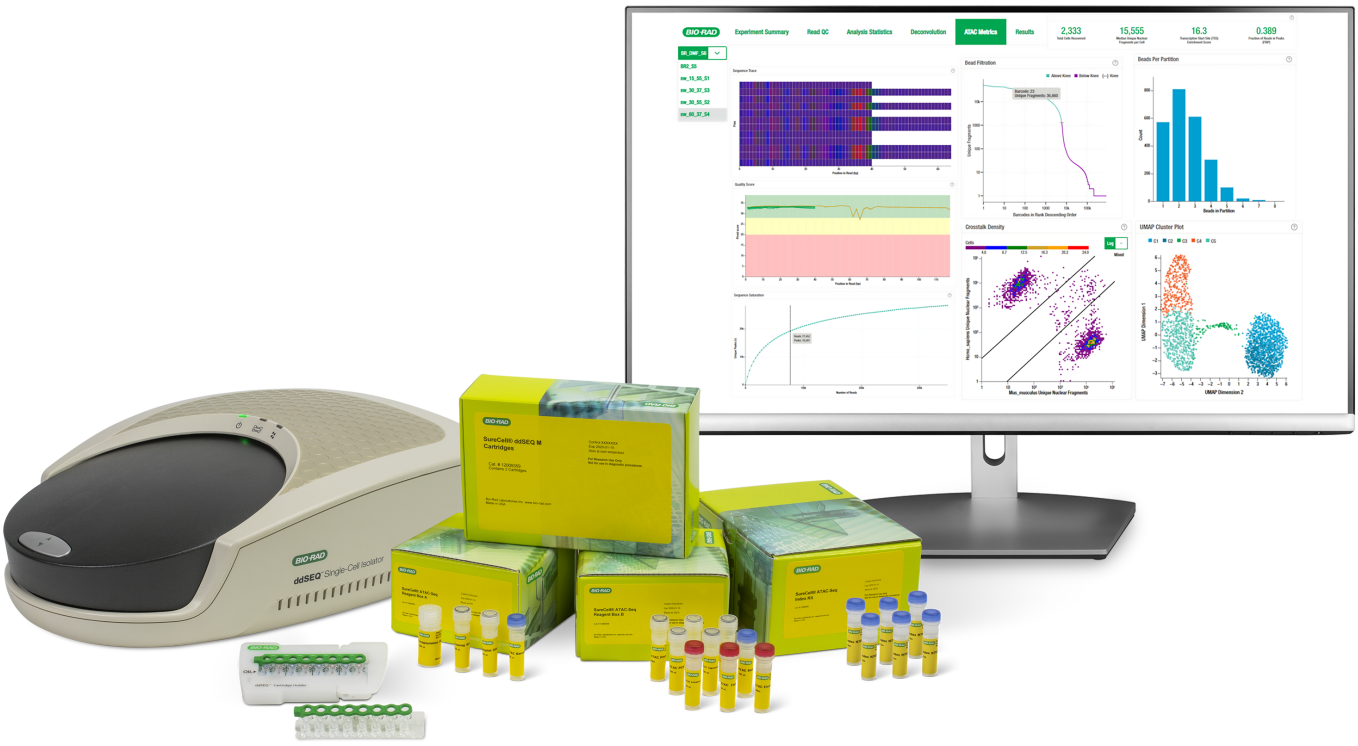

SureCell® ATAC-Seq Library Preparation Kit

User Guide

Version 1.0.1

BIO-RAD

SureCell ATAC-Seq Library Preparation Kit

User Guide

Version 1.0.1

Catalog No. 17004620
12009359
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12009360

BIO-RAD

The Bio-Rad Technical Support department in the U.S. is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific time.

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Revision History

Document	Date	Description of Change
SureCell ATAC-Seq Library Preparation Kit User Guide DIR#10000106678 Ver D	October 2023	Update with additional SME edits
SureCell ATAC-Seq Library Preparation Kit User Guide DIR#10000106678 Ver C	May 2023	Incorporate SME edits, remove Omnition Analysis Software on the cover, update legal notices and trademark symbols, correct page numbers, fix variable entries in text and footer
SureCell ATAC-Seq Library Preparation Kit User Guide DIR#10000106678 ATAC-Seq Library Preparation Kit User Guide DIR#10000106678 Ver B	March 2023	Reformat the document into TechCom template and update with minor edits and SME changes

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Table of Contents

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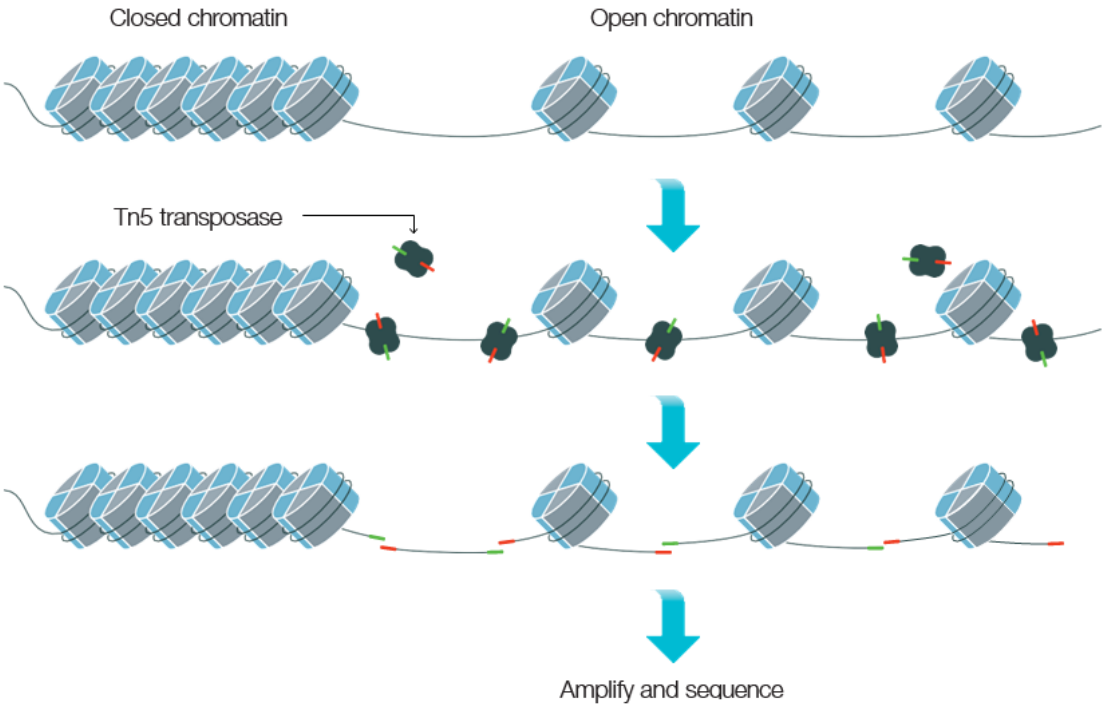
Chapter 1 Introduction

Single-Cell ATAC-Seq (Assay for Transposase-Accessible Chromatin using sequencing) provides a novel method for mapping genome-wide chromatin accessibility for thousands of individual single cells. The simple one day workflow provides easy access to epigenetic information, including transcriptional dynamics from cell to cell. Four samples can be processed per ddSEQ™ cartridge per run.

Nuclei isolated from single cells are tagged, using Tn5 transposase, at regions of open chromatin in bulk. Amplification from tagged sites within individual droplets generates a library of fragments that are representative of the original open chromatin on a per cell basis. Barcoding within individual droplets enables the reconstruction of single cell data from the barcode sequences after sequencing.

The following graphic shows sequencing adapters in red and green.



Key Features and Applications

Features

- In-depth investigations into DNA accessibility for various applications
- Scalable to large studies
- Single-day workflow with minimal hands-on time
- Sensitive for characterizing specific cell populations

Applications

- Identify cell types in a heterogeneous cell population using single-cell ATAC profiles
- Investigate cell-to-cell regulatory variation
- Understand epigenetic structure
- Quantify changes leading to gene expression and repression
- Provide insights into gene regulatory mechanisms underlying development, cancer heterogeneity, and drug response
- Validate scRNA-Seq studies

Data analysis is conducted using Omnition ATAC Analysis Software from Bio-Rad™, which takes raw sequencing files and then processes the data to provide QC metrics, experiment report, and output files.

Additional Resources

You can find support for this protocol at bio-rad.com, including background information, brochures, additional documentation, downloads, and compatible products.

Chapter 2 Required Consumables and Equipment

Table 1. SureCell ATAC-Seq Library Prep Kit contents

Name	Catalog number	Storage req	Associated document catalog number	Qty (each)	Cap color
ddSEQ M Cartridges	12009359	Room temp	12008720	2	N/A
SureCell ATAC-Seq Reagent Box A: (Catalog #12009358)					
Priming Solution	N/A	4° C	12008740	1	Clear
Droplet Disruptor	N/A	4° C	12008761	1	Clear
Encapsulation Oil	N/A	4° C	12008762	1	Clear
ATAC Barcode Mix	N/A	4° C	12008754	1	Blue
SureCell ATAC-Seq Reagent Box B: (Catalog #12009357)					
ATAC Barcode Buffer	N/A	–20° C	12008781	1	Blue
ATAC PCR Supermix	N/A	–20° C	12008782	1	Clear
ATAC Enhancer Enzyme	N/A	–20° C	12008784	1	Red
ATAC Primer Mix	N/A	–20° C	16005990	1	Clear
ATAC Tagmentation (TGMNT) Buffer	N/A	–20° C	12008775	1	Clear

Table 1. SureCell ATAC-Seq Library Prep Kit contents, continued

Name	Catalog number	Storage req	Associated document catalog number	Qty (each)	Cap color
ATAC Tagmentation Enzyme	N/A	–20° C	12008776	1	Clear
ATAC Enzyme Buffer	N/A	–20° C	12008778	1	Red
ATAC Enzyme	N/A	–20° C	12008779	1	Red
ATAC Sequencing Primer	N/A	–20° C	16005986	1	Clear

Table 2. Verified apparatus and equipment (not provided)

Apparatus/equipment	Catalog number	Supplier
Agilent 2100 Bioanalyzer	G2939B	Agilent
Qubit 4 Fluorometer	Q33238	Thermo Fisher Scientific
TC20 Automated Cell Counter	1450102	Bio-Rad
ddSEQ Single Cell Isolator (with ddSEQ Cartridge Holder included)	12004336	Bio-Rad
C1000 Thermal Cycler with deepwell module *or* PTC Tempo Thermal Cycler (deepwell)	1851197 12015392	Bio-Rad Bio-Rad
Eppendorf ThermoMixer C	5382000023	Eppendorf
Centrifuge 5418R (rotor of 1.5 ml tube required)	5401000137	Eppendorf
Centrifuge 5430 (rotor of MTP and 15 ml conical tube required)	022620509	Eppendorf
Magnetic Stand 96	AM10027	ThermoFisher Scientific
DynaMag-96 Side Skirted Magnet	12027	ThermoFisher Scientific
Rainin Pipet-Lite LTS Pipette L-1000XLS+	17014382	Mettler Toledo

Table 2. Verified apparatus and equipment (not provided), continued

Apparatus/equipment	Catalog number	Supplier
Rainin Pipet-Lite LTS Pipette L-200XLS+	17014391	Mettler Toledo
Rainin Pipet-Lite LTS Pipette L-100XLS+	17014384	Mettler Toledo
Rainin Pipet-Lite LTS Pipette L-20XLS+	17014392	Mettler Toledo
Rainin Pipet-Lite LTS Pipette L-2XLS+	17014393	Mettler Toledo
Rainin Pipet-Lite Multi Pipette L8-200XLS+	17013805	Mettler Toledo
Rainin Pipet-Lite Multi Pipette L8-50XLS+	17013804	Mettler Toledo
Rainin Pipet-Lite Multi Pipette L8-20XLS	17013803	Mettler Toledo
96-well Cooling Block	Any	Any

Table 3. Verified reagents (not provided)

Reagent	Catalog number	Supplier
Digitonin (20 mg/ml or 2%)	G9441	Promega
10% Bovine Serum Albumin in PBS	PI37525	Thermo Fisher Scientific
Tris-HCl pH 7.4, 1 M	T2194-100 ml	Sigma Aldrich
Resuspension buffer (10 mM Tris-HCl, pH 8.0, no EDTA), DNase free	T1173	Teknova
Tween 20, 10% solution	T0710	Teknova
$MgCl_2$, 1M	AM9530G	Thermo Fisher Scientific
NaCl, 5 M	AM9759	Thermo Fisher Scientific
1X PBS, pH 7.4	10010023	Thermo Fisher Scientific
NP-40 Surfact-Amps Detergent Solution	28324	Thermo Fisher Scientific
Ethanol, 80% (make fresh)	N/A	Any
Nuclease-free water	N/A	Any
Glycerol, 50% (v/v)	3290-16	Ricca Chemical

Table 4. Verified consumables (not provided)

Consumable	Catalog number	Supplier
Agencourt AMPure XP	A63880	Beckman Coulter
ddPCR 96-well plates	12001925	Bio-Rad
Optical Flat 8-cap strips	TCS0803	Bio-Rad
Microseal™ F PCR Plate Seal with pierceable foil	MSF1001	Bio-Rad
0.2 ml 8-Tube PCR Strips and Caps	76318-798	VWR
1.5 ml DNA LoBind Tubes	022431021	Eppendorf
35 µm Cell Strainer, Sterile (or cell strainer of appropriate pore size for cells of interest)	352235	Corning
Qubit Assay Tubes	Q32856	Life Technologies
Pipette Tips TR LTS 1000 µL F 768A/8	17014967	Mettler Toledo
Pipette Tips TR LTS 200µL F 960A/10	17014963	Mettler Toledo
Pipette Tips TR LTS 20µL S 960A/10	17014960	Mettler Toledo

Table 5. Commercial kits

Commercial kit	Catalog number	Supplier
Agilent High Sensitivity DNA Kit	5067-4626	Agilent
Qubit dsDNA Quantitation Assay, high sensitivity	Q32851	Thermo Fisher Scientific
TC20 Cell Counting Kit, with trypan blue	14500003	Bio-Rad
SureCell ATAC-Seq Index Kit	12009360	Bio-Rad

Chapter 3 SureCell ATAC-Seq Workflow

The following table provides a summary of workflow steps for this protocol.

Step	Workflow procedure
1	Prepare, count, and assess viability of single-cell suspension
2	Use OMNI-ATAC tagmentation OR Whole-Cell tagmentation
3	Prepare barcode and enzyme Suspension Mixes
4	Partition the nuclei
5	Perform ddSEQ barcoding and sample index PCR
	Pause point: Store at 4° C for 72 hrs, or store at –20° C long-term.
6	Perform emulsion breakage and first PCR cleanup
	Pause point: Store at 4° C for 72 hrs, or store at –20° C long-term.
7	Perform second PCR
	Pause point: Store at 4° C for 72 hrs, or store at –20° C long-term.
8	Perform second PCR cleanup
	Pause point: Store at 4° C for 72 hrs, or store at –20° C long-term.
9	Perform Library QC and sequencing
10	Perform data analysis

OMNI-ATAC Tagmentation Protocol vs. Whole-Cell Tagmentation Protocol

Isolation of nuclei from mammalian cells requires the use of detergents to disrupt the integrity of the cellular membrane. You can choose from two protocols, depending on cell type, as follows:

- The OMNI-ATAC tagmentation protocol has been optimized for cell lines and uses a combination of harsh detergents.
- The Whole-Cell tagmentation protocol uses a combination of gentle detergents and is more suitable for fragile cells, such as blood cells and PBMCs.

  Important: The Whole-Cell protocol is not ideal for cell lines and other cell types that are more resistant to lysis.

Depending on specific need, further optimization might be necessary to determine which tagmentation protocol is appropriate for the individual cell type or cell source. When working with a new cell/tissue type, Bio-Rad recommends optimizing this portion of the protocol.

Table 6. Protocols

Protocol	Cells verified by the current protocol
OMNI-ATAC	HEK-293, K562, NIH-3T3, GM12878, mouse embryonic stem cells
Whole-Cell	Peripheral blood mononuclear cells (PBMCs), bone marrow mononuclear cells

Key Workflow Considerations

Note the following:

- A single-cell suspension is required to start the protocol. Depending on sample type, you might need additional optimizations for cell dissociation. For more information, see Appendix A, Supporting Information.
- A minimum of 150,000 cells (ideally 300,000 cells) are required to start the workflow.
- Take extra precautions to minimize cell loss when performing cell preparation steps in the Cell Lysis and Tagmentation section. Cell loss at this step reduces cell numbers in the final library.
- You must load all sample ports in a cartridge with enzyme and barcode reaction mixtures. If fewer than four samples are available, fill the unused wells with 50% (v/v) glycerol solution in water before loading to the ddSEQ cartridge.
- A custom sequencing primer is provided for Read 1. You must use the custom primer in sequencing to obtain the correct cell barcode information.

  Important: You cannot combine the custom primer with other standard Read 1 sequencing primers, as it results in poor cluster quality and loss of reads.

Cell Preparation Guidelines

Note the following:

- Fully Dissociated Single Cells

  This protocol requires cells in single-cell suspension. Cell aggregates or doublets that are present in the suspension significantly increase the probability of doublets or multiplets during single-cell isolation on the ddSEQ Single Cell Isolator.

  Dissociation, straining, washing, and quantitation might require additional optimization and specific techniques appropriate to the individual cell type or cell source.

- Cell Viability

  The presence of dead or damaged cells can reduce recovery and impact performance due to ambient DNA and cellular debris. Bio-Rad recommends starting with cells that have viability above 80%.

  To assess viability, use the Bio-Rad TC20 Automated Cell Counter (Bio-Rad Catalog No. 1450102), along with 0.4% trypan blue (Bio-Rad Catalog No. 1450022). Before loading 10 µl on a cell counting slide, pipet-mix the trypan blue and cells at equal volumes . If viability is less than 80%, treat cells with DNase I to digest ambient DNA. For more information, see S1, Working with Low Viability Cells in Appendix A, Supporting Information.

- Centrifugation

  The tagmentation portion of this protocol requires pelleting of cells by centrifugation to remove supernatant. When placing tubes in the centrifuge during a pelleting step, note the orientation of the tube relative to the center of the centrifuge. The cells should pellet on the side of the tube farthest from the center of the centrifuge.

  When removing supernatant, use multiple pipeting steps. First, use a P200 or P1000 pipet to remove all the volume except 20 µl, and then use a P20 pipet to remove the remaining liquid. Do not disturb the pellet.

- Cell Counting

  Accurate cell counting is critical to achieve target cell throughput and avoid cell multiplets. Bio-Rad has validated and recommends the TC20 Automated Cell Counter (Bio-Rad Catalog No. 1450102) for counting cells with a round shape and a cell diameter between 6 and 50 µm.

  To avoid counting cell debris, size-based gating for automated counters or manual count might be required. Include both viable and non-viable cells in the total cell count.

Best Practices

Bio-Rad recommends the following:

- Before starting the workflow:
  - Read through the entire protocol to familiarize yourself with the workflow.
  - Ensure all required equipment is properly installed and functioning.
  - Ensure all required materials and reagents are available.
- Pause the protocol only at designated stopping points.
- Use separate laboratory areas for appropriate steps. Bio-Rad recommends performing the cell culture steps in a designated cell culture room and hood, and conducting pre-PCR and post-PCR steps in their respective zones.
- To avoid cross-contamination, change pipet tips between each sample and each reaction.
- When handling emulsions, use only specified plastic consumables.

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Chapter 4 Protocol

Buffer Preparation

Before starting cell preparation and tagmentation, prepare fresh buffers according to the tables in this section.

Note the following:

- If using the OMNI-ATAC protocol, prepare the buffers in Table 7 through Table 10.
- If using the Whole-Cell protocol, prepare only the buffer in Table 7.

Important: Keep all prepared buffers on ice.

Table 7. 1X PBS + 0.1% BSA (100 ml volume sufficient for 4 reactions)

Component	Stock concentration	Final concentration	Required volume
PBS, pH 7.4	1x	1x	99 ml
BSA	10% (w/v)	0.1% (v/v)	1 ml

Table 8. ATAC-RSB (10 ml volume sufficient for 4 reactions)

Component	Stock concentration, mM	Final concentration, mM	Required volume, µl
Nuclease-free water	N/A	N/A	9,850
Tris-HCl, pH 7.4	1,000	10	100
NaCl	5,000	10	20
$MgCl_2$	1,000	3	30
TOTAL			10,000

Table 9. ATAC-Lysis buffer (300 µl volume sufficient for 4 reactions)

Component	Stock concentration, mM	Final concentration, mM	Required volume, µl
ATAC-RSB (see Table 8)	N/A	N/A	291
NP-40	10	0.1	3
Tween 20	10	0.1	3
1% Digitonin*	1	0.01	3
TOTAL			300

* Digitonin is supplied at 2% in DMSO. Dilute 1:1 with nuclease-free water to make a 1% stock solution and keep at –20° C (good for up to 5 freeze-thaw cycles).

Table 10. ATAC-Tween buffer (5 ml volume sufficient for 4 reactions)

Component	Stock concentration, mM	Final concentration, mM	Required volume, µl
ATAC-RSB (see Table 8)	N/A	N/A	4,950
Tween 20	10	0.1	50
TOTAL			5,000

Cell Preparation

Guidelines

Before you begin this section, you must put the cells into solution with user-defined and optimized protocols.

Appendix A, Supporting Information, Section S2, provides methods and materials to put K562 and NIH-3T3 cells in suspension. For all other cell types, including adherent or suspension cell cultures, tissues, or FACS-sorted cells, you must implement your own protocols and supply necessary materials to create cell suspensions.

Note the following:

- To obtain an estimate of the total number, count the cells after they are in suspension.
- For samples with <1 million cells, follow instructions to minimize cell loss during wash steps.
- When they are not being handled, keep cells and nuclei on ice at all times.

Cell Washing and Filtering

Complete the following steps:

1. For every cell type that will be used, chill the cell strainers on ice in their collection tubes.

   The appropriate cell strainer varies according to the cell type. For K562 and NIH-3T3 cells, 35 µm strainers are adequate.

2. In a 15 ml conical tube, centrifuge cells at 300x g for 3 min.

   If starting with <1 million cells, spin the cells in a 1.5 ml microcentrifuge tube. A chilled centrifuge is not required.

3. Without disturbing the cell pellet, discard the supernatant:

   a. Using a serological pipet, remove all but ~1 ml.

   b. Using a P1000 pipet, remove the remaining liquid.

   Important: If starting with <1 million cells, use only a P1000 to remove the liquid.

4. Resuspend the cells in 1 ml of cold 1X PBS + 0.1% BSA. Using a P1000 pipet, pipet-mix 10–15x to break the cell clumps.

   Skip step 5 if you are starting with <1 million cells.

5. Add 9 ml of cold 1X PBS + 0.1% BSA and invert the 15 ml conical tube 3–5x.

6. Repeat steps 2–5 for a total of 2 washes.

7. Perform a cell count measurement to verify how much buffer is needed in the next resuspension step:

   a. Pulse vortex cells at medium speed 3x, 3 sec per pulse.

   b. Using a P20 pipet, remove 10 µl of the cell suspension and load onto a Bio-Rad TC20 counting slide.

   c. To obtain the count, insert the slide into the TC20 Cell Counter.

8. To obtain an approximate cell concentration of 1,000 cells/µl, spin down and resuspend the cell pellet in an appropriate volume of cold 1X PBS + 0.1% BSA.

   A range of 500–5,000 cells/µl is acceptable. Pipet-mix the cells 20x.

   Note: For cells that will be processed through the Whole-Cell Protocol, having >4,000 cells/µl (if possible) will streamline washes downstream.

9. To remove residual cell aggregates, filter the cells through the prechilled cell strainers on ice.

   Important: To account for dead volume in the cell strainer, filter at least 250 µl.

Cell Viability and Cell Count Measurements

Complete the following steps:

1. Pulse vortex cells at medium speed in the strainer tube 3x, 3 sec per pulse.
2. Immediately after mixing, use a P20 pipet to aspirate 10 µl from the middle of the cell suspension and transfer to a 1.5 ml tube.
3. Add 10 µl of 0.4% trypan blue and pipet 10x to mix well.
4. Immediately pipet the stained cell mix onto Bio-Rad TC20 counting slide.
5. To initiate the measurement, insert the slide into the TC20 Cell Counter.
6. Before proceeding to the next step, record the count and check that viability is at least 80%.

   If viability is <80%, refer to Section S1, Working with Low Viability Cells in Appendix A, Supporting Information.
7. Complete the following substeps to perform cell count measurements without adding trypan blue:
   a. Pulse vortex cells at medium speed in the strainer tube 3x, 3 sec per pulse.
   b. Using a P20 pipet, remove 10 µl from the middle of the cell suspension and load onto a Bio-Rad TC20 Cell Counter counting slide.
   c. Insert the slide into the TC20 Cell Counter to obtain the counts.
   d. Repeat steps a–c 3x for a total of 4 counts.
   e. To determine an accurate cell count, calculate the average of all 4 counts.

Cell Lysis and Tagmentation

Table 11. Advance preparation

Item	Cap color	Part number	Storage	Preparation
ATAC Tagmentation (TGMNT) Buffer	Clear	12008775	–20° C	Thaw at room temperature. Vortex vigorously to mix, and then centrifuge briefly. When thawed, keep on ice.
ATAC Tagmentation Enzyme	Clear	12008776	–20° C	Flick the tube to mix, then centrifuge briefly. Keep on ice.

OMNI-ATAC Protocol

Complete the following steps:

1. Before starting the protocol, ensure the microcentrifuge is cooled to 4° C and the thermomixer is pre-heated to 37° C.
2. For each sample:
   a. Before transferring, pulse vortex 3–5 sec each at medium high speed to ensure that cells are in suspension.
   b. Transfer a minimum of 150,000 (ideally 300,000) of the filtered cells prepared in Cell Preparation on page 22 to a 1.5 ml Eppendorf tube.
3. Place the tubes, with the hinges oriented upward, into the cooled microcentrifuge. Spin down at 500 RCF for 5 min at 4° C.
4. Remove all supernatant. Do not disturb the cell pellet.

   If the pellet is difficult to visualize, assume it is located on the same side as the tube hinge. Keep the cell pellet on ice.

   Important: It is essential to minimize cell loss at this step to ensure enough cells are available for the final reaction. Avoid disturbing the cell pellet and do not use a large volume pipet (for example, a P1000) to remove supernatant. Use a P100 or P200 pipet to aspirate supernatant, leaving approximately 20–30 µl. Switch to a P20 pipet to remove the remaining liquid.
5. Add 50 µl of cold ATAC-Lysis buffer and pipet-mix at least 15x, or until the cell pellet goes back into suspension.

6. Incubate on ice for 3 min.
7. Add 1 ml of cold ATAC-Tween Buffer to each tube. Invert the tubes 3x to mix.
8. Spin down at 500 RCF for 10 min at 4° C in a fixed angle centrifuge.
9. Once centrifugation from step 8 is finished, remove the supernatant from the tube. Do not disturb the nuclei pellet. Keep the nuclei pellet on ice.

   Important: It is essential to minimize nuclei loss at this step to ensure enough nuclei are available for the final reaction. Use a P200 or P1000 pipet to remove supernatant, leaving approximately 40 µl. Switch to a P20 pipet to remove the remaining liquid.
10. Resuspend the nuclei pellet in 20 µl cold 1X PBS + 0.1% BSA. Pipet-mix 10–15x, or until the pellet goes back into suspension. Keep on ice.
11. Count nuclei in the TC20 Automated Cell Counter:
    a. Immediately take 2.5 µl nuclei from the middle of the suspension and transfer to a 1.5 ml tube.
    b. Add 7.5 µl of 1X PBS + 0.1% BSA and pipet-mix 15x to thoroughly mix the diluted nuclei.
    c. Add 10 µl of 0.4% trypan blue to the diluted nuclei and pipet 10 x to mix well.
    d. Immediately pipet the stained nuclei mix onto the Bio-Rad TC20 counting slide.
    e. Insert the slide into TC20 Cell Counter to initiate measurement.
    f. Before proceeding to the next step, record the count and ensure that viability at this step is <10%.
    g. Pulse vortex the nuclei suspension 3x at medium speed, 3 sec per pulse.

       Important: Take the nuclei count measurement immediately after adding trypan blue and mixing (steps c–g). To improve accuracy, do not let the nuclei sit in trypan blue longer than 30 sec. Include a 4x dilution factor in the calculation of the final nuclei concentration.

12. Prepare the required number of OMNI-ATAC Tagmentation Mixes according to Table 12.

Table 12. OMNI-ATAC tagmentation mix

Component	Required volume, µl (1 rxn + 10% excess)	Required volume, µl (4 rxn + 10% excess)
ATAC Tagmentation (TGMNT) Buffer	11.00	44.00
ATAC Tagmentation Enzyme	2.75	11.00
Nuclease-free water	23.10	92.40
TOTAL	36.85	147.40

13. Mix and dispense:
    a. To mix, pulse vortex the OMNI-ATAC Tagmentation Mix.
    b. Keep on ice until you are ready to use it.
    c. For each sample, dispense 33.5 µl into a 1.5 ml Eppendorf Lo-Bind tube.
14. Add 60,000 nuclei to the pre-aliquoted OMNI-ATAC Tagmentation Mix, followed by 1X PBS + 0.1% BSA to a final volume of 50 µl.

    Use the following equations to calculate the volume of nuclei and 1X PBS + 0.1% BSA required for each reaction

    - Volume of nuclei suspension required, µl = 60,000 nuclei/nuclei concentration determined from count (nuclei/µl)
    - Volume of 1X PBS + 0.1% BSA required, µl = 16.5 µl – volume of nuclei suspension required (µl)

    Important: Before adding nuclei to the Tagmentation Mix, pulse vortex the nuclei suspension 3x, for 3–5 sec each, at medium-high speed. This ensures that the nuclei are evenly distributed and the correct number is added to the tagmentation reaction. In order to maintain correct concentrations of components in the tagmentation reaction, do not add more than 16.5 µl total volume of nuclei and 1X PBS + 0.1% BSA in the final reaction.
15. Set a P100 pipet to 30 µl and pipet up and down 10x to mix the tagmentation reaction.
16. Incubate at 37° C for 30 min in a ThermoMixer with 500 RPM mixing.
17. After the 37° C incubation, transfer the tagmentation reaction onto ice and proceed immediately to the first step in Prepare Solutions for Droplet Encapsulation on page 31.

Whole-Cell Protocol

Complete the following steps:

1. Before starting the protocol, ensure the microcentrifuge is cooled to 4° C and the thermomixer is pre-heated to 37° C.
2. Prepare the required number of Whole-Cell Tagmentation Mixes according to Table 13. Vortex to mix the solution and keep on ice.

Table 13. Whole-Cell Tagmentation Mix

Component and diluted stock	Required volume, µl (1 rxn + 10% excess)	Required volume, µl (4 rxn + 10% excess)
ATAC Tagmentation (TGMNT) Buffer	11.00	44.00
ATAC Tagmentation Enzyme	2.75	11.00
0.5% Digitonin*	1.10	4.40
5% Tween 20*	1.10	4.40
Nuclease-free water	20.90	83.60
TOTAL	36.85	147.40

* Dilute the Digitonin and Tween 20 before use, as specified below:

- Digitonin is supplied at 2% in DMSO. Dilute 1:4 with nuclease-free water to make a 0.5% stock solution.
- Tween 20 is supplied at 10% concentration. Dilute 1:1 with nuclease-free water to make 5% stock solution.

3. To ensure that filtered cells prepared in Cell Preparation on page 22 are in suspension, pulse vortex at medium speed 3x, for 3 sec each.
4. Do one of the following:
   - If the concentration of cells obtained in Cell Viability and Cell Count Measurements on page 24 is >3,600 cells/µl, skip to step 10.
   - If the concentration is <3,600 cells/µl, proceed to step 5.
5. Transfer a minimum of 150,000 (ideally 300,000) cells to a 1.5 ml Eppendorf tube.
6. Place the tubes, with the hinges oriented upward, into the cooled microcentrifuge. Spin down at 500 RCF for 5 min at 4° C.
7. Without disturbing the cell pellet, remove all supernatant. Keep the cell pellet on ice.

   Important: It is essential to minimize cell loss at this step to ensure enough cells are available for the final reaction. Avoid disturbing the cell pellet and do not use a large volume pipet (for example, a P1000) to remove supernatant. Use a P100 or P200 pipet to aspirate supernatant, leaving approximately 20–30 µl. Switch to a P20 pipet to remove the remaining liquid.

   Note: If the pellet is difficult to visualize, assume it is located on the same side as the tube hinge.
8. Resuspend the cell pellet in 20 µl cold 1X PBS + 0.1% BSA. Pipet-mix 10–15x, or until the cell pellet goes back into suspension. Keep on ice.
9. Count cells in the TC20 Automated Cell Counter:
   a. Pulse vortex the cell suspension at medium speed 3x, 3 sec per pulse.
   b. Immediately take 2.5 µl of cells from the middle of the suspension and transfer them to a 1.5 ml tube.
   c. Add 7.5 µl of 1X PBS + 0.1% BSA. To thoroughly mix the diluted cells, pipet-mix 15x.
   d. Add 10 µl of 0.4% trypan blue to the diluted cells and pipet 10x to mix.
   e. Immediately pipet the stained cell mix onto the Bio-Rad TC20 counting slide.
   f. To initiate the measurement, insert the slide into TC20 Cell Counter.
   g. Record the count and ensure that viability is still >80%, as measured in Cell Preparation on page 22.

      Important: Take the cell count measurement immediately after adding trypan blue and mixing (steps d–g). To improve accuracy, do not let cells sit in trypan blue longer than 30 sec. Include a 4x dilution factor in calculation of final cell concentration.

10. To mix, vortex the Whole-Cell Tagmentation Mix and dispense 33.5 µl into a 1.5 ml Eppendorf Lo-Bind tube.
11. Add 60,000 cells to the pre-aliquoted Whole-Cell Tagmentation Mix followed by 1X PBS + 0.1% BSA to final volume of 50 µl. To calculate the volume of cells and 1X PBS + 0.1% BSA required for each reaction, use the following equations.

    - Volume of cell suspension required, µl = 60,000 cells/cell concentration determined from count (cells/µl)
    - Volume of 1X PBS + 0.1% BSA required, µl = 16.5 µl – volume of cell suspension required (µl)

    Important: Before adding cells to the Tagmentation Mix, pulse vortex the cell suspension 3x, for 3–5 sec each, at medium-high speed. This ensures that the cells are evenly distributed and the correct number is added to the tagmentation reaction. In order to maintain correct concentrations of components in the tagmentation reaction, do not add more than 16.5 µl total volume of cells and 1X PBS + 0.1% BSA in the final reaction.
12. Set a P100 pipet to 30 µl and pipet up and down 10x to pipet-mix the tagmentation reaction.
13. In a ThermoMixer with 500 RPM mixing, incubate at 37° C for 30 min.
14. After the 37° C incubation, transfer the tagmentation reaction on to ice and proceed immediately to the next section.

Prepare Solutions for Droplet Encapsulation

Table 14. Advance preparation

Item	Cap color	Catalog number	Storage	Preparation
ATAC Enzyme Buffer	Red	12008778	–20° C	Thaw at room temperature. Vortex vigorously to mix, and then centrifuge briefly. When thawed, keep on ice.
ATAC Enzyme	Red	12008779	–20° C	Flick the tube to mix, and then centrifuge briefly. Keep on ice.
ATAC Enhancer Enzyme	Red	12008784	–20° C	Flick the tube to mix, and then centrifuge briefly. Keep on ice.
ATAC Barcode Buffer	Blue	12008781	–20° C	Thaw at room temperature. Vortex vigorously to mix, and then centrifuge briefly. Once thawed, keep on ice.
ATAC Barcode Mix	Blue	12008754	4° C	Centrifuge briefly, and then vortex vigorously to mix. Keep on ice.
SureCell ddSEQ Index Kit	Blue	12009360	–20° C	Thaw at room temperature. Vortex vigorously to mix, and then centrifuge briefly. Once thawed, keep on ice.

Prepare Barcode Suspension Mix

Complete the following steps.

1. Prepare the Barcode Reaction Mix:
   a. Use the components specified in Table 15.
   b. Thaw all components and mixes, and then keep them on ice.
   c. Immediately before adding, vortex all components.
   d. To thoroughly mix the solution, pulse vortex the completed Barcode Reaction Mix 3x, for 5 sec each, at medium speed.
   e. After all components are added, vortex the final mix.

   Important: Do not spin down the ATAC Barcode Mix after vortexing. This creates a concentration gradient that affects the amount of barcode input.

Table 15. Barcode Reaction Mix

Component	Catalog number	Required volume, µl (4 rxn + 10% excess)
ATAC Barcode Buffer	12008781	33.00
ATAC Barcode Mix	12008754	55.00
TOTAL		88.00

2. Label the wells of an 8-well PCR strip as A1, A2, B1, B2, C1, C2, D1, and D2.

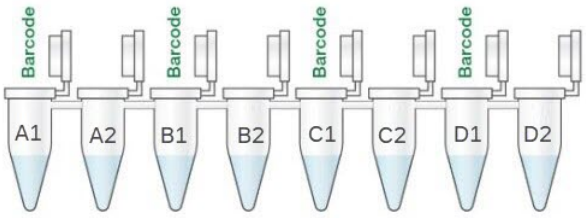

Fig. 1: 8-well strip with Barcode Reaction Mix

Note: This layout facilitates cartridge loading in Droplet Encapsulation on page 35.

3. Dispense 20 µl of the Barcode Reaction Mix into alternating wells of the 8-well PCR strip (A1, B1, C1, and D1). Leave the filled strip in a cold block on ice.
4. To the same wells (A1, B1, C1, and D1), add 5 µl of SureCell ddSEQ Sample Index, one per well, selected from 24 available indices.

   Important: Use a different index for each well and record the index name for each sample. This information will be required when setting up the sequencing run.
5. Centrifuge briefly to bring contents to the bottom of the tube.

   The completed Barcode Suspension Mix should have a total volume of 25 µl. Keep the mix on ice.

Prepare Enzyme Suspension Mix

Complete the following steps.

1. To prepare the Enzyme Reaction Mix
   a. Use the components specified in Table 16.
   b. Thaw all components and mixes, and then keep them on ice.
   c. Immediately before adding, vortex all components.
   d. To thoroughly mix the solution, pulse vortex the completed Enzyme Reaction Mix 3x, for 3 sec each, at medium speed.
   e. After all components are added, vortex the final mix.

Table 16. Enzyme Reaction Mix

Component	Catalog number	Required volume, µl (4 rxn + 20% excess)
ATAC Enzyme Buffer	12008775	60.00
Nuclease-free water	N/A	7.80
ATAC Enzyme	12008779	9.0
ATAC Enhancer Enzyme	12008784	4.80
TOTAL		81.60

2. Dispense 17 µl of the Enzyme Reaction Mix into alternating wells of the 8-well PCR strip (A2, B2, C2, and D2). Leave the filled tube strip in a cold block on ice.

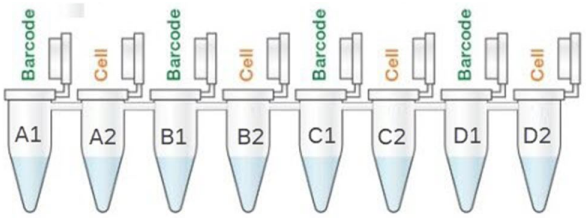

Fig. 2: 8-well strip with Enzyme Reaction Mix added

3. To the same wells (A2, B2, C2, and D2), add the required volume of tagmented nuclei and water to obtain the desired cell throughput per sample. Refer to Table 17 for volumes required.

   Important: Before adding to the Enzyme Reaction Mix, ensure the tagmented nuclei are suspended and homogenous.

4. Using a P100 pipet that is set to 30 µl, pipet the tagmented nuclei up and down 10x and immediately aspirate from the center to remove the required volume.

   Targeted cell throughput is an estimate. Actual cell throughput might vary, depending on variability in cell counts, cell viability, and differential loss during upstream tagmentation.

Table 17. Nuclei and water input volumes per sample

Targeted cell throughput, cells	Tagmented nuclei, µl (from OMNI-ATAC Protocol orWhole-Cell Protocol)	Nuclease-free water, µl
Empty well	N/A	8.0
≤ 500	0.64*	7.36*
501 to 1,000	1.56	6.44
1,001 to 3,000	4.69	3.31
3,001 to 5,000	8.00	N/A

*Pipeting 0.64 µl might result in error. Therefore, Bio-Rad recommends prior dilution of the tagmented nuclei. To dilute the tagmented nuclei, add 2 µl of the tagmentation reaction to 6 µl of water and pipet to mix. After mixing, add 2.56 µl of the diluted tagmented nuclei and 5.44 µl of nuclease-free water to the reaction per sample.

5. To mix the completed Enzyme Suspension Mix, set a P20 multichannel pipet to 20 µl and pipet up and down 5x. Leave the mix on ice and immediately proceed to Droplet Encapsulation on page 35.

Droplet Encapsulation

Guidelines

Note the following:

- Ensure the ddSEQ Single-Cell Isolator is installed according to manufacturer instructions and the power indicator is lit.
- Review pipeting guidelines in the ddSEQ Single-Cell Isolator Instruction Manual.
- Use Rainin pipets and corresponding tips to load the cartridge. Use of other tips can negatively affect ddSEQ cartridge performance.
- Before use, equilibrate the ddSEQ Priming Solution and ddSEQ Encapsulation Oil to room temperature.
- When loading reagents, ensure the ddSEQ cartridge is correctly inserted into the cartridge holder.
- ddSEQ cartridges are one-time use only. After use, apply proper laboratory protocols to dispose of the cartridge.

Best Practices: Loading Cartridges

Important: All sample chambers on the cartridge must be loaded before inserting the cartridge into the ddSEQ instrument. To ensure that no sample wells are left empty, confirm that the correct number of samples were prepared in Prepare Solutions for Droplet Encapsulation on page 31. If fewer than four samples are available, fill the unused wells with 50% (v/v) glycerol solution in water.

Bio-Rad recommends the following:

- Immediately before loading the cartridge, vortex or pipet-mix the Barcode Suspension Mix and Enzyme Suspension Mix.
- To avoid bubbles, press the pipet plunger only to the first stop when loading the cartridge with Barcode Suspension Mix and Enzyme Suspension Mix.
- Due to the viscosity of the solution, aspirate and dispense the Encapsulation Oil slowly.

Best Practices: Droplet Handling

Bio-Rad recommends the following:

- After droplet generation, immediately transfer droplets to the recommended plate (Bio-Rad ddPCR 96-well plate), chilled on ice.
- Slowly (3–5 sec count) aspirate the droplets and slowly (3–5 sec count) dispense the droplets down the side of the 96-well plate.
- When droplets are loaded into a plate, keep the plate chilled on ice when transferring to a thermal cycler.
- To avoid static generation when handling encapsulated samples:
  - Work in a clear, static-free area.
  - Do not use latex gloves when making or handling droplets.

Preparing the Cartridge

Complete the following steps.

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

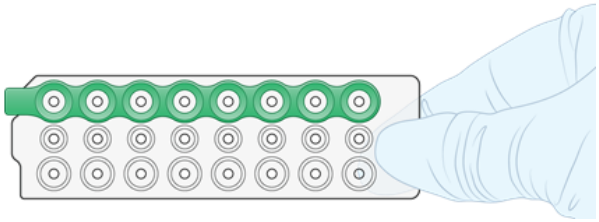

Fig. 3: Bio-Rad ddSEQ cartridge

2. Insert the cartridge into the cartridge holder:
   a. Lift the cartridge holder lever.
   b. Position the green gasket on the cartridge with the green stripe on the cartridge holder.
   c. Insert the tab under the rails, and then slide the cartridge into the holder.
   d. Before closing the lever, ensure the cartridge is fully inserted and lying flat against the bottom of the holder. If the lever does not close completely, remove and reinsert the cartridge.

Fig. 4: Insert cartridge into cartridge holder

The following graphic shows a correctly assembled cartridge and cartridge holder:

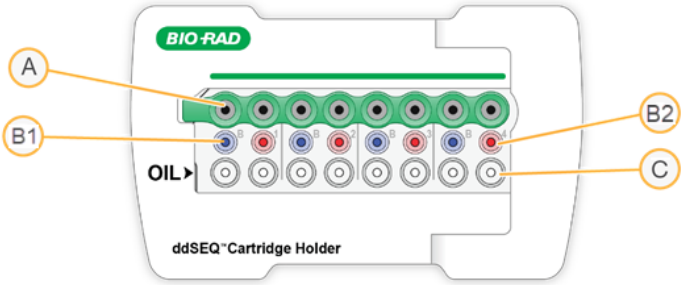

Fig. 5: Correctly assembled cartridge and cartridge holder

Row A) Encapsulated sample output wells
Row B) B1 – Barcode Suspension Mix input wells (Blue)
Row B) B2 – Enzyme Suspension Mix input wells (Red)
Row C) Encapsulation Oil input wells

The following graphic shows two examples of incorrect assembly that result in the latch not closing completely.

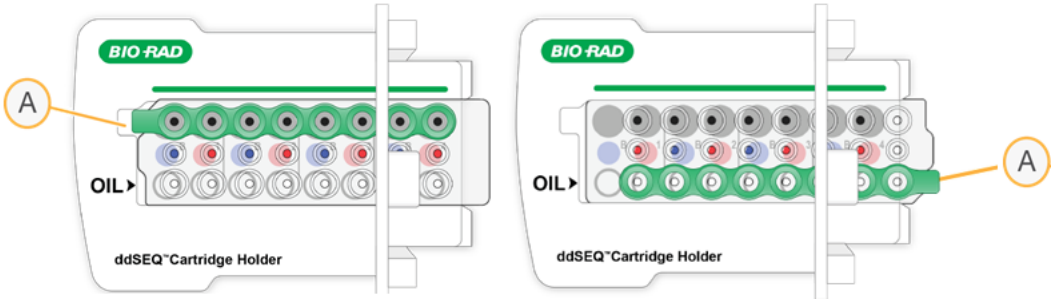

Fig. 6: Incorrect cartridge assembly

Row A) Image on the left shows the cartridge not fully inserted and the image on the right shows the cartridge upside down

Priming the Cartridge

Complete the following steps.

1. Using a P200 single-channel pipet, add 25 µl of room temperature ddSEQ Priming Solution to each well of an 8-tube strip.
2. Using a P20 multi-channel pipet, transfer 20 µl of ddSEQ Priming Solution from the 8-tube strip to each well in the second row of the cartridge (B), as shown in Fig. 7.

   Important: To avoid bubbles, press the pipet plunger only to the first stop.

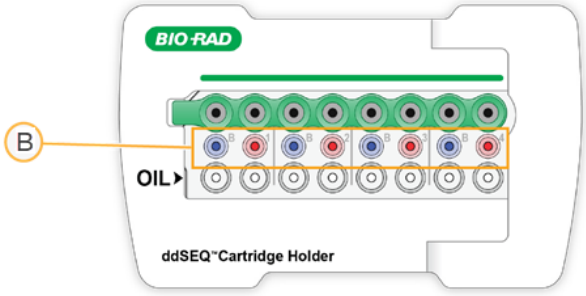

Fig. 7: ddSEQ Priming Solution wells

Row B) Priming Solution

3. Allow the ddSEQ Priming Solution to remain in the wells for 1 min.
4. Using a P20 multichannel pipet set to 25 µl, remove all 20 µl of Priming Solution in the well.
5. Repeat the aspiration of the liquid one time to ensure that all ddSEQ Priming Solution is removed from the wells.

   Important: Do not allow the ddSEQ Priming Solution to remain in the wells longer than 3 min. Ensure all ddSEQ Priming Solution is removed from the wells before proceeding to the next section.

Loading the Cartridge and Generating Droplets

Complete the following steps.

1. Using a P20 multi-channel pipet, pipet-mix the Barcode Suspension Mix and Enzyme Suspension Mix (previously prepared in an 8-tube strip) 10–15x.
2. Using the same tips, aspirate 20 µl of the Barcode Suspension Mix and Enzyme Suspension Mix and load into the alternating blue and red ports, respectively, in the middle row of the ddSEQ cartridge (Fig. 7 on page 39).

   Important: You must load all Barcode Suspension Mix and Enzyme Suspension Mix input wells. If fewer than four samples are available, fill the unused wells with 50% (v/v) glycerol solution in water.To avoid bubbles, press the pipet plunger only to the first stop.
3. To prepare the Encapsulation Oil, pour the oil into a multichannel pipet reservoir or add 90 µl to each well in an 8-tube strip.

   Tip: A bottle of Encapsulation Oil is enough for two cartridges.
4. Using a P200 multi-channel pipet, load 80 µl of room temperature Encapsulation Oil into each well of the bottom row (labeled OIL) of the ddSEQ cartridge.

   Important: Before loading, ensure the Encapsulation Oil is equilibrated to room temperature. To avoid bubbles, press the pipet plunger only to the first stop.
5. To open the ddSEQ Single-Cell Isolator, press the silver button on the top of the instrument.

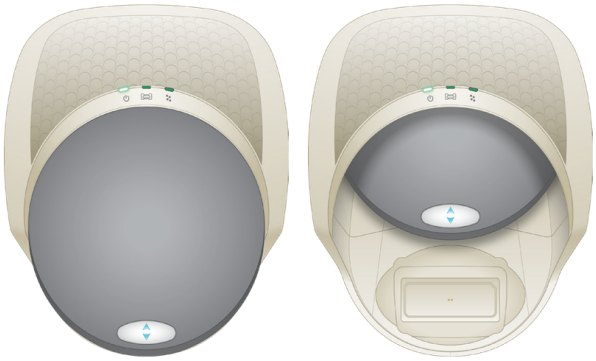

Fig. 8: Bio-Rad ddSEQ Single-cell Isolator

6. Place the cartridge holder into the instrument.

   To confirm that the cartridge holder is in the correct position, verify that the cartridge indicator light (middle light) is solid green. If the cartridge indicator light is not lit, reseat the cartridge holder on the magnetic plate.

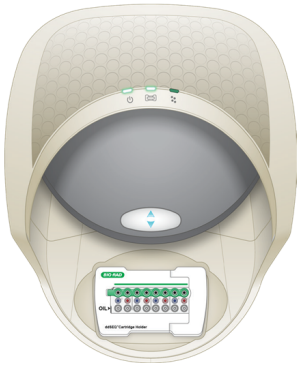

Fig. 9: ddSEQ cartridge loaded on ddSEQ Single-Cell Isolator

7. To close the instrument, press the silver button on the top of the ddSEQ Single-Cell Isolator.
   - Droplet generation begins automatically after the ddSEQ Single-Cell Isolator door is closed, and takes ~5 min
   - The droplet indicator flashes green to indicate that cell isolation is in progress.
   - When all 3 indicator lights are solid green, single-cell isolation is complete
8. To chill the plate, securely place the 96-well plate on a chilled 96-well cooling block while the cartridge is running.
9. After the run has completed, press the silver button on the front of the ddSEQ Single-Cell Isolator to open the instrument.

   If running another cartridge, wait for the instrument to reset (only the first light should be solid green) before loading the next cartridge.
10. Remove the cartridge holder from the ddSEQ Single-Cell Isolator. Samples that are successfully encapsulated appear cloudy in the output wells.

    Important: Check for wells that look clear or empty, as droplet generation might have failed.

11. Using a P50 multichannel pipet set at 43 µl, gently and slowly aspirate all encapsulated sample at a slight angle from the output wells.

    Important: Fast or harsh pipeting will break encapsulated samples. Pipet slowly to avoid yield loss.

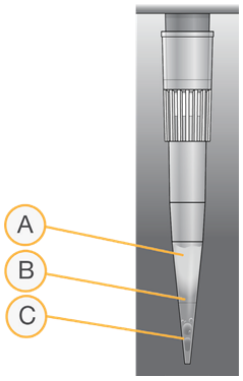

Fig. 10: Emulsion layers from output well

A) Aqueous layer
B) Oil layer
C) Oil and air bubbles

Note: Total emulsion volume transferred to each well is 35–40 µl, with approximately 5 µl of air.

12. Dispense the encapsulated sample as follows:

    a. Slowly dispense the encapsulated sample into the corresponding column of the plate, as shown in Fig. 11 on page 43. Dispensing should take approximately 5 sec.

    b. Wait 5 sec for remaining sample to collect at the tip of pipet.

       Important: Do not discard tips until all the encapsulated sample has been transferred to the plate. Discarding tips with sample results in yield loss.

c. Slowly dispense the remaining sample into the same column of the plate:

- Sample 1 from cartridge to rows A–B of the plate
- Sample 2 from cartridge to rows C–D of the plate
- Sample 3 from cartridge to rows E–F of the plate
- Sample 4 from cartridge to rows G–H of the plate

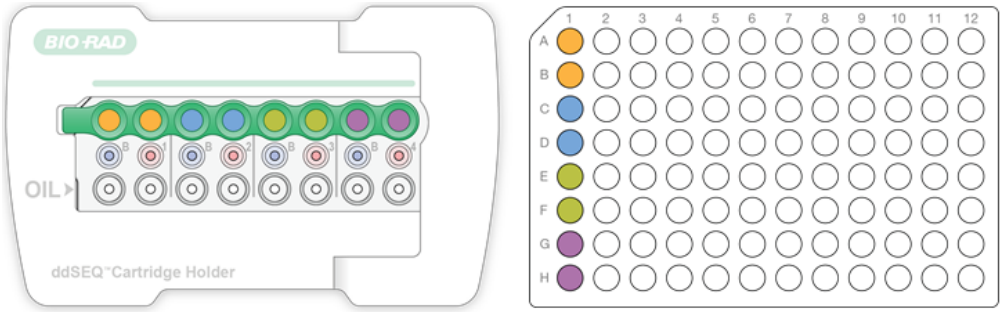

Fig. 11: Transferring encapsulated samples

13. Seal the plate with a Microseal F PCR Plate Seal or PCR Tube 8-Cap Strips.
14. If you have finished processing cartridges, proceed immediately to Barcoding and Amplification of Fragments on page 44.

Important: Do not invert the cartridge when removing it from the cartridge holder. Dispose of cartridges according to standard laboratory protocols.

Barcoding and Amplification of Fragments

Complete the following steps.

1. Place the 96-well plate on the deep well thermal cycler (P1000 or PTC Tempo).
2. Proceed with the incubation protocol in Table 18 and Table 19 until amplification is complete.

   Important: To maintain droplet integrity, always keep the plate in the 96-well cooling block on ice while transporting to the thermal cycler.

Table 18. ATAC first amplification (1)

Lid temperature	Reaction volume	Run time
105° C	50 µl	75 min

Table 19. ATAC first amplification (2)

Step	Temperature	Time	Ramp ° C/sec
1	37° C	30 min	2.0
2	85° C	10 min	2.0
3	72° C	5 min	2.0
4	98° C	30 sec	2.0
5	98° C	10 sec	2.0
6	55° C	30 sec	2.0
7	72° C	60 sec	2.0
8	Repeat steps 5–7, 7x (for a total of 8 cycles)		
9	72° C	5 min	2.0
10	4° C	Hold	2.0

This is a safe stopping point. You can store the sample at 4° C for up to 72 hr, or at –20° C for a longer period.

Emulsion Breakage and AMPure XP Cleanup

Guidelines

Note the following:

- Before use, ensure that the AMPure XP beads are at room temperature and thoroughly mixed.
- Prepare fresh 80% ethanol daily for all wash steps.
- During this step, sample wells contain separate oil and aqueous layers and you must mix the contents at the interface of the layers. When mixing, mix only in the specified layer.
- This procedure requires both a Magnetic Stand-96 (peg stand) and a DynaMag-96 Side Skirted Magnet. Both magnets are required in this protocol and are not interchangeable. Pay careful attention to which magnet is used for each step.

Table 20. Advance preparation

Item	Cap color	Catalog number	Storage	Preparation
Droplet disruptor	Clear	12008761	4° C	Immediately before use, vortex 3–5x to mix, and then centrifuge briefly. During use, you can keep this reagent at room temperature.

Procedure

Complete the following steps:

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

   Important: 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 emulsion layer on top.
3. Carefully remove the foil seal.

   Tip: If the foil seal is difficult to remove, carefully pierce openings above each sample with a pipet tip.

4. To add 20 µl of Droplet Disruptor, dispense slowly against the side of the well above each sample.

   Important: DO NOT mix or pipet Droplet Disruptor into the sample.

5. Wait 30 sec and then dispense 30 µl of nuclease-free water against the side of the well above each sample. DO NOT mix or pipet water into the sample.

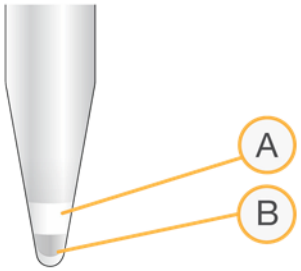

Fig. 12: Sample emulsion layers

A) Emulsion Layer
B) Oil Layer

6. Vortex the AMPure XP beads until they are well-dispersed.
7. Using either a multichannel pipet and beads poured into a reservoir, or a P100 single channel pipet, dispense 50 µl of AMPure XP beads into the upper aqueous layer of the samples.
8. Using either a P50 multichannel pipet or single channel P100 pipet, set the pipet to 50 µl and pipet-mix the AMPure XP beads in the aqueous layer only until the layer is evenly distributed (10–15x).

   Important: To mix the AMPure XP beads with the aqueous layer, aspirate the liquid from just above the interface, and then lift the tips and dispense near the top of the tube. Repeat until the entire aqueous layer is uniformly brown and any dark brown lines at the interface go into solution. If necessary, lift the plate to examine the quality of mix for the aqueous layer.

   After mixing, the samples have 2 distinct layers, an oil layer on the bottom of the well and a homogenous brown aqueous layer on the top.

Fig. 13: A lighter brown aqueous layer at the top indicates incomplete mixing

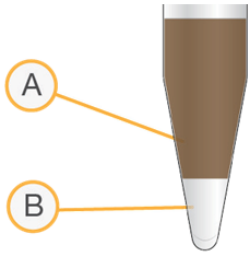

Fig. 14: Properly mixed

A) Mixed aqueous layer, homogenous
B) Oil layer

9. Incubate at room temperature for 5 min.
10. Place on a magnetic peg stand and wait 5 min.
11. Using a P200 multichannel pipet set to 200 µl, remove and discard all supernatant from each well (oil and aqueous layer). Do not disturb the bead pellet accumulated on the magnet.
12. Wash 2x as follows, keeping the plate on the magnetic peg stand:
    a. Add 200 µl of freshly prepared 80% ethanol to each well, pipeting the ethanol directly over the pellet.
    b. Incubate on the magnetic peg stand for 30 sec.
    c. Remove and discard all supernatant from each well.
    d. Repeat steps a–c to wash again.
13. Remove the plate from the magnetic peg stand and seal it with a Microseal F PCR Plate Seal or PCR Tube 8-Cap Strips.
14. To bring down any ethanol or liquid remaining on the sides of wells, centrifuge at 280 RCF for 10 sec.
15. Place the plate on the DynaMag-96 Side Skirted Magnet and wait 30 secs.
16. Using a P20 single channel pipet, remove residual 80% ethanol in each well.
17. Air-dry on the DynaMag-96 Side Skirted Magnet until there is no remaining liquid in each well.

    Important: Air-dry time varies, depending on the humidity and temperature of the room, but typically takes 3–5 min. Monitor the wells closely. If the magnetic bead pellet begins to crack, proceed immediately to the next step, as this is an indicator of over-drying and can lead to reduced yields.
18. Remove the sample plate from the DynaMag-96 Side Skirted Magnet.
19. Using a P50 multichannel pipet and reservoir, or P100 single channel pipet:
    a. Add 25 µl of Resuspension Buffer (10 mM Tris-HCl, pH 8) to each sample well to wet each pellet.
    b. Pipet to mix, making sure all beads are resuspended.

    Important: Confirm that solution is homogenous. Yield loss can occur if beads are not thoroughly resuspended.
20. Incubate at room temperature for 2 min.
21. Seal the plate using a Microseal F PCR Plate Seal or PCR Tube 8-Cap Strips and centrifuge at 280 RCF for 10 sec.
22. Place on a DynaMag-96 Side Skirted Magnet and wait 2 min.

23. Using a P100 single channel pipet, combine the 2 wells for each sample by transferring 20 µl of supernatant from each sample well to a new plate or 8-tube strip, as follows. Keep the sample on the DynaMag-96 Side Skirted Magnet during this step.

    - Sample 1, combine rows A–B in one well
    - Sample 2, combine rows C–D in one well
    - Sample 3, combine rows E–F in one well
    - Sample 4, combine rows G–H in one well

    Note: After transferring, the total volume of supernatant in each well of the new plate is 40 µl.

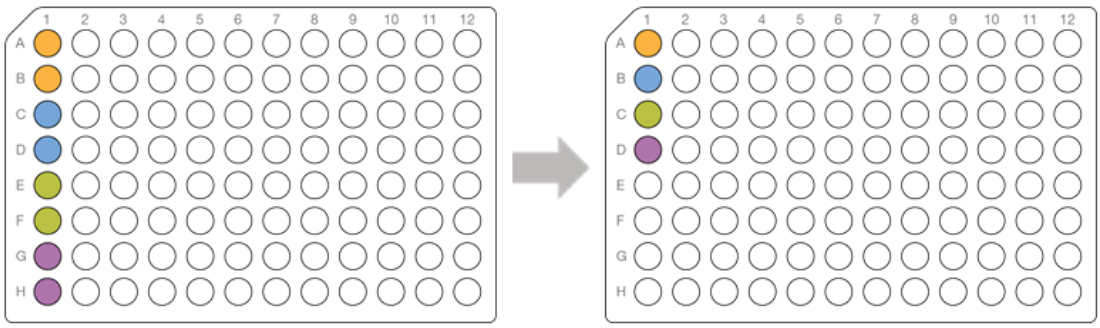

Fig. 15: Combined samples in new plate

24. Seal the plate or tubes. Vortex briefly to mix, then centrifuge using a tabletop centrifuge or plate centrifuge (500 RCF for 30 sec for plates).

This is a safe stopping point. You can store the sample at 4° C for up to 72 hr, or at –20° C for a longer period.

Second Amplification of Barcoded Fragments

Table 21. Advance preparation

Item	Cap color	Catalog number	Storage	Preparation
ATAC PCR Supermix	Clear	12008782	–20° C	Thaw at room temperature. Vortex vigorously to mix, and then centrifuge briefly. Once thawed, keep on ice.
ATAC Primer Mix	Clear	16005990	–20° C	Thaw at room temperature. Vortex vigorously to mix, and then centrifuge briefly. Once thawed, keep on ice.

Procedure

Complete the following steps.

1. To prepare the PCR master mix, add the following components in Table 22 to a 1.5 ml tube on ice. To mix, pulse vortex 3x at medium speed, 3 sec per pulse, and then centrifuge briefly.

Table 22. PCR Master Mix

Component	Catalog number	Required volume, µl (4 rxn, + 10% excess)
ATAC PCR Supermix	12008782	110.0
ATAC Primer Mix	16005990	8.8
Nuclease-free water	N/A	13.2
TOTAL		132

2. Dispense 30 µl of PCR Master Mix from step 1 into each well of a new 96-well plate.
3. Add 20 µl of purified sample to each well of the plate containing the PCR Master Mix.
4. Seal the plate using Microseal F PCR Plate Seal or PCR Tube 8-Cap Strips and vortex for 5 sec to mix.

5. Centrifuge at 500 RCF for 30 sec.
6. Place the 96-well plate on the thermal cycler and proceed with the PCR protocol specified in Table 23,Table 24, and Table 25 until amplification is complete.

Table 23. ATAC Second amplification (1)

Lid Temperature	Reaction volume	Run time
105° C	50 µl	Device dependent

Table 24. ATAC Second amplification (2)

Step	Temperature	Time	Ramp
1	98° C	30 sec	Default
2	98° C	10 sec	Default
3	55° C	30 sec	Default
4	72° C	60 sec	Default
5	Repeat steps 2–4. Refer to Table 25 for guidance on recommended number of cycles.		
6	72° C	5 min	Default
7	4° C	Hold	

Table 25. Recommended cycle number

Targeted cell throughput	Suggested total number of cycles
<2,000	9
2,000 to 3,000	8
3,001 to 5,000	7

This is a safe stopping point. You can store the sample at 4° C for up to 72 hr, or at –20° C for a longer period.

Second AMPure XP Cleanup

Guidelines

Note the following:

- Before use, ensure that AMPure XP beads are at room temperature and thoroughly mixed.
- Prepare fresh 80% ethanol daily for all wash steps.
- This procedure requires both a Magnetic Stand-96 (peg stand) and a DynaMag-96 Side Skirted Magnet.

  Important: Both magnets are required in this protocol and are not interchangeable.

Procedure

Complete the following steps:

1. Remove the 96-well plate from the thermal cycler.
2. Centrifuge the plate at 500 RCF for 30 sec.
3. Carefully remove the seal.
4. Vortex the AMPure XP beads until well-dispersed.
5. Add 50 µl of AMPure XP beads to each sample well. Pipet to mix. Ensure that all beads are resuspended and the mixture is homogenous.
6. Incubate at room temperature for 5 min.
7. Place on a magnetic peg stand and wait 5 min.
8. Using a P200 multichannel pipet set to 200 µl, remove and discard all supernatant from each well (~100 µl). Do not disturb the bead pellet accumulated on the magnet.
9. Wash 2x as follows, keeping the plate on the magnetic peg stand:
   a. Add 200 µl of freshly prepared 80% ethanol to each well.
   b. Incubate on the magnetic peg stand for 30 sec.
   c. Remove and discard all supernatant from each well.
   d. Repeat steps a–c to wash again.

10. Remove the plate from the magnetic peg stand and seal the plate with a Microseal F PCR Plate Seal or PCR Tube 8-Cap Strips.
11. To bring down any ethanol or liquid remaining on the sides of wells, centrifuge at 280 RCF for 10 sec.
12. Place on the DynaMag-96 Side Skirted Magnet and wait 30 sec.
13. Use a P20 single channel pipet to remove residual 80% ethanol in each well.
14. Air-dry on the DynaMag-96 Side Skirted Magnet until there is no remaining liquid in each well.

    Important: Air-dry time varies, depending on the humidity and temperature of the room, but typically takes 3–5 min. Monitor the wells closely. If the magnetic bead pellet begins to crack, proceed immediately to the next step, as this is an indicator of over-drying and can lead to reduced yields.
15. Remove the sample plate from the DynaMag-96 Side Skirted Magnet.
16. Using a P50 multichannel pipet and reservoir or P100 single channel pipet, add 22 µl of Resuspension Buffer (10 mM Tris-HCl, pH 8) to each sample well.
17. Pipet to mix. Ensure that all beads are resuspended.

    Important: Confirm the solution is homogenous. Yield loss can occur if beads are not thoroughly resuspended.
18. Incubate at room temperature for 2 min.

19. Seal the plate using Microseal F PCR Plate Seal or PCR Tube 8-Cap Strips.
20. Centrifuge at 280 RCF for 10 sec.
21. Place the plate on a DynaMag-96 Side Skirted Magnet and wait 2 min.
22. Transfer 20 µl of supernatant from each sample well to a new 96-well plate or 8-tube strip.

This is a safe stopping point. You can store the sample at 4° C for up to 72 hr, or at –20° C for a longer period.

Library Assessment and Quantification

Table 26. Required consumables and equipment (not provided)

Consumable/equipment	Catalog number	Supplier
Agilent 2100 Bioanalyzer	G2939BA	Agilent
High Sensitivity DNA Kit	5067-4626	Agilent
Qubit 4 Fluorometer	Q33238	Thermo Fisher Scientific
Qubit dsDNA Quantitation Assay, high sensitivity	Q32851	Thermo Fisher Scientific

Procedure

Complete the following steps.

To determine library concentration

1. Using the Qubit dsDNA HS Quantitation Assay, follow the manufacturer's instructions to prepare the Qubit working solution and standard #1 and #2 solutions.
2. To prepare 1 µl of undiluted library samples, mix with the appropriate volume of Qubit working solution.
3. Using the Qubit 4 Fluorometer, select dsDNA High Sensitivity as the assay type, and then perform a reading of standards and samples.
4. Record the library concentration in ng/µl.

To evaluate library quality

1. Using the manufacturer's instructions, run 1 µl of undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip.
2. Using the Agilent Technology 2100 Bioanalyzer Software, modify the region from 200-8000 bp.
3. Record the final library size in bp.

   Note: Typical libraries show a broad size distribution of ~250–2,000 bp and an average fragment length of 400–800 bp.

   Tip: If library concentration is >30 nM, the Bioanalyzer might be saturated. Dilute libraries 1:5 with RSB and rerun the chip.

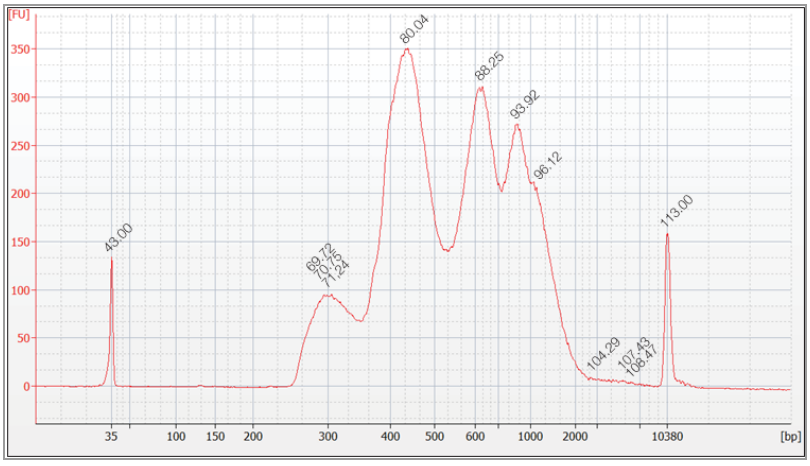

Fig. 16: Library size distribution on Agilent 2100 Bioanalyzer

Sequencing Guidelines

Guidelines

Note the following:

- Use the provided ATAC Sequencing Primer (Bio-Rad Catalog No. 16005986) as the custom Read 1 primer.
- Ensure that a custom Read 1 sequencing primer is specified as part of the run during setup. This is crucial for successful single-cell sequencing.

  Important: Do not mix the ATAC Sequencing Primer with other standard Read 1 primers, as it results in poor cluster quality and loss of reads.
- The ATAC Sequencing Primer is concentrated at 50 µM and must be diluted according to the custom sequencing primer documentation for your instrument.
- If the BaseSpace Prep Tab is not used, confirm that Illumina Experiment Manager, Version 1.13 or later, is used to set up the sequencing sample sheet.

  Note: Version 1.13 or later has the appropriate index sequences for sample demultiplexing.
- If you are demultiplexing outside of the BaseSpace Sequence Hub, confirm that bcl2fastq Version 2.18 or later is used for FASTQ Generation.

Table 27. Prepare the following consumables

Item	Catalog Number	Storage	Preparation
ATAC Sequencing Primer	16005986	–20° C	Thaw at room temperature. Vortex vigorously to mix, and then centrifuge briefly. This reagent can be kept at room temperature during use.

Custom Primer Guides

- NextSeq System Custom Primers Guide (document #15057456)

Modify SureCell ATAC-Seq Loading Concentrations

Use Table 28 to assist in determining your loading concentration. Recommendations in this table are based on quantification with the Agilent 2100 Bioanalyzer or Thermo Fisher Qubit 4 Fluorometer, and are recommended to maximize data output and retain sequencing quality. If you are quantifying with another method, further optimizations might be required.

For information, click the following hyperlinks to see pages for library quantification and unit conversion:

Converting ng/µl to nM when calculating dsDNA library concentration - Illumina Knowledge

Library quantification and manual normalization of Nextera XT libraries - Illumina Knowledge

Table 28. Determine loading concentration

Instrument	Kit	Loading concentration (pM)	PhiX
MiSeq	MiSeq Reagent Kit v3 (150 cycles)	8	0
NextSeq550	NextSeq High Output Kit v2.5 (150 cycles)	1.5	0
NextSeq2000	NextSeq 2000 P3 Reagents (200 Cycles)	450	0
NovaSeq6000	NovaSeq 6000 S2 Reagent Kit v1.5 (200 cycles)	260–300	0

Sequencing Run Parameters

SureCell ATAC-seq libraries use a custom Read 1 sequencing primer and are run using paired-end sequencing with single indexing. The supported number of cycles for each read is shown in Table 29.

Important: Due to a compatibility issue, you cannot mix the custom Read 1 primer with other Read 1 sequencing primers in the same port.

Table 29. Sequencing run parameters for NextSeq 550, NextSeq 2000, and NovaSeq 6000

Sequencing read	Recommended number of cycles	Custom primer
Read 1	118 cycles	Yes
i7 index	8 cycles*	No
i5 index	0 cycles	No
Read 2	40 cycles	No

* i7 index cycles are required only if you are combining more than one sample in the same sequencing run.

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Appendix A Supporting Information

S1: Working with Low Viability Cells

To obtain high quality data, this protocol requires the input of viable single cells in suspension. Using cells with <80% viability can lead to reduced cell throughput, and more noise in sequencing data, due to ambient DNA from dead or damaged cells.

DNase I treatment to digest ambient DNA from dead or damaged cells is a common method for increasing cell viability.

Guidelines

Note the following:

- This protocol assumes that cells are already in single-cell suspension in complete media. If using tissue, further optimizations might be required to dissociate starting material into single cells before proceeding.
- DNase I needs divalent cations; therefore, treat cells in appropriate culture media that lacks EDTA.

Table 30. Other consumables and equipment required (not provided)

Reagent/equipment	Catalog number	Supplier
Culture media (without EDTA) compatible with cell type	Varies	Varies
DNase I	Any	Any

Prepare Reagents

- Prepare culture medium with DNase I at a final concentration of 200 U/ml.
- If using powdered DNase, resuspend in Hanks Balanced Salt Solution.

Procedure

Complete the following steps:

1. Centrifuge cells at 300 RCF for 3 min at room temperature.
2. Without disturbing the cell pellet, remove all supernatant.
3. Resuspend cells in 5 ml of culture medium with DNase I.

   Tip: To minimize cell loss if you are starting with <1 M cells, resuspend in 1 ml (instead of 5 ml) of culture medium with DNase I in a 1.5 ml tube.
4. Incubate at 37° C for 30 min.
5. After 30 min, proceed immediately with Cell Preparation on page 22.

S2: Mixed Species Control (K562 & NIH-3T3)

This section contains a set of verified procedures, and required materials, to prepare fresh or cryopreserved K562 (human) and NIH-3T3 (mouse) cell lines for analysis as a mixed-species single-cell suspension.

You can use this protocol as a control in parallel with other samples. It also detects mixed-species crosstalk, thereby enabling the assessment of doublet cells. Upon completion of the protocol, cells are ready for Cell Preparation.

Guidelines

Note the following:

- These procedures have been verified for cryopreserved or fresh K562 and NIH-3T3 cells only. Further optimizations might be required for other cell types.
- If starting from fresh cells, proceed with Section S2.1 to prepare the cells. After completion of Section S2.1, skip to Section S2.3.
- If starting from cryopreserved cells, skip to Section S2.2 to thaw and prepare cells, and then continue to Section S2.3.

Table 31. Cell lines and culture media

Supplier	Cell line	Catalog number	Compatible media
ATCC	K-562 (human)	CCL-243	IMDM + 10% FBS + antibiotic
ATCC	NIH-3T3 (mouse)	CRL-1658	DMEM + 10% FBS + antibiotic

List of Materials not Included in Tables 1–3

- 37° C water bath
- Complete growth medium based on the ATCC recommendation for the respective cell type

  Important: Store at 4° C. Before cell handling and preparation, you must warm up the complete growth medium to 37° C.
- Gibco 1X PBS (Catalog No.10010023) + 0.1% BSA solution

  Important: Store this solution at 4° C at all times. While performing cell dissociation, store the solution on ice. If working under a hood, take out a large volume from the refrigerator right before use.
- Gibco TrypLE Express Enzyme (Catalog No. 12604013)

  Note: This is required only if you are starting from fresh cells.
- Gibco 1X PBS (Catalog No. 10010023)

  Note: This is required only if you are starting from fresh cells.
- Corning Life Sciences Cell Strainer CP ST 5 ml 500/CS (Catalog No. 352235), 35 µm
- Vortex mixer
- Microscope
- Biological safety cabinet (hood)
- Cell centrifuge
- Standard cell culture lab equipment

S2.1: Using Fresh Cells

Cell Dissociation Protocol for Fresh NIH-3T3 Cells

This protocol is optimized for cells cultured in a T75 cell culture flask.

Complete the following steps:

1. Warm the required media from the refrigerator to 37° C (~30 min).
2. Remove the flask of NIH-3T3 cells from the incubator and check the cells under the microscope.

   Tip: Use cells at a confluency between 60–90%.
3. Without touching the surface of the flask, carefully remove all the media.
4. Rinse attached cells using 7 ml of 1X PBS.
5. Remove and discard all the PBS.
6. For a regular T-75 flask, add 2.5 ml of TrypLE Express to cover the entire surface of the flask.
7. Place flask in the incubator for 3–5 min.
8. Remove the flask and verify that cells have detached or are starting to detach.

   Important: Do not tap the flask to detach cells. Allow the protease to detach cells and dissociate them.
9. To neutralize the TrypLE Express, add 7.5 ml of warm medium to the flask.
10. Using a serological pipet, pipet up and down 10–15x to break the cell clumps.
11. Transfer the cell suspension to a 15 ml conical tube.

Cell Preparation for Fresh K562 Cells

Complete the following steps.

1. Remove the flask of K562 cells from the incubator and check the cells under the microscope.
2. Using a serological pipet, pipet up and down to mix the cells in the flask.
3. Transfer the cell suspension to a 15 ml conical tube.
4. After completing cell preparation for fresh NIH-3T3 and K562 cells, skip to Section S2.3.

S2.2: Using Cryopreserved Cells

Complete the following steps in parallel to prepare both K562 and NIH-3T3 cells.

1. Warm the required complete growth media from the refrigerator to 37° C.
2. Before starting this protocol, set the water bath to 37° C and ensure that it has reached the desired temperature.
3. Remove a cryovial of frozen cells from liquid nitrogen storage.

   Important: When handling liquid nitrogen, wear cryogloves, safety glasses, a lab coat, and close-toed shoes and carefully follow all instructions and precautions.
4. Place the cryovial in the 37° C water bath and let it thaw for *no longer than* 1–3 min.
5. When the cells are almost completely thawed, and only a tiny ice crystal remains in the tube, remove the cryovial from the water bath.

   Important: Do not leave the vial in the water bath for longer than 5 min.
6. Add 1 ml of warmed media dropwise (1 drop per sec).
7. Mix the cells by gently pipeting 10x and transfer the entire volume to a 15 ml conical tube.
8. Rinse the cryovial with 1 ml warm complete growth media.
9. Add the rinse medium dropwise (1 drop per 3 sec) to the 15 ml conical tube while gently swirling the tube.
10. Add 9 ml of warm complete growth media slowly to the 15 ml conical tube (about 0.5 ml per sec).
11. Gently invert the conical tube 4x to mix.
12. Centrifuge the cells at 300x g for 3 min.

    Important: Do not use a chilled centrifuge.
13. Without disturbing the cell pellet, discard the supernatant as follows:
    a. Using a serological pipet, remove all but ~1 ml.
    b. Using a P1000 pipet, remove the remaining liquid.
14. Resuspend the cell pellet in 10 ml of warm complete growth media.
15. Proceed immediately to Section S2.3.

S2.3: K562 and NIH-3T3 Cell Preparation

Complete the steps in Cell Preparation on page 22 and then complete the following critical additional notes and steps:

1. At the beginning of the cell preparation procedure, place 2 (1 for each cell type) 35 µm cell strainers, in their collection tubes, on ice.
2. You must perform all steps from Cell Washing and Filtering on page 23 to Cell Viability and Cell Count Measurements on page 24 on each cell type individually.

   Important: Do not mix cells until all steps in Cell Viability and Cell Count Measurements are completed.
3. After the steps in Cell Viability and Cell Count Measurements are completed, mix together an equal number of K562 and NIH-3T3 cells, as follows:
   - For a single sample, mix 75,000 cells from each species to yield a total of 150,000 cells.
   - For technical replicates, make additional aliquots of 150,000 mixed species cells.
4. Store all mixed-species aliquots on ice.

Appendix B Ordering Information

Table 32. Ordering information

Catalog number	Description
12004336	ddSEQ Single Cell Isolator, which includes the instrument and associated component consumables
17004620	SureCell ATAC-Seq Library Prep Kit, which includes ■ SureCell ATAC-Seq Reagent Box A ■ SureCell ATAC-Seq Reagent Box B ■ ddSEQ M Cartridges
12009360	SureCell ATAC-Seq Index Kit

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France *00 800 00 24 67 23* ***Germany*** *00 800 00 24 67 23* ***Hong Kong*** *852 2789 3300* ***Hungary*** *00 800 00 24 67 23* ***India*** *91 124 4029300* ***Israel*** *0 3 9636050*
Italy *00 800 00 24 67 23* ***Japan*** *81 3 6361 7000* ***Korea*** *82 080 007 7373* ***Luxembourg*** *00 800 00 24 67 23* ***Mexico*** *52 555 488 7670*
The Netherlands *00 800 00 24 67 23* ***New Zealand*** *64 9 415 2280* ***Norway*** *00 800 00 24 67 23* ***Poland*** *00 800 00 24 67 23* ***Portugal*** *00 800 00 24 67 23*
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