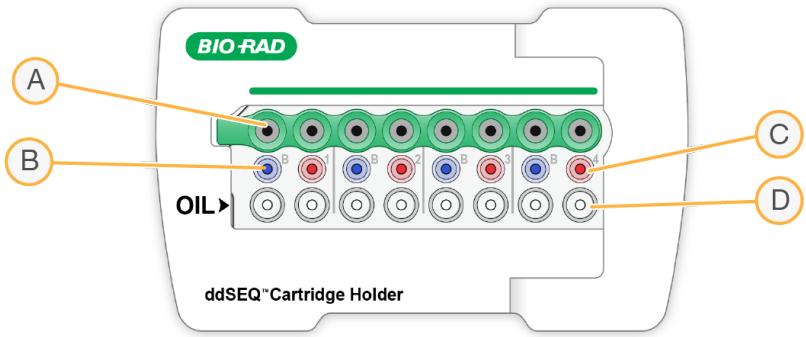
Figure 4 Insert Cartridge Into Cartridge Holder

Figure 5 Incorrectly Assembled Cartridge and Cartridge Holder



A Cartridge not fully inserted
B Cartridge oriented incorrectly

Figure 6 Assembled Cartridge and Cartridge Holder



A Encapsulated sample output wells
B Barcode Suspension Mix input wells (Blue)
C Nuclei Suspension Mix input wells (Red)
D Encapsulation Oil input wells

Procedure

Prime Cartridge

Prime the ddSEQ cartridge to prepare fluidics for single cell isolation.

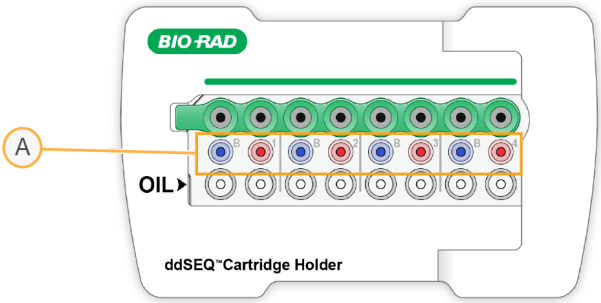
1 Use a P200 single channel pipette to add 25 µl of ddSEQ Priming Solution to each well of an 8-tube strip.

2 Use a P20 multichannel pipette to add 20 µl of ddSEQ Priming Solution from the 8-tube strip to each well of the second row of the cartridge as shown in Figure 7.

NOTE

Use a multichannel pipette to avoid missing wells during cartridge priming.

Figure 7 ddSEQ Priming Solution Wells



A ddSEQ Priming Solution wells

3 Allow the ddSEQ Priming Solution to remain in wells for 1 minute, then remove with a P20 multichannel pipette set to 20 µl.
Do not allow the ddSEQ Priming Solution to remain in wells longer than 3 minutes.

CAUTION

ddSEQ Priming Solution interferes with single cell isolation. Make sure all ddSEQ Priming Solution is removed from the wells.

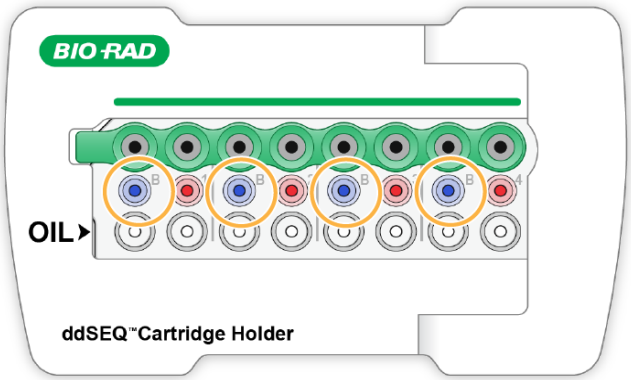
Load Cartridge

Proper mixing of the barcode suspension mix and nuclei suspension mix provides even distribution into encapsulated droplets.

1 Vortex the barcode suspension mix for 1 second, and repeat 3 times.

2 Using a P20 single channel pipette, load20 µl of the barcode suspension mix into the bottom of the **B** ports (Blue). Depress the pipette plunger only to the first stop to avoid bubbles.

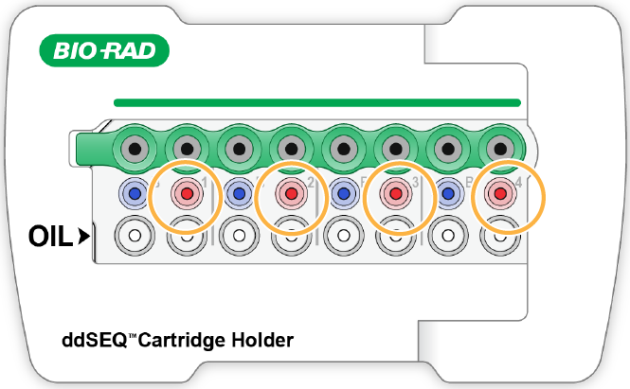
Figure 8 Barcode Suspension Mix Wells



3 Pipette mix the entire volume of the nuclei suspension mix 20 times to create a homogeneous single nuclei suspension.

4 Using a P20 single channel pipette, load 20 µl of cell suspension mix into the bottom of the red ports, numbered 1–4.
Depress the pipette plunger only to the first stop to avoid bubbles.

Figure 9 Cell Suspension Mix Wells



5 Pour the Encapsulation Oil into a multichannel pipette reservoir. Using a P200 multichannel pipette, load 80 µl of Encapsulation Oil into each well of the bottom row of the cartridge labeled OIL.
Depress the pipette plunger only to the first stop to avoid bubbles.

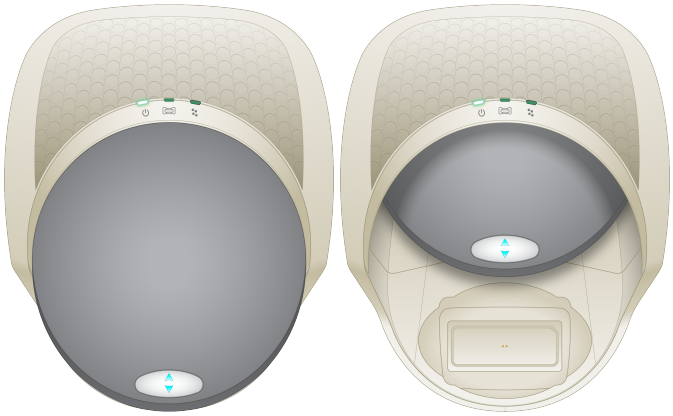
NOTE
One bottle of Encapsulation Oil is enough for 2 cartridges.

6 Keep the loaded cartridge in the cartridge holder for single nuclei isolation on the ddSEQ Single-Cell Isolator.

Generate Single Cell Droplets

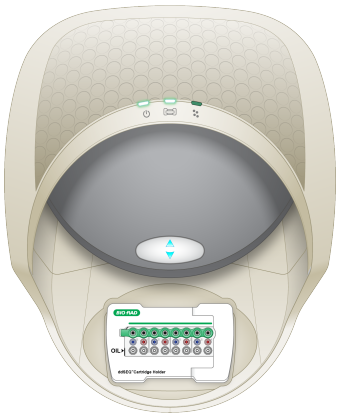
1 Press the silver button on the top of the ddSEQ Single-Cell Isolator to open the instrument.

Figure 10 Bio-Rad ddSEQ Single-Cell Isolator



2 Place the cartridge holder into the instrument. Make sure that the cartridge indicator light is solid green to confirm that the cartridge holder is in the correct position. If the cartridge indicator light is not lit, reseat the cartridge holder on the magnetic plate.

Figure 11 ddSEQ Cartridge Loaded on ddSEQ Single-Cell Isolator



3 Press the silver button on the top of the ddSEQ Single-Cell Isolator to close the instrument.
Single-nuclei isolation begins automatically after the ddSEQ Single-Cell Isolator door is closed and takes approximately 5 minutes. The droplet indicator flashes green to indicate that nuclei isolation is in progress. Single-cell isolation is complete when all three indicator lights are solid green.

CAUTION

Do not proceed until all three indicator lights are solid green.

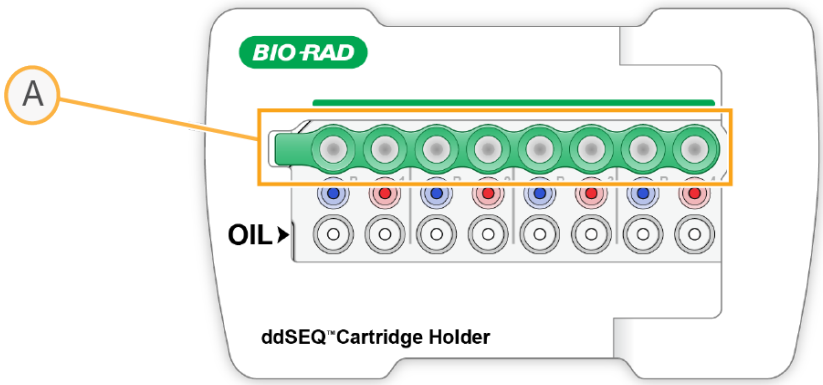
4 Press the silver button on the front of the ddSEQ Single-Cell Isolator to open the instrument.

NOTE

After the door opens, the instrument continues to make noise for ~5 seconds while it resets.

5 Remove the cartridge holder from the ddSEQ Single-Cell Isolator.
Successfully encapsulated samples appear cloudy in the output wells. Check for wells that look clear or empty, as droplet generation may have failed. Note clear or empty wells in the *Lab Tracking Chart* on page 42.

Figure 12 Encapsulated Samples in Output Wells



A Output wells

NOTE

Proceed ***immediately*** to the next step.

Transfer Samples

Encapsulated samples are transferred to a 96-well cooling block and kept cold until starting reverse transcription. Keeping the encapsulated samples cold at this step ensures stability of barcoded RNA and encapsulated droplets.

1 Chill a 96-well plate by securely placing it on a chilled 96-well cooling block.

NOTE

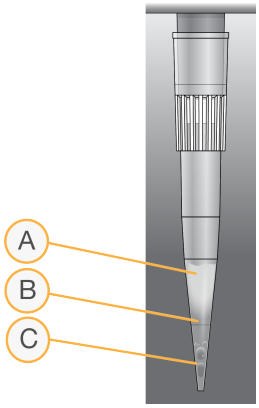
Avoid static generation while handling encapsulated samples.

2 Use a P50 multichannel pipette set at 43 µl to gently and slowly aspirate all encapsulated sample from the output wells.
Fast or harsh pipetting will break the encapsulated samples. Pipette very slowly to avoid yield loss.

CAUTION

Using a single channel pipette to individually transfer encapsulated samples will result in uneven sample volumes.

Figure 13 Emulsion Layers



A Aqueous layer
B Oil layer
C Oil + air bubbles

NOTE

The total emulsion volume transferred to each well is 35–40 µl and ~5 µl of air.

3 Dispense the encapsulated sample as follows.

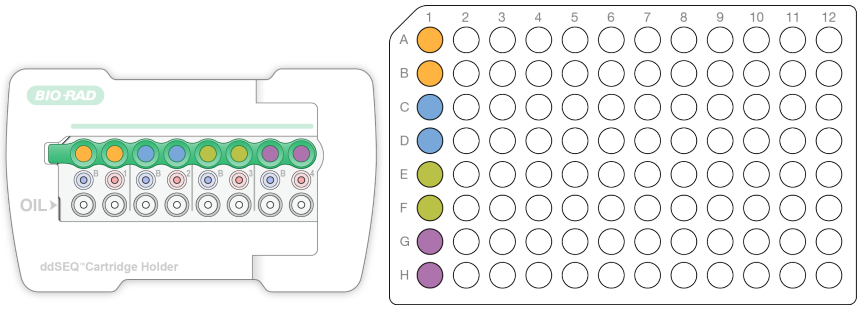
a Very slowly dispense the encapsulated sample into the corresponding column of the plate, as shown in Figure 14. Dispense should take approximately five seconds.

CAUTION

Do not discard tips until all of the encapsulated sample has been transferred to the plate. Discarding tips with sample will result in yield loss.

b Wait five seconds for remaining encapsulated sample to collect at the tip of the pipette.
c Slowly dispense the remaining encapsulated sample into the same column of the plate.

Figure 14 Transferring Encapsulated Samples



4 Cover sample wells using an 8-tube strip cap and keep samples on the 96-well cooling block until *Reverse Transcribe Samples* on page 22.

CAUTION

Plastic plate seals can generate static and impact encapsulated samples. Use 8-tube strip caps to seal wells. Other plate seals may generate static and adversely affect encapsulated samples.

5 If you are processing a second cartridge, proceed to *Prepare Cartridge* on page 16.
Add encapsulated samples from each additional cartridge to a new column of the same 96-well plate.

NOTE

If you are processing more than four cartridges, contact Illumina Technical Support for a modified protocol.

6 If you have finished processing cartridges, proceed to *Reverse Transcribe Samples* on page 22.
Liquid remaining in the input wells after droplet generation is due to flushing sample from the input wells—this is not left over sample.
When removing the cartridge from the cartridge holder, do not invert the cartridge.
Dispose of cartridges according to standard laboratory procedures.

Reverse Transcribe Samples

This step reverse transcribes samples on a thermal cycler.

Guidelines

- Keep the plate on the 96-well cooling block while transporting to the thermal cycler.
- Work in a clear, static-free area, and avoid static generation while handling encapsulated samples.

Preparation

1 Save the following Reverse Transcription (RT) program on a thermal cycler:
   - Choose the preheat lid option and set to 105°C
   - Set the reaction volume to 50 µl
   - 37°C for 30 minutes
   - 50°C for 60 minutes
   - 85°C for five minutes
   - Hold at 4°C

Procedure

1 Place the 96-well plate on the thermal cycler and run the RT program.

> **CAUTION**
> Do not vortex or spin down the plate before placing on the thermal cycler.

2 Remove Purification Beads (SPB) from storage and bring to room temperature for the *Clean Up First Strand Synthesis* on page 24.

Break Emulsion

This step breaks the individual droplets containing barcoded sample cells for further sample processing.

Consumables

- Droplet Disruptor
- Nuclease-free water
- Rainin pipettes
  - P20 single channel and P200 single channel

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
Droplet Disruptor	2°C to 8°C	Vortex 3–5 times immediately before use to mix, then centrifuge briefly. This reagent can be kept at room temperature during use.

Procedure

1 Remove the 96-well plate from the thermal cycler.

> **CAUTION**
> Do not vortex or spin down the plate after removing it from the thermal cycler.

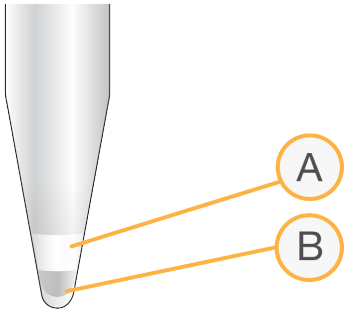
2 Visually examine the samples which should all have equal volumes. Each sample has 2 distinct layers, an oil layer on the bottom and an aqueous layer on top. Note if any wells have only one layer in the *Lab Tracking Chart* on page 42.

3 Remove the 8-tube strip caps carefully to avoid cross-sample contamination.

4 Add 20 µl of Droplet Disruptor by dispensing slowly against the side of the well above each sample.
Do not mix or pipette Droplet Disruptor into the sample.

5 Wait 30 seconds, then add 100 µl of water by dispensing against the side of the well above each sample.
Do not mix or pipette water into the sample.

Figure 15 Sample Emulsion Layers



A Emulsion layer
B Oil layer

NOTE

Proceed ***immediately*** to the next step.

Clean Up First Strand Synthesis

This step uses Purification Beads (SPB) to purify the first strand product (library cDNA), provides a selection step that removes short fragments (unbound barcodes), and combines the two output volumes from each sample into a single well.

Consumables

- Resuspension Buffer (RSB)
- Purification Beads (SPB)
- Freshly prepared 80% ethanol (EtOH)
- Pipettes
  - P20 single channel and P200 single channel and multichannel
- 96-well plate seal

Equipment

- Magnetic peg stand (Thermo Fisher, catalog # AM10027)
- DynaMag 96 Side Magnet (Thermo Fisher, catalog # 12331D) or the DynaMag 96 Side Skirted Magnet (Thermo Fisher catalog # 12027)

About Reagents

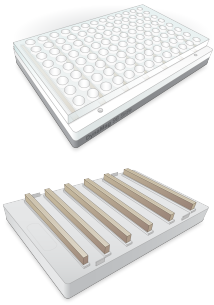
- See *Handling Purification (Magnetic) Beads* on page 4 for details about working with Purification Beads (SPB).

About Samples

- Sample wells contain separate oil and aqueous layers during this step. When mixing, mix only in the specified layer.

About Magnets

- This procedure uses two types of magnetic stands. Both are needed in this protocol and are not interchangeable.

Magnet Type	Illustration	Step Procedure
Magnetic Peg Stand		Use this magnet with *Bind* on page 25 and *Wash* on page 26.
DynaMag 96 Side Magnet or DynaMag 96 Side Skirted Magnet		Use either DynaMag magnet with *Elute* on page 27.

Guidelines

- Use a single channel pipette to transfer Purification Beads (SPB) to sample wells. Using a multichannel pipette reservoir and a multichannel pipette results in inadequate Purification Beads (SPB) reagent volume needed to complete this protocol.
- This process requires both a magnetic peg stand and either DynaMag side magnet.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
Resuspension Buffer (RSB)	2°C to 8°C	Can be used after removing from 2°C to 8°C. Do not discard until the protocol is complete.
Purification Beads (SPB)	2°C to 8°C	Let stand for 15 minutes to bring to room temperature.

2 Prepare fresh 80% ethanol from absolute ethanol.

Procedure

Bind

1 Vortex Purification Beads (SPB) until well-dispersed.

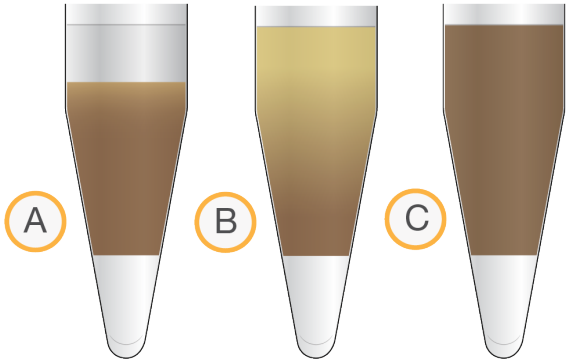
2 Use a P200 single channel pipette to add 90 µl Purification Beads (SPB) to the samples by dispensing slowly above the aqueous layer without mixing. Do not dispense into the oil layer at the bottom of the well.

3 Use a P200 single channel pipette, set to 50 µl, to pipette mix Purification Beads (SPB) ***in the aqueous layer only*** until the layer is evenly distributed (10-15 times).
After mixing, the samples have two distinct layers: an oil layer on the bottom of the well and a homogeneous brown aqueous layer on the top.

4 Lift the plate to examine the quality of mix for the aqueous layer closely.

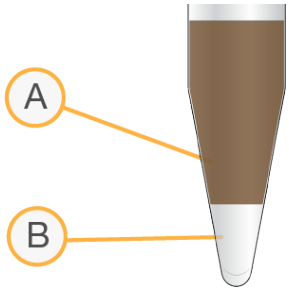
> **CAUTION**
> The aqueous layer should not appear clear at the top. If parts of the aqueous layer still appear clear or a lighter brown, continue to mix until the entire aqueous layer is homogenously brown.

Figure 16 Mixing States From Initial State to Properly Mixed State



A Initial state with a clear aqueous layer at the top
B Not properly mixed, indicated by a lighter brown aqueous layer at the top
C Properly mixed with an entirely homogenous brown aqueous layer

Figure 17 Mixed Aqueous Layer and Oil Layer



A Mixed aqueous layer
B Oil layer

5 Incubate at room temperature for 10 minutes.

6 Place on a magnetic peg stand and wait 10 minutes. Use a magnetic peg stand until *Elute* on page 27.

NOTE

The liquid might not be completely clear of beads due to retention of beads in the aqueous and oil layers.

7 Use a P200 single channel pipette, set to 200 µl, to remove and discard all supernatant from each well. Use a fresh pipette tip to go into the well again to discard approximately 20–30 µl more of supernatant.

Wash

1 Wash 2 times as follows.

a Add 200 µl freshly prepared 80% EtOH to each well.
b Incubate on the magnetic peg stand for 30 seconds.
c Remove and discard all supernatant from each well.
d Repeat steps a - c to wash again.

2 Seal the plate and centrifuge at 280 × g for 10 seconds to bring down any ethanol or liquid remaining on sides of wells.

3 Place on a magnetic peg stand and wait 30 seconds.

4 Use a P20 single channel pipette to remove residual 80% EtOH from each well.

5 Air-dry on the magnetic peg stand for 5 minutes.

Elute

1 Remove the sample plate from the magnetic peg stand.

2 Use a P200 single channel pipette to add 35 µl Resuspension Buffer (RSB) to each sample well. Pipette to mix, making sure all beads are resuspended.

CAUTION

Yield loss can occur if beads are not thoroughly resuspended.

3 Incubate at room temperature for 2 minutes.

4 Seal the plate and centrifuge at 280 × g for 10 seconds.

5 Place on a DynaMag 96 side magnet and wait 2 minutes.

NOTE

You may proceed to the next step even if the solution is not completely clear after 2 minutes.

Combine Wells From Sample and Transfer

1 Using a P200 single channel pipette, combine the 2wells for each sample into a single well by transferring 34 µl of supernatent from each sample well to a new plate, as follows. Keep the sample plate on the DynaMag 96 side magnet during this step.

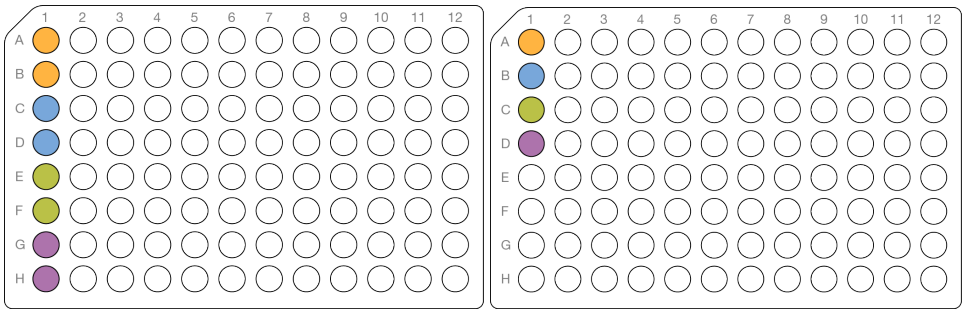
- Sample 1, rows A—B to row A of the corresponding column in the new plate.
- Sample 2, rows C—D to row B of the corresponding column in the new plate.
- Sample 3, rows E—F to row C of the corresponding column in the new plate.
- Sample 4, rows G—H to row D of the corresponding column in the new plate.

After transferring, the total volume of supernatant in each well of the new plate is 68 µl.

CAUTION

Each pair of sample wells in succession represent ***one*** sample. Proper pooling is critical for library prep indexing and sample processing.

Figure 18 Combining Barcoded Samples



NOTE

Proceed ***immediately*** to the next step.

Synthesize Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand to generate double stranded cDNA.

Consumables

- Second Strand Buffer (SSB)
- Second Strand Enzyme (SSE)
- Pipettes
  - P20 single channel and P200 multichannel

About Reagents

- Second Strand Enzyme (SSE) is viscous and pipettes slowly. Ensure that the specified volume is obtained.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
Second Strand Buffer (SSB)	-25°C to -15°C	Thaw on ice. Vortex to mix, and then centrifuge briefly. Keep on ice until use.
Second Strand Enzyme (SSE)	-25°C to -15°C	Thaw on ice. Pipette mix and then centrifuge briefly. Keep on ice until use.

2 Save the following Second Strand Synthesis (SSS) program on the thermal cycler:
- Turn off the heated lid function
- Set the reaction volume to 80 µl
- 16°C for 2 hours
- Hold at 4°C

Procedure

1 Prepare Second Strand Synthesis Master Mix by adding the following to a 1.7 ml tube on ice. Pipette 10 times to mix.

Second Strand Synthesis Component	Volume (µl) for 1 Cartridge (4 Samples)	Volume (µl) for 2 Cartridges (8 Samples)
Second Strand Buffer (SSB)	36	72
Second Strand Enzyme (SSE)	18	36

2 Using a P20 single channel pipette, add 12 µl of Second Strand Master Mix to each sample well.

3 Using a P200 multichannel pipette set to 40 µl, pipette to thoroughly mix each sample well.

4 Seal the plate and centrifuge at 280 × g for 10 seconds.

5 Place on the preprogrammed thermal cycler and run the Second Strand Synthesis (SSS) program.

SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 4°C overnight or store at -25°C to -15°C for up to two days.

Clean Up cDNA

This process uses Purification Beads (SPB) to purify the library DNA and provides a selection step that removes short library fragments.

Consumables

- Resuspension Buffer (RSB)
- Purification Beads (SPB)
- Freshly prepared 80% ethanol (EtOH)
- Pipettes
  - P20 single channel and P200 single channel and multichannel
- 96-well plate seal

Equipment

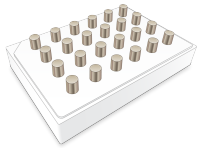
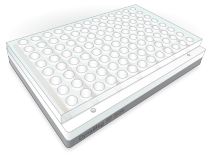
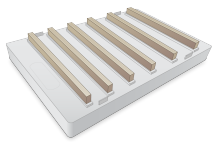
- Magnetic peg stand (Thermo Fisher, catalog # AM10027)
- DynaMag 96 Side Magnet (Thermo Fisher, catalog # 12331D) or the DynaMag 96 Side Skirted Magnet (Thermo Fisher catalog # 12027)

About Reagents

- See *Handling Purification (Magnetic) Beads* on page 4 for details about working with Purification Beads (SPB).

About Magnets

- This procedure uses two types of magnetic stands. Both are needed in this protocol and are not interchangeable.

Magnet Type	Illustration	Step Procedure
Magnetic Peg Stand		Use this magnet with *Bind* on page 30 and *Wash* on page 30.
DynaMag 96 Side Magnet or DynaMag 96 Side Skirted Magnet	 	Use either DynaMag magnet with *Elute* on page 30.

Guidelines

- Use a single channel pipette to transfer Purification Beads (SPB) to sample wells. Using a multichannel pipette reservoir and a multichannel pipette results in inadequate Purification Beads (SPB) reagent volume needed to complete this protocol.

- This process requires both a magnetic peg stand and either DynaMag side magnet.

Preparation

1 Prepare the following consumables:

Item	Storage	Instructions
Resuspension Buffer (RSB)	2°C to 8°C	Do not discard until the protocol is complete.
Purification Beads (SPB)	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% ethanol from absolute ethanol.

Procedure

Bind

1 Centrifuge sample plate at 280 × g for 30 seconds.

2 Vortex Purification Beads (SPB) until well-dispersed.

3 Use a P200 single channel pipette to add 44 µl Purification Beads (SPB) to each sample well. Pipette mix until evenly distributed (10–15 times).

4 Incubate at room temperature for 5 minutes.

5 Place on a magnetic peg stand until the liquid is clear (~5 minutes). Use a magnetic peg stand until *Elute* on page 30.

6 Use a P200 pipette, set to 120 µl, to remove and discard all supernatant from each well.

Wash

1 Wash 2 times, as follows.

   a Add 200 µl freshly prepared 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

2 Air-dry on the magnetic peg stand for 5 minutes.

3 Using a P20 pipette, remove residual 80% EtOH from each well.

Elute

1 Remove from the magnetic peg stand.

2 Using a P20 single channel pipette, add 11 µl Resuspension Buffer (RSB) to each sample well. Pipette to mix, making sure all beads are resuspended.

> **CAUTION**
> Yield loss can occur if beads are not thoroughly resuspended.

3 Incubate at room temperature for 2 minutes.

4 Seal the plate and centrifuge at 280 × g for 10 seconds to bring entire solution to the bottom of the well.

5 Place on a DynaMag 96 side magnet and wait until the liquid is clear (~2 minutes).

6 Transfer 10 µl of supernatant from each sample well to a new sample well of a 96-well plate.

Check Libraries

Perform the following procedure for quality control analysis on your sample library. cDNA yields at > 2 ng are sufficient to proceed to *Tagment cDNA* on page 31. Yields lower than > 2 ng will result in lower final library yields insert size due to overtagmentation. Expected cDNA yields for PBMC samples are in the 2–3 ng range.

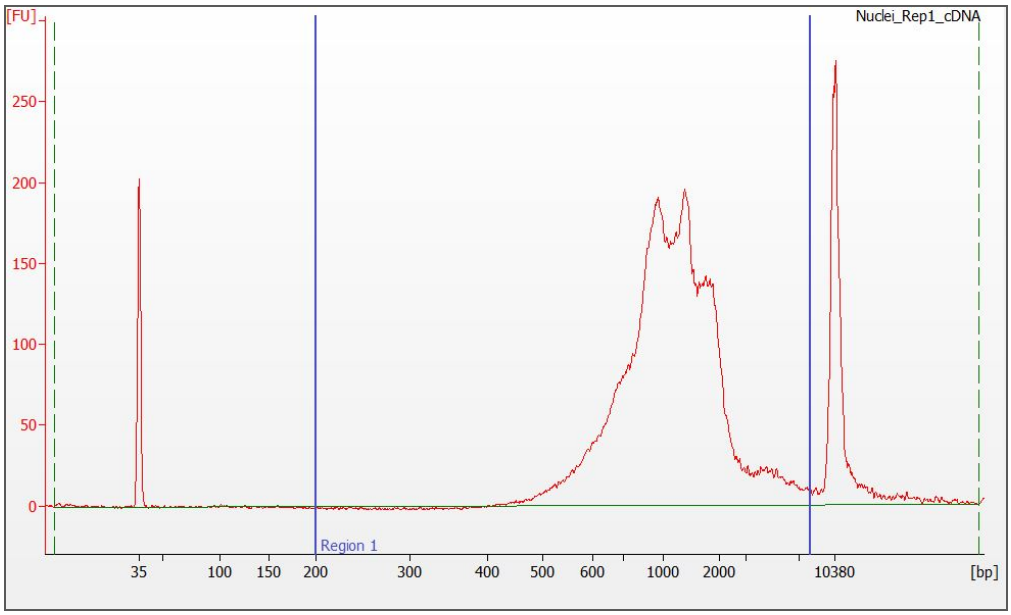
1 Run 1 µl of undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip.

2 Drag the blue regions to capture the 200-8000 bp range.
Typical libraries have sizes ranging from ~400–8000 bp, with a trace profile similar to Figure 19.

> **CAUTION**
> Lower cDNA yields will lead to suboptimal library preparation in subsequent steps and is not recommended.

3 Record the cDNA library fragment size and cDNA yield. See *Lab Tracking Chart* on page 42.
An example of the resulting cDNA prepared using this protocol is shown in Figure 19.

Figure 19 Example of Bioanalyzer cDNA Product



Tagment cDNA

This step uses the Nextera SureCell transposome to tagment cDNA, which is a process that simultaneously fragments and tags DNA with adapter sequences in a single step.

Consumables

- Tagment Buffer (TCB)
- Tagment Enzyme (TCE)
- Tagment Stop Buffer (TSB)
- 96-well plate seal
- Pipettes
  - P20 single channel and P200 single channel

Preparation

1 Prepare the following consumables.

Item	Storage	Volume (µl)
Tagment Buffer (TCB)	-25°C to -15°C	Thaw on ice. Vortex to mix, and then centrifuge briefly.
Tagment Enzyme (TCE)	-25°C to -15°C	Thaw on ice. Gently invert the thawed tubes 3–5 times, and then centrifuge briefly.
Tagment Stop Buffer (TSB)	15°C to 30°C	Check for precipitates. If present, vortex until all particulates are resuspended.

2 Save the following Tagmentation Program (TGM) on the thermal cycler.
- Choose the preheat lid option (105 degrees)
- Set the reaction volume to 40 µl
- 55°C for 5 minutes
- Hold at 4°C

Procedure

1 Prepare Tagmentation Mix in a 1.7 ml tube on ice as follows. Pipette 10 times to mix.

Tagmentation Mix Component	Volume (µl) for 1 Cartridge (4 Samples)	Volume (µl) for 2 Cartridges (8 Samples)
Tagment Buffer (TCB)	88	176
Tagment Enzyme (TCE)	44	88

2 Add 30 µl of Tagmentation Mix to each sample well. Mix with pipette.

3 Seal the plate and centrifuge at 280 × g for 10 seconds.

4 Place on the preprogrammed thermal cycler and run the TGM program.

5 Remove the plate from the thermal cycler as soon as the temperature reaches 4° C. Do not leave the PCR plate on the thermal cycler for longer than 6 minutes.

6 Remove the seal carefully to avoid cross-sample contamination.

7 Use a P20 pipette to add 10 µl of Tagment Stop Buffer to each well. Pipette to mix with a P200 pipette.

8 Seal the plate and centrifuge at 280 × g for 10 seconds.

9 Incubate at room temperature for 5 minutes.

Amplify Tagmented cDNA

This step uses a 15-cycle PCR program to amplify tagmented cDNA and add DNA adapters required for cluster generation. To ensure that your libraries produce high-quality sequencing results, use the specified number of PCR cycles.

Consumables

- Tagmentation PCR Mix (TPM)
- Tagment PCR Adapter (TPP1)
- DNA Adapters (N7XX)
- 96-well plate seal

- Pipettes
  - P20 single channel and P200 single channel and multichannel

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
DNA Adapters	-25°C to -15°C	Only remove adapters being used. Thaw at room temperature for 20 minutes. Vortex to mix, and then centrifuge briefly.
Tagmentation PCR Mix (TPM)	-25°C to -15°C	Thaw on ice. Gently invert the thawed tubes 3–5 times, and then centrifuge briefly.
Tagment PCR Adapter (TPP1)	-25°C to -15°C	Thaw at room temperature for 20 minutes. Vortex to mix, and then centrifuge briefly.

2 Save the following Library Amplification (LA) program on the thermal cycler:
- Choose the preheat lid option and preheat to 105°C
- Set the reaction volume to 100 µl
- 95°C for 30 seconds
- 15 cycles of:
  - 95°C for 10 seconds
  - 60°C for 45 seconds
  - 72°C for 60 seconds
- 72°C for 5 minutes
- Hold at 4°C

Procedure

1 Arrange the DNA Adapters in a tube rack. Use a different index for each sample well. Record the DNA Adapter index used for each sample well.
This information will be required when setting up the sequencing run.

2 Using a P200 single channel pipette, add 30 µl of Tagmentation PCR Mix (TPM) to each of the tagmented samples.

3 Using a P20 single channel pipette, add 10 µl of Tagment PCR Adapter (TPP1) to each of the tagmented samples.

4 Using a P20 single channel pipette, add 10 µl of each DNA Adapter to each tagmented sample.
DNA Adapters are 1 time use and do not require new orange caps.

5 Use a P200 multichannel pipette to mix 10–15 times.

6 Seal the plate and centrifuge at 280 × g at 20°C for 30 seconds.

7 Place on the preprogrammed thermal cycler and run the LA program.

SAFE STOPPING POINT

If you are stopping, leave the plate on the thermal cycler at 4°C overnight or store at -25°C to -15°C for up to two days.

Clean Up Libraries

This process uses Purification Beads (SPB) to purify the library DNA and provides a selection step that removes short library fragments.

Consumables

- Resuspension Buffer (RSB)
- Purification Beads (SPB)
- Freshly prepared 80% ethanol (EtOH)
- Pipettes
  - P20 single channel and P200 single channel and multichannel
- 96-well plate seal

Equipment

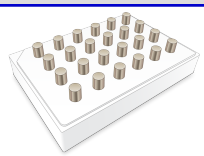
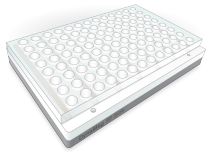
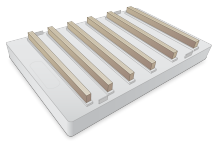
- Magnetic peg stand (Thermo Fisher, catalog # AM10027)
- DynaMag 96 Side Magnet (Thermo Fisher, catalog # 12331D) or the DynaMag 96 Side Skirted Magnet (Thermo Fisher catalog # 12027)

About Reagents

- See *Handling Purification (Magnetic) Beads* on page 4 for details about working with Purification Beads (SPB).

About Magnets

- This procedure uses two types of magnetic stands. Both are needed in this protocol and are not interchangeable.

Magnet Type	Illustration	Step Procedure
Magnetic Peg Stand		Use this magnet with *Bind* on page 35 and *Wash* on page 35.
DynaMag 96 Side Magnet or DynaMag 96 Side Skirted Magnet	 	Use either DynaMag magnet with *Elute* on page 35.

Guidelines

- Use a single channel pipette to transfer Purification Beads (SPB) to sample wells. Using a multichannel pipette reservoir and a multichannel pipette results in inadequate Purification Beads (SPB) reagent volume needed to complete this protocol.
- This process requires both a magnetic peg stand and either DynaMag side magnet.

Preparation

1 Prepare the following consumables.

Item	Storage	Instructions
Resuspension Buffer (RSB)	2°C to 8°C	Do not discard until the protocol is complete.
Purification Beads (SPB)	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% ethanol from absolute ethanol.

Procedure

Bind

1 Centrifuge sample plate at 280 × g for 30 seconds.

2 Vortex Purification Beads (SPB) until well-dispersed.

3 Using a P200 single channel pipette, add 58 µl of Purification Beads (SPB) to each sample well. Pipette to mix, making sure that all beads are resuspended.

4 Incubate at room temperature for 5 minutes.

5 Place on a 96-well magnetic peg stand until the liquid is clear (~5 minutes). Use a magnetic peg stand until *Elute* on page 35.

6 Remove and discard all supernatant from each well.

Wash

1 Wash 2 times, as follows.

   a Add 200 µl freshly prepared 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

2 Using a P20 pipette, remove residual 80% EtOH from each well.

3 Air-dry on the magnetic peg stand for 5 minutes.

Elute

1 Remove from the magnetic peg stand.

2 Using a P200 pipette, add51 µl of Resuspension Buffer (RSB) to each sample well. Pipette mix until beads are thoroughly resuspended.

3 Incubate at room temperature for 2 minutes.

4 Apply the seal and centrifuge at 280 × g for 10 seconds to bring entire solution to the bottom of the well.

5 Place on a DynaMag 96 side magnet until the liquid is clear (~2 minutes). Use a DynaMag 96 side magnet until *Second Bind* on page 35.

6 Transfer 50 µl of supernatant from each sample well to a new sample 96-well plate.

Second Bind

1 Vortex Purification Beads (SPB) until well-dispersed.

2 Add 30 µl of Purification Beads (SPB) to each sample well. Use a P200 to pipette until evenly distributed (10–15 times).

3 Incubate at room temperature for 5 minutes.

4 Place on a magnetic peg stand until the liquid is clear (~5 minutes). Use a magnetic peg stand until *Second Elute* on page 36.

5 Remove and discard all supernatant from each well.

Second Wash

1 Wash 2 times, as follows.

   a Add 200 µl freshly prepared 80% EtOH to each well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

2 Using a P20 pipette, remove residual 80% EtOH from each well.

3 Air-dry on the magnetic peg stand for 5 minutes.

Second Elute

1 Remove the 96-well plate from the magnetic peg stand.

2 Add Resuspension Buffer (RSB) to each sample well. Using a P200, pipette to mix until beads are resuspended.

3 Incubate at room temperature for 2 minutes.

4 Seal the plate and centrifuge at 280 × g for 10 seconds to bring entire solution to the bottom of the well.

5 Place on a DynaMag 96 side magnet until the liquid is clear (~2 minutes).

6 Transfer of supernatant from each sample well to a new 96-well plate.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to seven days.

Assess Libraries

Accurately quantify DNA libraries to ensure optimum cluster densities on the flow cell.

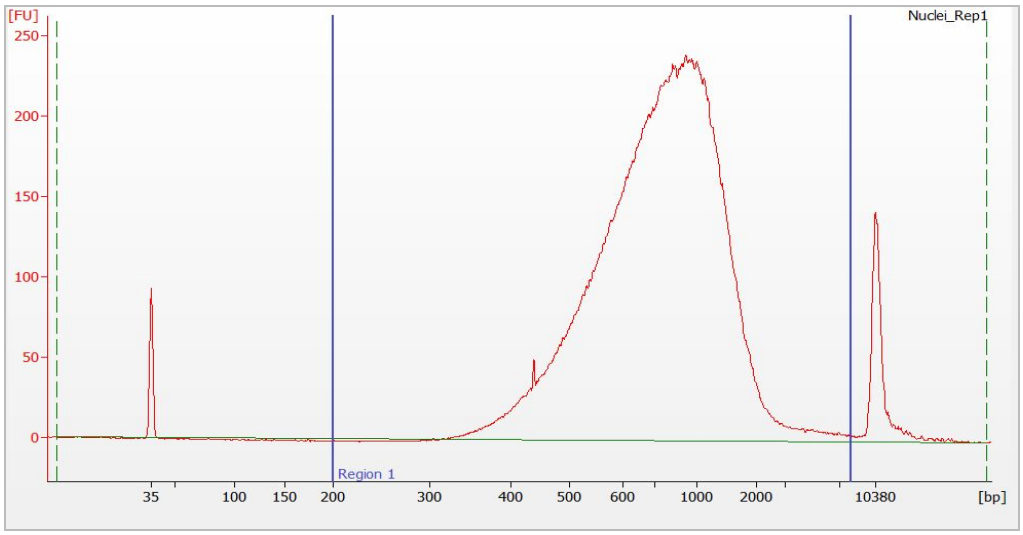
1 Run 1 µl undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip.

2 Determine the concentration of the library using the Agilent Technology 2100 Bioanalyzer.

3 Select the **Region Analysis** tab.

4 Drag the blue region lines to capture the 200–8000 bp region. Record the final library fragment size and final library yield. See *Lab Tracking Chart* on page 42.
The following figure shows an example trace of a successfully sequenced library. Typical libraries show a broad size distribution ~300–1000 bp. A wide variety of libraries can be sequenced with average fragment sizes as small as 450 bp or as large as 1200 bp.

Figure 20 Sample Library Size Distributions



5 Expected library yield is 2–10 nM. Depending on actual library yield, normalize samples to 2 nM.

Prepare for Sequencing

Use the SureCell Sequencing Primer for your sequencing runs. The Sequencing Primer (SP) is concentrated at 50 µM and must be diluted according to the custom sequencing primer documentation for your instrument. See *Additional Resources* on page 1.

Confirm that Illumina Experiment Manager v1.13 or later is used to set up the sequencing sample sheet if the BaseSpace Prep Tab is not used. Version 1.13 or later has the appropriate UMI settings and index sequences for sample demultiplexing. If demultiplexing outside of BaseSpace Sequence Hub, confirm that bcl2fastq v2.18 or later is used for FASTQ generation.

NOTE

SureCell Sequencing Primer is compatible with this library and PhiX only.

Consumables

- Sequencing Primer (SP) (50 µM)
- [Optional] PhiX Control v3

Custom Primer Guides

- *NovaSeq System Custom Primers Guide (document # 1000000022266 v00)*
- *NextSeq System Custom Primers Guide (document # 15057456)*
- *HiSeq System Custom Primers Guide (document # 15061846)*
- *MiSeq System Custom Primers Guide (document # 15041638)*
- *MiniSeq System Custom Primers Guide (document # 1000000002700 v01)*

Modify SureCell Loading Concentrations

Use this table to help you determine your SureCell loading concentration. Loading concentrations presented in this tabled are based on Bioanalyzer quantification. If you are quantifying with another method, you may need to optimize the loading concentration.

Instrument	Resource	Loading Concentration
NextSeq	*System Custom Primers Guide (document # 15057456)*	3 pM
HiSeq 2500	*System Custom Primers Guide (document # 15061846)*	18–20 pM
HiSeq 3000/4000	*System Custom Primers Guide (document # 15061846)*	350–400 pM
MiSeq	*System Custom Primers Guide (document # 15041638)*	25–32 pM
MiniSeq	*System Custom Primers Guide (document # 1000000002700)*	Not provided
NovaSeq	*System Custom Primers Guide (document # 1000000022266)*	Not provided

Sequence in Your Lab

1 Follow the instructions for using custom primers for a sequencing run on your instrument. See *Additional Resources* on page 1.

CAUTION

For NextSeq runs connected to BaseSpace Sequence Hub, select the SureCell WTA 3′ Library Prep kit during Prep Tab setup to ensure that the Custom Primer R1 option is automatically selected on the Planned Runs screen. This option ***must be selected*** or the sequencing run fails.

NOTE

[Optional] Add a 1% PhiX control spike-in as a positive control for alignment and error rate calculations. For more information, see the PhiX Control v3 support page on the Illumina website.

Sequence Using an Outside Lab

1 Consult with your sequencing lab about dilution of Sequencing Primer (SP) and dilute accordingly.

2 Send Sequencing Primer (SP) with the quantified libraries.
The lab adds the Sequencing Primer (SP) to the appropriate sequencing reagents for the Illumina instrument used for sequencing.

3 [Optional] Add a 1% PhiX control spike-in as a positive control for alignment and error rate calculations. For more information, see the PhiX Control v3 support page on the Illumina website.

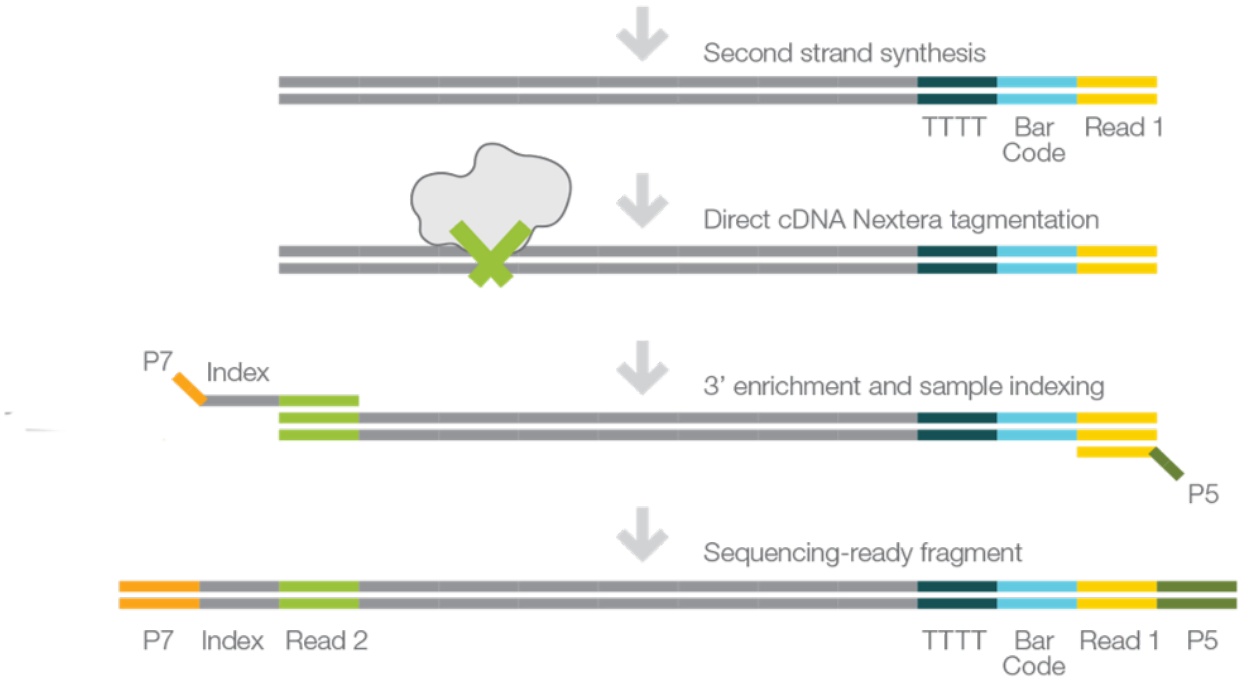
Supporting Information

Introduction

The protocol described in this guide assumes that you have reviewed the contents of this section, confirmed workflow contents, and obtained all required consumables and equipment.

How Does the SureCell WTA 3' Assay Work?

Single cells or nuclei are individually partitioned into subnanoliter droplets on a disposable cartridge using the Bio-Rad ddSEQ™ Single-Cell Isolator. Cell lysis and cell barcoding of mRNA transcripts takes place in each droplet during reverse transcription. Then droplets are disrupted, and the barcoded cDNA is pooled for second strand synthesis in bulk. Double-stranded cDNA is tagmented by Nextera SureCell transposome to add primer binding sites for subsequent indexing and amplification by PCR. Final libraries are purified and ready for sequencing on Illumina sequencing platforms.



Verified Protocol: Mixed Species Control

Introduction

This is a verified protocol for culturing and preparing HEK-293 (human) and NIH-3T3 (mouse) cell lines for analysis as a mixed species cell suspension. This protocol can be used as a control in parallel with other samples. It also enables the assessment of doublet cells by detecting mixed-species crosstalk. Upon completion of the protocol, cells are ready to begin step 9 in *Prepare, Count, and Assess Viability of Single-Cell Suspension* on page 8.

Cell Lines and Culture Media

Cell Line	Species	Supplier	Catalog Number	Culture Conditions
HEK-293	Human	ATCC®	CRL-1573	EMEM + 10 % FBS + antibiotic
NIH-3T3	Mouse	ATCC®	CRL-1658	DMEM + 10 % FBS + antibiotic

List of Materials

- Complete Growth Medium based on the ATCC recommendation for the respective cell type and stored at 4°C. Complete Growth Medium needs to be warmed up to 37°C before the cell handling and preparation.
- PBS + 0.1% BSA solution; this solution needs to be stored at 4° C at all times. While performing cell dissociation, the solution needs to be stored on ice. If working under a hood, take out a large volume from the refrigerator right before use.
- PBS is stored at room temperature and can be used as is.
- Corning Life Sciences Cell Strainer CP ST 5ML 500/CS (catalog # 352235)
- Trypan Blue Solution 0.4%
- Bio-Rad TC20
- Bio-Rad TC20 counting slides, dual chamber for cell counter
- VWR Analog Vortex Mixer
- Pipettes and pipette tips
- Microscope, hood, centrifuge, standard cell culture lab equipment
- TypLE Express

Cell Dissociation Protocol

Follow these steps in parallel to perform cell dissociation on both the HEK-293 and NIH-3T3 cell lines.

1. Warm the required media from the refrigerator to 37° C (approximately 30 minutes).
2. Remove the flask from the incubator and check the cells under the microscope. Confluency should be between 60–90%.
3. Carefully remove all the media without touching the surface of the flask.
4. Add PBS to briefly rinse attached cells and discard the PBS.
5. For a regular T-75 flask, add 2.5 ml of Trypsin-EDTA to cover the entire surface of the flask.
6. Place flask in the incubator for between 3 - 5 minutes; remove the flask and check to see if cells have detached or are starting to detach.

> **CAUTION**
> Do not tap the flask to detach cells as this may increase the chance of cell aggregation.

7 Add 1 ml of warm medium to the flask. Using a P1000 pipette, break the cell clumps by pipetting up and down 10–15 times.

8 Add 9 ml of media to the flask to neutralize the TrypLE Express.

Cell Counting Protocol

Follow these steps in parallel to perform cell counting on both the HEK-293 and NIH-3T3 cell lines.

Assess concentration and viability of the PBMC suspension after the washes and final resuspension in PBS+0.1% as described in *Prepare, Count, and Assess Viability of Single-Cell Suspension* on page 8.

1 Transfer each cell suspension into a Falcon tube. Centrifuge at 250 × g for three minutes.

2 Discard supernatant. Suspend the cells in 1 mL cold PBS + 0.1% BSA. With a P1000 pipette, break the cell clumps by pipetting up and down 10-15 times. Then add 9 ml of cold PBS + 0.1% BSA to bring the volume to 10 ml. Spin at 250 × g for three minutes.

> **NOTE**
> Keep the cell suspension on ice for the remainder of the protocol.

3 Discard supernatant. Suspend cells in 1 ml of cold PBS + 0.1% BSA to reach an approximate cell concentration above 2500 c/µl. (Estimated volume range of 250–1500 µl).

4 Dissociate cells by pipetting vigorously (at least 40 times) and then filter cells through the 35 µm cell strainer to remove cell aggregates.

5 Perform cell viability measurement as follows:

a Vortex the cell tube for a few seconds.
b Use a P20 pipette to take 10 µl from the middle of the cell suspension, and then add 10 µl of trypan blue 0.4%.
c Pipette 10 times to mix, then load onto Chamber A of the Bio-Rad TC20 counting slides.
d Measure the cell viability (should be ≥ 95%).

> **NOTE**
> Make sure the cell suspension is stored on ice during this step.

6 Perform cell concentration measurement as follows:

a Vortex the cell tube for a few seconds.
b Use a P20 pipette to take 10 µl from the middle of the cell suspension, and immediately load it onto Chamber B of the TC20 chip.
c Measure the cell counts in Chamber B on the TC20. This step should be performed without adding trypan blue.
d Vortex the cell tube for a few seconds.
e Use a P20 pipette to take 10 µl from the middle of the cell suspension and immediately load it onto Chamber A of the TC20 chip.
f Take another 10 µl from the middle of the cell suspension and immediately load it onto Chamber B of the TC20 chip.
g Measure the cell counts in Chamber A and B on the TC20. This step should be performed without adding trypan blue.

> **NOTE**
> Make sure the cell suspension is stored on ice during this step.

7 Calculate the average cell concentration with the three measurements performed in step 6 to arrive at an accurate cell count.

8 Proceed to step 9 in *Prepare, Count, and Assess Viability of Single-Cell Suspension* on page 8.

Lab Tracking Chart

Record lab tracking information and sample observations throughout the protocol. The following template can be used to record process control results such as cDNA yield and final library yield.

Have this information available when contacting Illumina Technical Support. In addition to the observation form, record the following information if you have unexpected results.

- A detailed description of the problem
- The steps that were performed immediately before the problem occurred
- The expected results
- The observed results

Lab Tracking ID	Step: Cell Preparation	Cell Samples			
		Sample 1	Sample 2	Sample 3	Sample 4
1	Cell confluency (%)				
2	Cell Viability (%)				
3	Cell concentration (cell / µl)				
4	Single-cell suspension (visually confirmed – Yes/No)				

	Step: After Droplet Generation	Cartridge ports							
		1a	1b	2a	2b	3a	3b	4a	4b
4	Visibly cloudy droplets observed in output wells after single-cell isolation (Yes/No)								
5	Both oil and aqueous layers visible in PCR plate (Yes/No)								

	Step: After cDNA synthesis	cDNA library			
		Sample 1	Sample 2	Sample 3	Sample 4
6	Average cDNA fragment size (bp)				
7	cDNA yield (ng)				

	Step: Final library QC	Final library samples			
		Sample 1	Sample 2	Sample 3	Sample 4
8	Average library fragment size (bp)				
9	Total library yield (nM)				

Acronyms

Acronym	Definition
BSA	Bovine Serum Albumin
PBS	Phosphate-Buffered Saline
RSB	Resuspension Buffer
RTE	Reverse Transcription Enzyme
SPB	(Sample) Purification Beads
SSB	Second Strand Buffer
SSE	Second Strand Enzyme
TCB	Tagment Buffer
TCE	Tagment Enzyme
TPM	Tagmentation PCR Mix
TPP1	Tagment PCR Adapter
TSB	Tagment Stop Buffer

Kit Options

Make sure that you have all the reagents identified in this section before proceeding to the library preparation procedures. Kits are available in the following configurations.

Consumable	Catalog #
SureCell WTA 3’ Library Prep Kit for the ddSEQ System (1 Library Prep, 8 Samples)	20014279
SureCell WTA 3’ Library Prep Kit for the ddSEQ System (1 Library Prep, 24 Samples)	20014280

NOTE

Certain components of the kit are stored at a temperature that differs from the shipping temperature. Store kit components at the specified temperature.

CAUTION

The SureCell WTA 3' includes Sequencing Primer (SP). Include the Sequencing Primer (SP) with the library when sending to an outside lab for sequencing.

SureCell WTA 3’ Library Prep Kit for the ddSEQ System - 8 Samples

Box 1

Quantity	Description
2	ddSEQ Cartridges

Box 2—Store at 2°C to 8°C

Quantity	Reagent	Acronym
1	Encapsulation Oil	N/A
1	3′ Barcode Mix (Blue Cap)	N/A
1	Droplet Disruptor	N/A
1	Resuspension Buffer	RSB
2	Purification Beads	SPB
1	Tagment Stop Buffer	N/A
1	ddSEQ Priming Solution	N/A

Box 3—Store at -25°C to -15°C

Quantity	Reagent	Acronym
1	Barcode Buffer (Blue Cap)	N/A
1	Cell Suspend Buffer (Red Cap)	N/A
1	RNA Stabilizer (Red Cap)	N/A
1	DTT (Red Cap)	N/A
1	Enzyme Enhancer	N/A
1	RT Enzyme (Red Cap)	RTE
1	Second Strand Buffer	SSB
1	Second Strand Enzyme	SSE
1	Tagment Buffer	TCB
1	Tagment Enzyme	TCE
1	Tagmentation PCR Mix	TPM
1	Tagment PCR Adapter	TPP1
1	Sequencing Primer	SP

Box 4—Store at -25°C to -15°C

Quantity	Reagent	Acronym
8	DNA Adapters	N701–N707, N718

SureCell WTA 3’ Library Prep Kit for the ddSEQ System - 24 Samples

Box 1 (Quantity 3)

Quantity	Description
2	ddSEQ M Cartridges

Box 2 (Quantity 3) — Store at 2°C to 8°C

Quantity	Reagent	Acronym
1	Encapsulation Oil	N/A
1	3′ Barcode Mix	N/A
1	Droplet Disruptor	N/A
1	Resuspension Buffer	RSB
2	Purification Beads	SPB
1	Tagment Stop Buffer	TSB
1	ddSEQ Priming Solution	N/A

Box 3 (Quantity 3) — Store at -25°C to -15°C

Quantity	Reagent	Acronym
1	Barcode Buffer (Blue Cap)	N/A
1	Cell Suspend Buffer (Red Cap)	N/A
1	RNA Stabilizer (Red Cap)	N/A
1	DTT (Red Cap)	N/A
1	Enzyme Enhancer	N/A
1	RT Enzyme (Red Cap)	RTE
1	Second Strand Buffer	SSB
1	Second Strand Enzyme	SSE
1	Tagment Buffer	TCB
1	Tagment Enzyme	TCE
1	Tagmentation PCR Mix	TPM
1	Tagment PCR Adapter	TPP1

Box 4 — Store at -25°C to -15°C

Quantity	Reagent	Acronym
24	DNA Adapters	N701–N707, N710–N712, N714–N716, N718–N724, N726–N729

Consumables and Equipment

Make sure that you have the required user-supplied consumables and equipment before starting the protocol.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

NOTE

- Use a dedicated set of consumables and equipment for pre-PCR and post-PCR procedures.
- The SureCell WTA 3' library prep protocol requires different magnetic stands during library clean-up procedures.

Consumables

Consumable	Supplier
20 µl racked pipette tips	Rainin, item # 17002928
20 µl multichannel pipettes	Rainin, item # 17013803
20 µl single channel pipettes	Rainin, item # 17014392
50 µl multichannel pipettes	Rainin, item # 17013804
200 µl racked pipette tips	Rainin, item # 17002428
200 µl multichannel pipettes	Rainin, item # 17013805
200 µl single channel pipettes	Rainin, item # 17014391
Cell strainer 35 µm (500/case)	Corning Life Sciences, product #352235
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, product # E7023
Nuclease-free water	General lab supplier
Bio-Rad ddPCR Plate	Bio-Rad, catalog # 12001925
TC20 slides	Bio-Rad, catalog # 145003
Trypan blue	Bio-Rad, catalog # 1450021
8-tube strip	General lab supplier
Flat cap strips	Bio-Rad, catalog # TCS-0803
Multichannel pipette reservoir	General lab supplier
BSA (Bovine Serum Albumin)	General lab supplier
1 x PBS (Phosphate-Buffered Saline)	General lab supplier
DEPC	General lab supplier
1M Tris-HCL, pH 7.4	General lab supplier
NaCl	General lab supplier
MgCl2	General lab supplier
IGEPAL CA-630	General lab supplier
SUPERas IN RNase Inhibitor	Thermo Fishe catalog # AM2694

Equipment

Equipment	Supplier
ddSEQ Single-Cell Isolator	Bio-Rad, catalog # 12004336
One of the following 96-well thermal cyclers: • T100 Thermal Cycler • C1000 Touch Thermal Cycler	 Bio-Rad, catalog # 1861096 Bio-Rad, catalog # 1851197
TC20 automated cell counter	Bio-Rad, catalog # 145-0102
Magnetic peg stand-96	Thermo Fisher, catalog # AM10027
DynaMag-96 side magnet DynaMage-96 side skirted magnet	Thermo Fisher, catalog # 12331D Thermo Fisher, catalog # 12027
Microplate centrifuge	Eppendorf, catalog # 5804 Eppendorf, catalog # 5430
Vortexer	General lab supplier