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Illumina Single Cell 3′ RNA Prep, T10

Product Documentation

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Introduction

The Illumina Single Cell 3′ RNA Prep, T10 kit uses particle-templated instant partitions (PIPs) to combine single cell or nuclei suspensions with barcoded beads to produce sequencing-ready libraries in less than two days. The assay supports eight individual reactions. Each reaction profiles ~ 10,000 cells or nuclei.

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Consumables & Equipment

The Illumina Single Cell 3′ RNA Prep, T10 protocol requires the following Illumina-supplied and user-supplied consumables and equipment.

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

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

Product Contents

The protocol requires one capture and barcoding kit, one library preparation kit, and one of the following starter equipment kits. The index adapters plate is only required to pool more than eight libraries together in a sequencing run.

If you are purchasing the Illumina Single Cell Vortexer and Accessories kit, a third-party dry bath is required. Refer to *User-Supplied Consumables & Equipment* on page 5 for more information on the third-party dry bath and accessories supported with this workflow.

Component	Kit Name	Illumina Catalog #
Library prep with capture and barcoding*	Illumina Single Cell 3′ RNA Prep, T10 (8 samples, 10,000 cells/sample)	20135691
Capture and barcoding only	Illumina Single Cell 3′ RNA Capture, T10 (8 samples, 10,000 cells/sample)	20132791
Library prep only	Illumina Single Cell Library Prep (8 reactions)	20132789
[Optional] Index adapters	Illumina Single Cell Unique Dual Indexes (96 Indexes, 96 Samples)	20132788
Starter equipment	Illumina Single Cell Prep Starter Equipment	20132796
	Illumina Single Cell Vortexer and Accessories	20145920

* Bundled capture and barcoding and library prep reagent kit option

Reagent Kit Contents

Each capture and barcoding kit comprises four boxes and an accessories bag. Each library prep reagent kit comprises two boxes. The following tables list the contents of each box and bag.

Illumina Single Cell 3′ RNA Capture, T10 Contents

Illumina Single Cell 3′ RNA Capture, T10, Box 1, Store at 15°C to 30°C

Quantity	Reagent	Description
1	PAR	Partitioning Reagent
1	CLB3	Chemical Lysis Buffer 3
1	DPR	Departitioning Reagent

Illumina Single Cell 3′ RNA Capture, T10, Box 2, Store at 2°C to 8°C

Quantity	Reagent	Description
1	IPB2	Illumina Purification Beads 2
1	BBF	Breaking Buffer
1	PWB1	Wash Buffer
1	CEB	cDNA Extraction Buffer

Illumina Single Cell 3′ RNA Capture, T10, Box 3, Store at -25°C to -15°C

Quantity	Reagent	Description
1	NSB	Nuclei Suspension Buffer
1	RTEM	RT Enzyme Mix
2	RTAM	RT Additive Mix
1	PMM4	4X PCR Master Mix
1	WTAP	WTA Primer
1	RNI	RNase Inhibitor (40 U/µl)
1	CSB	Cell Suspension Buffer
1	BSA	25% BSA

Illumina Single Cell 3′ RNA Capture, T10, Box 4, Store at -85°C to -65°C

Quantity	Reagent	Description
8	PIP	Particle-Templated Instant Partition
4	TSO1	Template-Switch Oligo

Illumina Single Cell 3′ RNA Capture, T10, Accessories Bag

Quantity	Component
32	1 ml syringe

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Quantity	Component
32	22G blunt-bottom syringe needle
16	0.5 ml Safe-Lock PCR Clean tubes, emulsion safe
24	1.5 ml Safe-Lock PCR Clean tubes, emulsion safe

Illumina Single Cell Library Prep Contents

Illumina Single Cell Library Prep, Box 1, Store at 2°C to 8°C

Quantity	Reagent	Description
1	IPB2	Illumina Purification Beads 2
1	NFW	Nuclease-Free Water

Illumina Single Cell Library Prep, Box 2, Store at -25°C to -15°C

Quantity	Reagent	Description
1	LAM	Library Adapter Mix
1	LPB	Library Prep Buffer
1	LPE	Library Prep Enzymes
1	LPMA	Library Prep Mix A
1	PMM4	4X PCR Master Mix
1	UDI8	Illumina Single Cell Prep UDI Mix Strip

Starter Equipment Kit Contents

The following tables list the contents of each starter equipment kit option.

Illumina Single Cell Prep Starter Equipment Kit Contents

Quantity	Component Name	Used for Kit Size
1	PIPseq Vortex Mixer	All
1	PIPseq rotating vortex assembly for 5 ml tubes	T100
1	PIPseq rotating vortex assembly for 1.5 ml tubes	T20
1	PIPseq rotating vortex assembly for 0.5 ml tubes	T2 and T10
2	Rotating vortex base assembly	All
1	US Power Supplies for Vortex Mixer	All
1	Vortexer gap cover	All

Quantity	Component Name	Used for Kit Size
2	PIPseq combination 1.5 ml and 0.5 ml tube stand	T2,T10,T20
1	PIPseq guide rack	T2
2	International Plug Converter	All
1	PIPseq Dry Bath 5 ml block	T100
1	PIPseq Dry Bath 1.5 ml block	T20
1	PIPseq Dry Bath 0.5 ml block	T2 and T10
1	US Power Supplies for PIPseq Dry Bath	All
1	PIPseq Dry Bath with heated lid, which includes the following materials: • 2.5 mm Allen key wrench, silver • PIPseq Dry Bath Stylus • Replacement tips for PIPseq Dry Bath Stylus • 5x20 mm 5A 250V Fast blow fuse for PIPseq Dry Bath • Dry block removal tool, white	All

Illumina Single Cell Vortexer and Accessories Kit Contents

Quantity	Component Name	Used for Kit size
1	PIPseq Vortex Mixer	All
1	PIPseq rotating vortex assembly for 5 ml tubes	T100
1	PIPseq rotating vortex assembly for 1.5 ml tubes	T20
1	PIPseq rotating vortex assembly for 0.5 ml tubes	T2 and T10
2	Rotating vortex base assembly	All
1	US Power Supplies for Vortex Mixer	All
1	Vortexer gap cover	All
2	PIPseq Combination 1.5 ml and 0.5 ml tube stand	T2,T10,T20
1	PIPseq guide rack	T2
2	International Plug Converter	All

User-Supplied Consumables & Equipment

The following sections provide information on the required user-supplied consumables and equipment.

The specific brands of plastic consumables have been tested for stability of PIP emulsions. Substituting these materials can adversely affect performance.

Consumables

Consumable	Supplier
0.2 ml PCR 8-tube strips and caps	One of the following suppliers: • Greiner Bio-One, catalog # 673210 (tubes) and catalog # 373270 (caps) • AXYGEN, catalog # PCR-0208-A (tubes) and catalog # PCR-02CP-A (caps) • USA Scientific, catalog # 1402-1700 • Millipore Sigma, catalog # BR781327
0.5 ml Safe-Lock PCR Clean tubes, emulsion safe	One of the following suppliers: • VWR, catalog # 62111-750 • Fisher Scientific, catalog # 05-402-93 • Eppendorf, catalog # 022363719
1.5 ml Safe-Lock PCR Clean tubes, emulsion safe	One of the following suppliers: • VWR, catalog # 62111-752 • Fisher Scientific, catalog # 05-402-94 • Eppendorf, catalog # 022363212
[Fresh cells] 5 ml screw-cap tubes, sterile, PCR Clean, emulsion safe	One of the following suppliers: • Eppendorf, catalog # 30122321 • Fisher Scientific, catalog # 13-864-407
[Cryopreserved cells] 15 ml polypropylene centrifuge tubes, sterile	Corning, catalog # 430790 or general lab supplier
Absolute ethanol, molecular biology grade	General lab supplier
[Isolated nuclei input] BSA, molecular biology grade	General lab supplier
Cell counting materials (eg, AO/PI)	General lab supplier
Nuclease-free water	Thermo Fisher, catalog # AM9937 or general lab supplier
Nuclease-free 1X low EDTA TE Buffer (IDTE), pH 8.0, 10 mM Tris, 0.1 mM EDTA	IDT, catalog # 11-05-01-09 or general lab supplier
Pipette tips, filtered, low-retention, sterilized, 20 µl	One of the following suppliers: • Rainin, catalog # 30389226 • Filtrous, catalog # PTF-LS-0020 • VWR, catalog # 89031-352

Consumable	Supplier
Pipette tips, filtered, low-retention, sterilized, 200 µl	One of the following suppliers: • Rainin, catalog # 30389240 • Filtrous, catalog # PTF-LS-0200 • VWR, catalog # 89031-374
Pipette tips, filtered, low-retention, sterilized, 1000 µl	One of the following suppliers: • Rainin, catalog # 30389213 • Filtrous, catalog # PTF-LS-1000 • VWR, catalog # 89031-414
Pipette tips, wide-bore, filtered, low-retention, sterilized, 200 µl	Rainin, catalog # 30389241
Pipette tips, wide-bore, filtered, low-retention, sterilized, 1000 µl	Rainin, catalog # 30389218
Quantification consumables, depending on system: • Bioanalyzer High Sensitivity DNA Kit • Tapestation High Sensitivity D5000 (for cDNA) • Tapestation High Sensitivity D1000 (for NGS library)	One of the following suppliers, depending on system: • Agilent, catalog # 5067-4626 • Agilent, catalog # 5067-5592 and 5067-5593 (HSD5000) • Agilent, catalog # 5067-5584 and 5067-5585 (HSD1000)
Qubit 1X dsDNA High Sensitivity Assay Kit	Thermo Fisher, catalog # Q33230 or Q33231
Qubit Assay Tubes	Thermo Fisher, catalog # Q32856
Solid waste container*	General lab supplier
[Isolated nuclei input] RNase Inhibitor	One of the following suppliers: • Thermo Fisher, catalog # AM2694 • Sigma, catalog # RNAINH-RO • Solis Biodyne, catalog # 06-26-4000U • Watchmaker Genomics, catalog # 7K0088
[Cryopreserved cells] Thawing media (use thawing media that is recommended for your sample type)	General lab supplier

* Determine the appropriate waste container in accordance with the applicable regional, national, and local laws and regulations.

Equipment

Equipment	Supplier
0.2 ml/1.5 ml Magnetic Separation Combo Rack	Permagen, MSR1224B or equivalent from alternative supplier
Benchtop centrifuge with swinging bucket rotor, suitable for 5/15 ml tubes	General lab supplier, recommend Thermo Scientific ST40
Benchtop mini microcentrifuge 2000 × g, suitable for 0.5/1.5 ml tubes and PCR tube strips	Thermo Fisher, catalog # 75004061 or general lab supplier
Hemocytometer or automated cell counter	General lab supplier
Ice bucket or cold blocks, suitable for 0.2/0.5 ml PCR tubes and 1.5 ml microcentrifuge tubes	General lab supplier
Micropipettes, 1–1000 µl capabilities (Rainin LTS-tip compatible pipettes are required if you are using the recommended Rainin pipette tips)	General lab supplier
Quantification system, either of the following: • Bioanalyzer 2100 • Tapestation 4200	One of the following suppliers, depending on system: • Agilent, catalog # G2940CA • Agilent, catalog # G2991BA
Qubit Fluorometer	Thermo Fisher
Thermal cycler	General lab supplier
Vortexer	General lab supplier
[Optional] Dry bath* The following components must be ordered separately: • Heated lid • 0.5 ml block and transfer rack	Eppendorf, catalog # 5382000023 or 5383000027 Eppendorf, catalog # 5308000003 Eppendorf, catalog # 5361000031

* The dry bath and its associated components are only required if you purchased the Illumina Single Cell Vortexer and Accessories starter equipment kit.

Equipment Preparation

This section provides instructions for preparing equipment for use with the Illumina Single Cell 3′ RNA Prep, T10 kit. For additional dry bath and vortex mixer documentation, including detailed installation, and operation instructions, refer to the Illumina Single Cell 3' RNA Prep pages on the Illumina support site. Make sure that you are familiar with the operation of all equipment before starting the protocol.

Starter equipment documentation:

- *PIPseq Vortex Mixer Operating Instructions (document # 200064269)*
- *PIPseq Vortexer Adapter Installation (document # 200064267)*
- *Dry Bath Incubator with Heated Lid Operating Instructions (document # 200064268)*

PIPseq Vortexer Operation

The protocol requires a vortexer with a 0.5 ml vortexer head. Make sure that the appropriate head is installed with the thumbscrew inserted into the front of the vortexer.

Review the process for rotating the vortex head from horizontal to vertical tube position. The process should be completed in less than 30 seconds.



Horizontal tube position



Vertical tube position

Dry Bath Operation

The protocol requires a dry bath with 0.5 ml tube block. Make sure that the appropriate block is installed.

The dry bath steps specified in this protocol are specific to the PIPseq dry bath sold as part of the Illumina Single Cell Prep Starter Equipment Kit. If you are using a user-supplied Eppendorf dry bath, you must manually program the lysis and reverse transcription protocols. Refer to Eppendorf's documentation for more detailed information on how to operate their products.

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Dry Bath Lid Control

Lid temperature is set manually and is independent from the dry bath programs. Understand how to configure lid mode and temperature before starting the protocol.

- The dry bath interface displays **LidOn** if the lid temperature control is not active. Select **LidOn** to activate the lid temperature control. When the lid temperature control is active, the interface displays **LidOff**.
- For nuclei lysis and reverse transcription, use the specific temperature control mode to set the lid temperature to 105°C. When set to this mode, the lid temperature box shows the set value (for example, 105.0).
- For cell lysis, use the offset temperature control mode to set the lid temperature to 5°C higher than the heat block. When set to this mode, the lid temperature box shows the temperature offset (for example +5.0).
- To change the lid mode, select **Edit**, and then select **LidMode** to toggle between 105°C and +5.0, and then select **Save/Return**.
- The lid temperature only climbs to 105°C during incubation temperatures of 35°C or higher.

⚠ The device can reach high temperatures and cause burns. Exercise caution while using the device, and do not touch the metal block or the heated lid when they are hot.

Dry Bath Programs

The dry bath is preprogrammed with nuclei lysis, cell lysis, and reverse transcription protocols.

ℹ Preheat and hold settings are displayed as 00:00 on the dry bath.

Program A (Cell Lysis)

- Preheat to 25°C
- 25°C for 15 minutes
- 37°C for 45 minutes
- 25°C for 10 minutes
- Hold at 20°C

Program B (Nuclei Lysis)

- Preheat to 66°C
- 66°C for 45 minutes
- 25°C for 10 minutes
- Hold at 20°C

Program C (Reverse Transcription)

- Preheat to 25°C

- 25°C for 30 minutes
- 42°C for 90 minutes
- 85°C for 10 minutes
- Hold at 4°C

Sample Preparation

The Illumina Single Cell 3′ RNA Prep protocol requires a high-quality single cell or nuclei suspension as input. Derive cells or nuclei from cell cultures, dissociated tissues, cell sorting, or other isolation methods.

The following guidelines apply to sample preparation:

- Minimize the presence of dead cells or aggregates to ensure the highest quality data.
- Minimize the amount of time that it takes for sample preparation through capture and lysis, and add RNase inhibitor at 0.4–1 U/µl final concentration to buffers being used in lengthy steps (dissociation, enrichment, sorting collection tubes, or sample suspension buffers).
- Use higher RNase inhibitor concentrations for challenging sample types.
- When processing many samples together at once, Illumina recommends processing sample preparations in batches through capture and lysis to prevent mRNA degradation.

Refer to the *Prepare Cell Suspension* on page 18 or *Prepare Nuclei Suspension* on page 21 for sample preparation instructions depending on input type. Start capture and lysis as soon as possible after diluting samples to the target concentration.

Cell and Nuclei Input Recommendations

This protocol describes the steps to profile ~10,000 cells or nuclei in each reaction. Illumina recommends an input of ~17,000 cells or nuclei into each reaction to recover ~10,000 cells or nuclei with a multiplet rate of < 8%. The optimal input concentration is 4250 live cells or nuclei/µl when adding 4 µl cells or nuclei plus 1 µl (40 units) RNase inhibitor into each PIP tube. If it is not possible to concentrate to 4250 cells or nuclei/µl, you can input a larger volume as long as the total loading volume including RNase inhibitor is ≤ 6 µl. Increased cell or nuclei input increases the observed multiplet rate.

Working with Fixed Cells

Fixed cells generated according to *DSP-Methanol Fixation for Cells Protocol Instructions* are compatible with this workflow. Before starting the fixation protocol, complete a 1 ml wash step using Cell Suspension Buffer, then resuspend in Cell Suspension Buffer that does not include additional BSA or RNase inhibitor to remove any inhibitors or other contaminants. This fixative yields comparable performance as fresh cells. Fixed cells can be stored for up to seven days at -25°C to -15°C. Alternative fixation approaches (for example, formaldehyde) have not been validated and are not recommended.

Working with Fixed Nuclei

Fixed nuclei are compatible with this workflow. Refer to *DSP-Methanol Fixation for Nuclei Protocol Instructions* for instructions on fixing isolated nuclei. Before starting the fixation protocol, nuclei must be resuspended in Nuclei Suspension Buffer (NSB) without BSA or RNase inhibitor. Illumina

recommends substituting NSB working solution for the wash steps in the alternative nuclei fixation protocol (at least 1 ml of NSB working solution without BSA or RNase inhibitor in up to two wash steps and the final resuspension before fixation). Fixed nuclei can be stored for up to two months at -25°C to -15°C.

Protocol

This section describes the Illumina Single Cell 3′ RNA Prep, T10 protocol. Safe stopping points are indicated at the end of applicable steps.

- Review the planned complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Before proceeding, confirm the kit contents and make sure that you have the necessary components, equipment, and consumables. For a complete list, refer to *Consumables & Equipment* on page 2.
- Follow the protocol in the order described using the specified parameters. Unless a safe stopping point is specified, move immediately to the next step.
- Avoid extended pauses until RNA is converted into double-stranded cDNA.

Illumina Single Cell 3′ RNA Workflow



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Tips and Techniques

Avoiding Cross-Contamination

- When adding or transferring samples or reagent master mixes, change tips between ***each sample*** unless instructed otherwise.
- Use separate areas for pre-PCR and post-PCR processes. PCR products can contaminate reagents, instruments, and samples, delaying normal operations and causing inaccurate results.
  - No material from a post-PCR work space should be moved into a pre-PCR workspace.
  - For some reagents and consumables, multiple sets may be required for pre-PCR and post-PCR area to avoid carryover contamination.
  - Complete the initial steps of this workflow in a pre-PCR area. To avoid PCR contamination, complete steps starting from the thermal cycling step in cDNA amplification in a post-PCR area (refer to *Amplify cDNA* on page 35 for more details). Complete all remaining steps in the post-PCR workspace.
  - If it is not possible to maintain two separate workspaces for pre and post-PCR work, Illumina recommends keeping two separate sets of consumable materials and to maintain strict workplace sterility. Make sure all benches and equipment are regularly cleaned with a bleach solution or a commercial DNA degrader to reduce contaminating amplicons. Always wear clean gloves.

Handling Reagents and RNA

- Keep thawed reagents on ice until needed unless otherwise specified. Promptly return all reagents to storage after use.
- When not in use, close lids and avoid touching the rims of tubes to limit contamination.
- Maintain a clean work space. Frequently decontaminate surfaces using a 70% isopropyl alcohol cleaning solution and work in the biosafety cabinet when instructed. Always wear appropriate PPE and handle reagents carefully.
- To avoid RNase contamination, occasionally clean surfaces using an RNase inactivation solution.

Handling Beads

- Do not overdry beads. Proceed to the next step when the magnetic beads have air dried to the point of only a slight gloss remaining, but before they show cracks. This can take as few as 2 minutes depending on the tube size and humidity level.

Handling PIPs

- PIPs are transparent and difficult to see when pelleted or resuspended.
- Dispense PIPs slowly to ensure that they do not stick to pipette tips.
- When removing supernatant from pelleted PIPs, do not disrupt or aspirate PIPs.

Centrifugation

- Complete all centrifuge steps in the Illumina Single Cell 3′ RNA workflow after sample preparation with a benchtop minifuge using speeds of 2000 × g.
- Always use the power button to turn the benchtop minifuge off for a gradual stop to maintain well packed PIPs. Do not apply standard brakes, like opening the lid.
- Use 0.5 ml tube inserts in the benchtop minifuge to prevent tube opening.

Thermal Cycler Lid Pressure

- Follow the manufacturer's instructions for using PCR tube strips with your brand of thermal cycler. Thermal cyclers with a lot of lid pressure can cause the 8-tube strip to collapse during PCR amplification steps when only one is used. Use empty tube strips as braces surrounding both sides of the 8-tube strip to help balance the lid pressure for brands that do not include plastic frame supports.

PCR 8-Tube Strip Lids

- When vortexing 8-tube strips, apply pressure to the lid to maintain a tight seal.
- Replace the lids on the 8-tube strip after each thermal cycler program using the 105°C lid setting to prevent lid damage.

Multichannel pipetting

- When centrifuging tubes in order to separate PIPs from supernatant, position all of the tubes in the same orientation within the centrifuge. This causes the PIPs to slope in the same direction, making it easier to use a multichannel pipette.
- When splitting multiple samples into separate wells, split each sample into adjacent wells in the 8-tube strip. This makes it easier to recombine the samples using a multichannel pipette.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

1. Remove sample from storage.
2. Remove third-party reagents from storage.

   Third-Party Reagents

Reagent	Storage	Instructions
RNase Inhibitor*	Varies for third-party reagents	Keep on ice. Refer to the manufacturer instructions.
[Cryopreserved cells] Thawing media	Varies for third-party reagents	Refer to the manufacturer instructions.

   * For cell and nuclei suspensions, use user-supplied RNase Inhibitor.

3. Remove the reagents from the box and thaw as follows.

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15°C to 30°C Storage

Reagent	Box Name	Instructions
PAR	Illumina Single Cell 3′ RNA Capture, T10, Box 1	Use at room temperature.
CLB3	Illumina Single Cell 3′ RNA Capture, T10, Box 1	Use at room temperature.

PAR may have a cloudy appearance. This does not affect assay performance.

-25°C to -15°C Storage

Reagent	Box Name	Instructions
[Cell preparation] CSB	Illumina Single Cell 3′ RNA Capture, T10, Box 3	Thaw and warm in a water bath set to 37°C. **[Optional]** If preparing fresh cells, CSB can be cooled on ice after thawing and used cold in step 5c.
[Nuclei preparation] NSB	Illumina Single Cell 3′ RNA Capture, T10, Box 3	Use at room temperature.
RNI*	Illumina Single Cell 3′ RNA Capture, T10, Box 3	Keep on ice.
[Nuclei preparation] BSA	Illumina Single Cell 3′ RNA Capture, T10, Box 3	Thaw on ice.

* For capture and lysis, use Illumina-supplied RNI.

-85°C to -65°C Storage

Reagent	Box Name	Instructions
PIP	Illumina Single Cell 3′ RNA Capture, T10, Box 4	Thaw one tube per sample on ice. Stable on ice for up to 5 hours.

Prepare Cell Suspension

This is an example protocol for preparing single-cell suspensions from mammalian fresh or cryopreserved cells for capture and lysis. If you are preparing single cell suspensions from tissues or fragile cell types, additional dissociation or cell handling steps might be required. Perform cell preparation in a biosafety cabinet using best practices for working in a sterile environment.

The following procedure steps might differ based on your cell type or expected number of input cells:

- Centrifugation steps 3 and 5f
- Cell resuspension in step 6
- Final cell dilution in step 9

If you are using an alternative cell preparation protocol, use this protocol as guidance. In your alternative protocol, it is critical to achieve high cell viability and to perform the final cell dilution with the CSB provided in the Illumina Single Cell 3′ RNA Prep, T10 Kit.

Consumables

- CSB (Cell Suspension Buffer)
- **[Cryopreserved cells]** Thawing media
- **[Fresh cells]** 5 ml screw-cap tube
- **[Cryopreserved cells]** 15 ml conical tube
- 1000 µl wide-bore, low-retention, pipette tips
- Cell counting materials

Preparation

1. **[Cryopreserved cells]** Prepare thawing media as follows.
   a. Label a 15 ml conical tube ***Cell Stock***.
   b. Add 9 ml thawing media to the ***Cell Stock*** tube.
   c. Warm to 37°C in a water bath.
2. **[Fresh cells]** Prepare fresh cells as follows.
   a. Label a sterile 5 ml screw-cap tube ***Cell Stock***.
   b. Transfer cells to the ***Cell Stock*** tube.
3. CSB—Confirm reagent is fully thawed and continue warming to 37°C in a water bath.

Procedure

If you are starting from fresh cells, proceed to step 2.

1. **[Cryopreserved cells]** If starting from cryopreserved cells, thaw as follows.
   a. Retrieve cryopreserved cell vial from liquid nitrogen tank and immediately place in 37°C water bath.
   b. Let the cell vial stand in the water bath until a small amount of ice remains (1–2 minutes).
   c. Remove the cell vial and the ***Cell Stock*** conical tube from the water bath.

   The remaining frozen cells thaw at room temperature (~ 1 minute).

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d. Decontaminate the outside of the cell vial and ***Cell Stock*** tube with 70% isopropyl alcohol, and then move into the biosafety cabinet.
e. Using a 1000 µl wide-bore pipette tip, slowly pipette the cell suspension in the cell vial to mix.
f. Transfer the cell suspension from the cell vial to the ***Cell Stock*** tube.
g. Cap the ***Cell Stock*** tube, and then gently invert 5 times to mix.

2. Using the following table, choose the centrifugation speed and time recommended to pellet your specific cell type.

Cell Input	Speed	Time (mins)
Most mammalian cell types	200 × g	5
Small cell types (PBMCs)	350 × g	5
Other cell types	Optimize	Optimize

3. Using a centrifuge with a swinging bucket rotor (70–80% maximum braking speed), centrifuge the ***Cell Stock*** tube at the determined speed and time for your cell type at room temperature.
4. Without disturbing the pellet, remove and discard the supernatant.

> The following wash step removes assay inhibitors from the cell suspension (FBS, BSA > 1%, high salt or calcium, carryover organic solvents). Failure to remove inhibitory reagents can cause cDNA QC failure.

5. Wash cells in the ***Cell Stock*** tube as follows.
   a. Remove CSB from the water bath.
   b. Decontaminate the outside of the CSB tube with 70% isopropyl alcohol, and then move into the biosafety cabinet.
   c. Add 2 ml of warm CSB to the ***Cell Stock*** tube, and then place the remaining CSB on ice to cool.
   d. Using a 1000 µl wide-bore pipette tip, slowly pipette 5 times to mix.
   e. Using the following table, choose the centrifugation speed and time recommended to pellet your specific cell type.

Cell Input	Speed	Time (mins)
Most mammalian cell types	200 × g	3
Small cell types (PBMCs)	350 × g	3
Other cell types	Optimize	Optimize

   f. Using a centrifuge with a swinging bucket rotor (70–80% maximum braking speed), centrifuge the ***Cell Stock*** tube at the determined speed and time to pellet your cells at room temperature.
   g. Without disturbing the pellet, remove and discard the supernatant.
6. Using a standard-bore pipette tip, resuspend cells in 100–500 µl chilled CSB to achieve the target concentration. Gently pipette 10–15 times to mix.

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For very high cell concentrations, perform a serial dilution using up to 500 ul chilled CSB to achieve the target concentration.

Use the minimum force and number of pipette mixes necessary to create a homogeneous cell suspension. If clumps are visible, increase the amount of force used during pipette mixing.

7. Place the ***Cell Stock*** tube on ice.
8. Use a cell counter to determine cell concentration.

   Cells should have > 90% viability to proceed. If necessary, perform additional wash steps to remove debris and improve viability. Always perform a final count.

9. Using a wide-bore pipette tip, prepare 4250 live cells/µl suspension in CSB to achieve an input volume of 4 µl (17,000 live cells).
   If it is not possible to concentrate cells to 4250 cells/µl, a larger volume can be input as long as the total loading volume including RNI is ≤ 6 µl during *Capture and Lysis* on page 23.
10. Repeat the cell count described in step 8 to confirm the final concentration in the prepared suspension. Adjust concentration and repeat count as necessary.
11. After the required concentration is achieved, place the cell suspension on ice and proceed immediately to *Capture and Lysis* on page 23.

Prepare Nuclei Suspension

Illumina recommends preparing nuclei suspensions using the Illumina Single Cell Nuclei Isolation Kit to ensure consistent, high-quality nuclei across frozen mammalian tissues. Refer to the *Illumina Single Cell Nuclei Isolation Product Documentation* for instructions. After preparing the nuclei suspension, place samples on ice and proceed immediately to *Capture and Lysis* on page 23.

Do not freeze isolated nuclei.

If you are not proceeding directly to capture and lysis, Illumina recommends fixing nuclei to prevent mRNA degradation. Refer to *Working with Fixed Nuclei* on page 12 for details.

If using an alternative nuclei isolation protocol, refer to *Alternative Nuclei Isolation Protocol Guidance* on page 21.

Alternative Nuclei Isolation Protocol Guidance

When using an alternative nuclei isolation protocol, follow these guidelines to make sure that the samples are compatible with this workflow.

- When preparing nuclei extraction and lysis buffers, include RNase inhibitor at a final concentration of 0.8 U/µl and protease inhibitor at a final concentration of 1X.
- It is critical to perform the final nuclei suspension in NSB Working Solution using the NSB provided in the kit. Use the following table to prepare at least 1 ml NSB Working Solution per sample.

Refer to *DSP-Methanol Fixation for Nuclei Protocol Instructions* for the volume to use for the final suspension before fixation.

Component Name	NSB Working Solution for Fresh Nuclei (µl)	NSB Working Solution for Fixed Nuclei (µl)
Nuclei Suspension Buffer	167	167
Nuclease-Free Water	773	833
25% BSA	40	Not applicable
RNase Inhibitor (40 U/µl)	20*	Not applicable
Total	1000	1000

* Alternatively, you can use 40 µl RNase inhibitor if provided at 20 U/µl and 753 µl nuclease-free water. Refer to RNase inhibitor recommendations in *User-Supplied Consumables & Equipment* on page 5.

- After nuclei extraction is complete, centrifuge nuclei suspension at 500 × g for 5 minutes at 4°C to pellet. Using a 200 µl pipette, remove and discard supernatant without disturbing the pellet.

  Using a fixed angle centrifuge for pelleting nuclei for wash steps can result in excessive sample loss. Use tubes compatible with swinging bucket rotor centrifuges to ensure a flat pellet.

- Substitute NSB Working Solution for the wash steps in the alternative nuclei isolation protocol (at least 1 ml of NSB Working Solution in up to two wash steps). If you are unable to remove less than 100 µl supernatant during removal, increase the wash volume to 2 ml.

  This kit only includes enough BSA for the final nuclei suspension, so make sure to add user-supplied BSA (1% final concentration) and RNase inhibitor (0.8 U/µl final concentration) if needed for the wash steps.

  The wash step removes reagents inhibitory to the assay from the nuclei suspension (eg, FBS, BSA > 1%, high salt or calcium, carryover organic solvents). Failure to remove inhibitory reagents can cause cDNA QC failure.

- Wash nuclei and perform a final resuspension in NSB Working Solution as the final steps of your alternative isolation protocol as follows.
  - Add 1 ml of NSB Working Solution, mix well and then centrifuge at 500 × g for 5 minutes at 4°C.
  - Without disturbing the pellet, remove supernatant and resuspend nuclei using NSB Working Solution.

Refer to the *Illumina Single Cell Nuclei Isolation Product Documentation* for recommendations on the resuspension volume per mg tissue for various tissue types. Illumina does not recommend processing more than 50 mg of tissue per sample at once to achieve optimal lysis.

Following completion of the alternative protocol, place samples on ice and proceed immediately to *Capture and Lysis* on page 23. If you are not proceeding directly to capture and lysis, Illumina recommends fixing nuclei to prevent mRNA degradation. Refer to *Working with Fixed Nuclei* on page 12 for details.

Capture and Lysis

Perform capture and lysis in a biosafety cabinet using best practices for working in a sterile environment.

Consumables

- CLB3 (Chemical Lysis Buffer 3)
- PAR (Partitioning Reagent)
- RNI (RNase Inhibitor)
- PIP (Particle-Templated Instant Partition)
- 0.5 ml Safe-Lock PCR Clean tubes (emulsion safe)
- 200 µl wide-bore, low-retention, pipette tips
- 1000 µl wide-bore, low-retention, pipette tips
- 1 ml syringe
- 22G blunt bottom syringe needle
- 0.5/1.5 ml combination tube stand

Preparation

1. Prepare CLB3 as follows.
   a. Check for the formation of crystals in the tube.
      If crystals are observed, place on the dry bath set to 25°C to equilibrate the tube to room temperature.
   b. Vortex for 5 seconds, and then centrifuge briefly.
2. Prepare RNI as follows.
   a. Flick to mix, and then centrifuge briefly.
   b. Keep on ice.
3. Prepare PIP tubes as follows.

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a. Centrifuge for 5 seconds.
b. Keep on ice.

4. For each sample, label one 0.5 ml Safe-Lock PCR Clean tube ***CLE***.
5. Depending on your sample type, select one of the following options to set the lid temperature on the dry bath:
   - For cell lysis, select **Prog.A**, and then manually set the lid temperature to +5.0 as follows.
     a. Select **Edit**.
     b. Select **LidMode** to toggle to +5.0.
     c. Select **Save/Return**.
     d. Select **Yes** to save files.
   - For nuclei lysis, select **Prog.B**, and then manually set the lid temperature to 105°C as follows.
     a. Select **Edit**.
     b. Select **LidMode** to toggle to 105°C.
     c. Select **Save/Return**.
     d. Select **Yes** to save files.

   Refer to *Dry Bath Operation* on page 9 to review program parameters.
6. Select **Run** to preheat the dry bath to the hold temperature for the applicable lysis program.
7. Select **LidOn** to preheat the dry bath lid.
   When the lid temperature control is active, the interface displays **LidOff**.

> The dry bath steps specified in this protocol are specific to the PIPseq dry bath sold as part of the Illumina Single Cell Prep Starter Equipment Kit. If you are using a user-supplied Eppendorf dry bath, you must manually program the protocols. Refer to Eppendorf's documentation for more detailed information on how to operate their products.

Procedure

> Dispense reagents slowly in the following steps to avoid forming bubbles.

1. Using a 2 µl or 10 µl pipette, slowly add 1 µl (40 units) RNI directly into the PIPs in each PIP tube.
2. Using a wide-bore 200 µl or 1000 µl pipette tip, slowly pipette the cell or nuclei suspension 10 times to mix.
3. Immediately add 4 µl cell or nuclei suspension at 4250 cells/µl (17,000 total) directly into the PIPs in each PIP tube.
   If it was not possible to concentrate cells at 4250 cells/ul, adjust the input volume to reach 17,000 total cells. Refer to Cell Preparation for more details.
4. Using a 200 µl pipette set to 75 µl, slowly pipette 10 times to mix.

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Remove the pipette tip by dispensing the final stroke along the side wall of the tube slowly to avoid PIP loss.

> To mix multiple PIP tubes at once, place tubes in the 0.5 ml side of the combination tube stand, and then use a pipette tip on every other channel of a 200 µl multichannel pipette in an alternating configuration.

5. Using a 1000 µl pipette, add 320 µl PAR down the side wall of each PIP tube.
6. Tightly cap PIP tubes and place in the vortex adapter head in the horizontal tube position. Make sure that the tubes are fully inserted into the adapter.

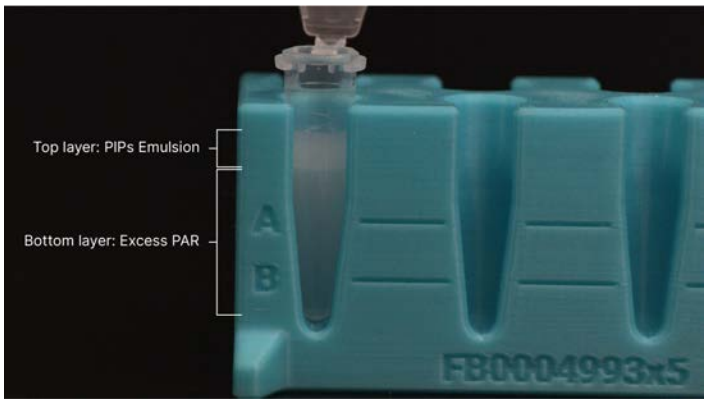


7. Vortex at 3000 rpm and stop after 15 seconds by selecting the **Run Stop** button on the vortexer.
8. Rotate the vortex adapter into the vertical tube position, and then vortex at 3000 rpm for 2 minutes.

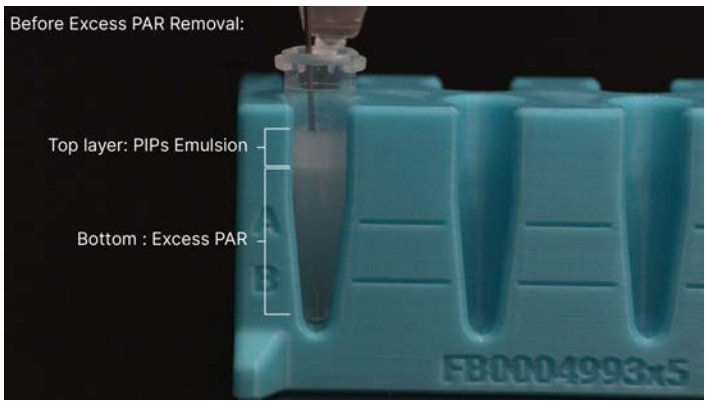


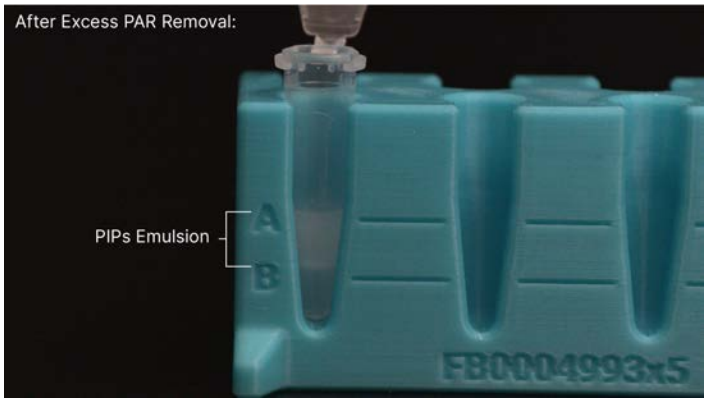
9. Place tubes in the 0.5 ml side of the combination tube stand and wait until the emulsion separates into two distinct layers (~30 seconds).
   The top layer contains PIPs and cells (PIPs emulsion). The bottom layer contains excess PAR.

> Do not centrifuge the emulsion. Centrifuging the emulsion results in elevated background noise and reduced assay sensitivity.



10. Using a 22G blunt bottom syringe needle on a 1 ml syringe, remove and discard the excess PAR (bottom layer) as follows.





    a. Slowly lower the syringe needle through the PIPs emulsion to the bottom of the tube .
    b. Wait 5 seconds.
    c. Aspirate the bottom layer until the bottom of the PIPs emulsion is even with the B stand mark on the combination tube stand. Do not aspirate any PIPs emulsion.
    d. While removing the needle, wipe it on the inside of the tube.
    e. Discard the syringe and needle in a biohazard sharps container.
11. Set PIP tubes aside at room temperature.

Do not place the PIP tubes on ice for the remainder of the Capture and Lysis steps to avoid precipitation of the lysis reagents.

12. Combine the following volumes individually in each ***CLE*** tube to prepare the Chemical Lysis Emulsion (CLE):
    - CLB3 (90 µl)
    - PAR (270 µl)

    The total volume of CLE per sample is 360 µl.

    Do not prepare the CLE in bulk.

13. To form the emulsion, vortex mix the ***CLE*** tubes for 10 seconds.

    To vortex multiple tubes at a time, CLE tubes can be placed in the combination tube stand and vortexed by holding the stand on a flat-head vortex mixer for 10 seconds.

14. Using a 1000 µl pipette, immediately transfer the entire volume of one ***CLE*** tube to each PIP tube.
15. Gently invert 10–15 times to mix.
16. Place PIP tubes on the preheated dry bath.
17. Select **Skip**, and then select **Yes** to proceed with the applicable lysis program.
    If you are immediately proceeding to mRNA isolation, prepare reagents while the lysis program is running.

SAFE STOPPING POINT

If you are stopping, leave on the dry bath at 20°C for up to 4 days. Do not refrigerate or freeze samples.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

1. Remove the reagents from the box and thaw as follows.

15°C to 30°C Storage

Reagent	Box Name	Instructions
DPR (pink)	Illumina Single Cell 3′ RNA Capture, T10, Box 1	Use at room temperature.

2°C to 8°C Storage

Reagent	Box Name	Instructions
BBF	Illumina Single Cell 3′ RNA Capture, T10, Box 2	Let stand for 30 minutes to bring to room temperature.
PWB1	Illumina Single Cell 3′ RNA Capture, T10, Box 2	Keep on ice.

-25°C to -15°C Storage

Reagent	Box Name	Instructions
RTAM	Illumina Single Cell 3′ RNA Capture, T10, Box 3	Thaw on ice.
RTEM	Illumina Single Cell 3′ RNA Capture, T10, Box 3	Keep on ice.

-85°C to -65°C Storage

Reagent	Box Name	Instructions
TSO1	Illumina Single Cell 3′ RNA Capture, T10, Box 4	Thaw one tube per two samples on ice.

Isolate mRNA

Consumables

- BBF (Breaking Buffer)
- DPR (Departitioning Reagent) (pink)
- PWB1 (Wash Buffer)
- 1.5 ml Safe-Lock PCR Clean tubes (emulsion safe)
- 1 ml syringe
- 22G blunt bottom syringe needle
- 0.5/1.5 ml combination tube stand

Preparation

1. Prepare BBF as follows.
   a. Invert to mix.
2. Prepare PWB1 as follows.
   a. For each sample, label one 1.5 ml tube ***Wash***.
   b. Aliquot 1 ml PWB1 into each ***Wash*** tube.
   c. Keep ***Wash*** tubes on ice.

Procedure

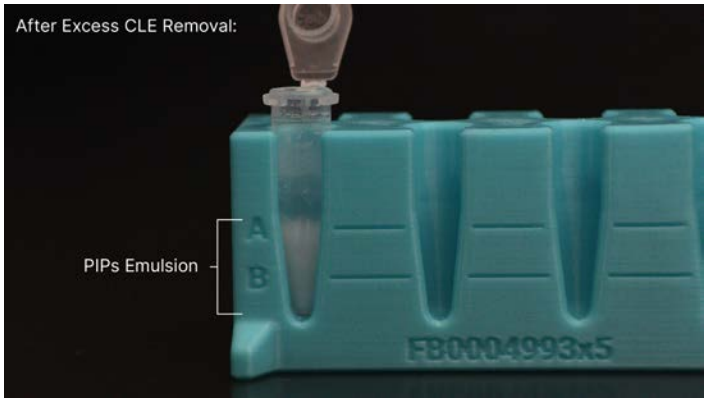
Break Emulsions

1. Remove PIP tubes from the dry bath.

It is standard for condensation to appear in the tubes following removal from the dry bath. Do not centrifuge the emulsion.

2. Place PIP tubes into the combination tube stand.
3. Using a 22G blunt bottom syringe needle on a 1 ml syringe, remove and discard the excess CLE (bottom layer) as follows.





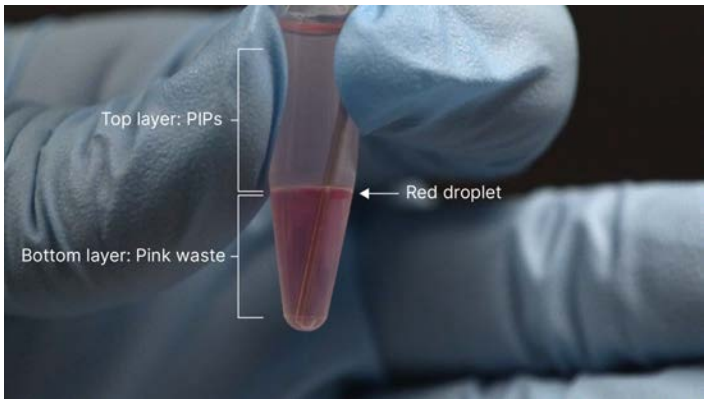
   a. Slowly lower the syringe needle through the emulsion to the bottom of the tube.
   b. Wait 5 seconds.
   c. Aspirate the bottom layer until the top of the emulsion is below the A marker on the combination tube stand.

      Remove as much of the bottom layer as possible without discarding any of the top layer.

   d. While removing the needle, wipe it on the inside of the tube.
   e. Discard the syringe and needle in a biohazard sharps container.
4. Add the following volumes to each PIP tube, along the side wall of the tube, ***in the order listed***:
   - BBF (clear) (300 µl)
   - DPR (pink) (100 µl)
5. Securely close the tubes, and then fully invert 10–20 times to break the emulsion.
6. Centrifuge for 10 seconds.

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7. Make sure that the emulsion is completely broken by visually confirming a distinct interface between the pink-colored bottom layer and the homogeneous aqueous top layer containing the PIPs.
8. Check for any inhomogeneity in the top layer, which is a sign of a partially unbroken emulsion.
   a. If the emulsion is partially unbroken, add 10 µl additional DPR and repeat steps 5 and 6.
   b. If the emulsion remains partially unbroken, add 50 µl additional DPR and repeat steps 5 and 6.
9. Using a new syringe, remove all of the pink-colored bottom layer. If a red droplet is observed, remove it with the pink-colored bottom layer.



10. Discard the syringe and needle in a biohazard sharps container.
11. Centrifuge for 10 seconds.
12. Using a new syringe, remove residual pink-colored bottom layer.

> It is critical to ensure all of the pink-colored bottom layer is removed. It is acceptable to remove a small amount of the top layer to ensure complete removal. If there are unbroken emulsions or pink waste remaining, it can inhibit reverse transcription and cause lower cDNA yield and fragment sizes.

13. Discard the syringe and needle in a biohazard sharps container.
14. Place PIP tubes on ice, and then proceed immediately to the next step.

Wash PIPs

1. Using a single 200 µl pipette tip, slowly transfer the entire volume from each PIP tube into a chilled ***Wash*** tube. Do not discard the PIP tubes.

> When transferring PIPs, make sure that no PIPs are left in the pipette tip by mixing up and down at least 3 times in the ***Wash*** tube.

2. Briefly centrifuge PIP tubes to collect remaining PIPs.
3. Transfer all remaining PIPs from each PIP tube to the corresponding ***Wash*** tube.
4. Place ***Wash*** tubes into the combination tube stand.
5. Vortex mix by holding the stand horizontally on a flat-head vortex mixer for ~3 seconds.

6. Centrifuge for 1 minute.
7. Return ***Wash*** tubes to the combination tube stand.
8. Without disturbing the pellet, use a 1000 µl pipette to remove supernatant to the 3 volume mark on the stand.

   The pellet is translucent at this step. Removing excess volume can result in loss of PIPs.

9. Perform an additional wash as follows.
   a. Add 1200 µl 1X PWB1.
   b. Vortex mix by holding the stand horizontally on a flat-head vortex mixer for ~3 seconds.
   c. Centrifuge for 1 minute.
   d. Return ***Wash*** tubes to the combination tube stand.
   e. Without disturbing the pellet, remove supernatant to the 3 volume mark on the combination stand.
10. Repeat steps 9 a–e to wash PIPs a **third** time. Use 1000 µl 1X PWB1.
11. Repeat steps 9 a–e to wash PIPs a **fourth** time. Use 1000 µl 1X PWB1.

Normalize PIP Volume

This step normalizes sample concentrations for cDNA synthesis.

Consumables

- 0.5 ml Safe-Lock PCR Clean tubes (emulsion safe)

Preparation

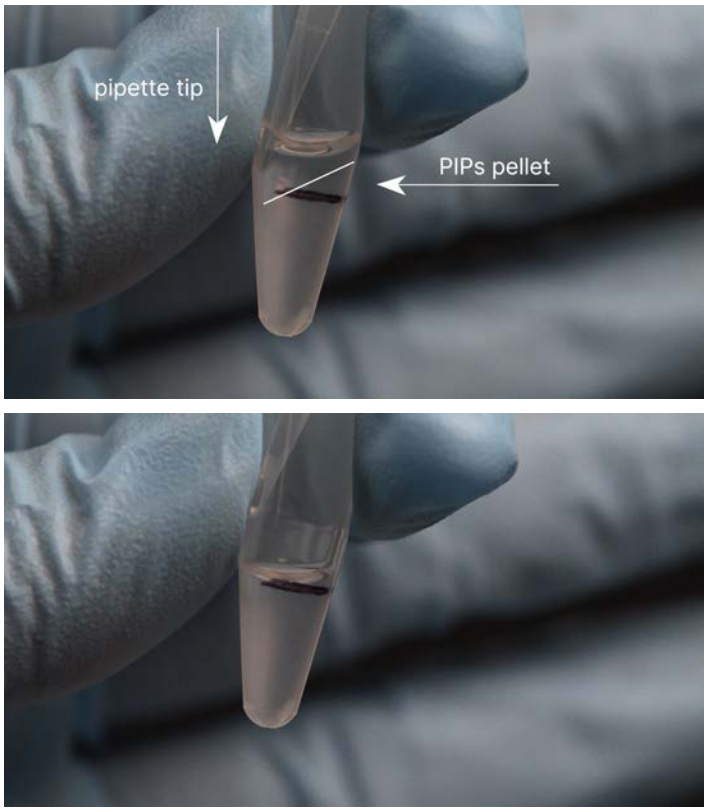
1. For each sample, label one 0.5 ml Safe-Lock PCR Clean tube ***RT***. Use a permanent marker to mark the 0.1 ml volume engraving on the tube.

Procedure

1. Using a 200 µl pipette, transfer each ***Wash*** volume (~200 µl) to the corresponding ***RT*** tube.

   Process two reactions at a time during the following centrifuge and supernatant removal steps to ensure optimal PIP pelleting.

2. Centrifuge for 1 minute.
3. Keeping the tube at an angle, use a 200 µl pipette to immediately remove the supernatant down to the 0.1 ml mark on the tube.
   Do not disrupt packed PIPs. If PIPs are disrupted, repeat step 2.



4. Place ***RT*** tubes on ice and proceed immediately to cDNA synthesis.

Synthesize cDNA

Consumables

- RTAM (RT Additive Mix)
- RTEM (RT Enzyme Mix)
- TSO1 (Template-Switch Oligo)

Preparation

1. Prepare the following consumables:
   - RTAM—Pipette to mix.
   - RTEM—Pipette to mix.
   - TSO1—Vortex to mix, and then centrifuge briefly.
2. Select **Prog.C** and then manually set the lid temperature to 105°C on the dry bath as follows.
   a. Select **Edit**.
   b. Select **LidMode** to toggle to 105°C.

c. Select **Save/Return**.

d. Select **Yes** to save files.

Refer to *Dry Bath Operation* on page 9 to review program parameters.

3. Select **Run** to preheat the dry bath to 25°C for the reverse transcription program.
4. Confirm the dry bath displays **LidOff**, which will begin preheating the lid.
   When the lid temperature control is active, the interface displays **LidOff**. The lid temperature only climbs to 105°C during incubation temperatures of 35°C or higher.

> The dry bath steps specified in this protocol are specific to the PIPseq dry bath sold as part of the Illumina Single Cell Prep Starter Equipment Kit. If you are using a user-supplied Eppendorf dry bath, you must manually program the protocols. Refer to Eppendorf's documentation for more detailed information on how to operate their products.

Procedure

1. Combine the following volumes to create RT Master Mix:

Reagent	Volume Per Reaction (µl)	4 Reactions (µl)	8 Reactions (µl)
RTAM	80.0	352.0	704.0
TSO1	8.0	35.2	70.4
RTEM	12.0	52.8	105.6
Total	100.0	440.0	880.0

   These volumes include 10% overage.
2. Gently pipette to mix.
3. Briefly centrifuge to bring liquid to the bottom of the tube.
4. Add 100 µl RT Master Mix to each ***RT*** tube.
5. Flick to disperse the pellet.
6. Pulse vortex for 5 seconds to mix.
7. Centrifuge briefly to bring liquid to the bottom of the tube.

> If PIPs are pelleted in the centrifuge, repeat steps 6 and 7.

8. Place ***RT*** tubes on the preheated dry bath.
9. Select **Skip**, and then select **Yes** to proceed with the cDNA Synthesis program, starting with incubation at 25°C for 30 minutes.

SAFE STOPPING POINT

If you are stopping, leave on the dry bath at 4°C or store at 2°C to 8°C overnight.

Condensation might occur during overnight incubation at 4°C. This does not affect assay performance.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

1. Remove the reagents from the box and thaw as follows.

2°C to 8°C Storage

Reagent	Box Name	Instructions
PWB1	Illumina Single Cell 3′ RNA Capture, T10, Box 2	Keep on ice.

-25°C to -15°C Storage

Reagent	Box Name	Instructions
PMM4	Illumina Single Cell 3′ RNA Capture, T10, Box 3	Keep on ice.
WTAP	Illumina Single Cell 3′ RNA Capture, T10, Box 3	Thaw on ice.

Wash PIPs for cDNA Amplification

Consumables

- PWB1 (Wash Buffer)
- Nuclease-free water (customer provided)
- 0.5/1.5 ml combination tube stand (blue)

Preparation

1. Prepare 0.5X concentration PWB1 as follows.
   a. Combine the following volumes. Multiply each volume by the number of samples.
      - PWB1 (660 µl)
      - Nuclease-free water (660 µl)

      These volumes produce 1320 µl 0.5X PWB1 per sample, which includes overage.
   b. Gently invert to mix.

Procedure

1. Remove ***RT*** tubes from the dry bath.
2. Centrifuge for 1 minute.
3. Without disturbing the pellet, remove and discard 130 µl supernatant.
4. Wash PIPs as follows.
   a. Add 450 µl 0.5X PWB1 to each ***RT*** tube.
   b. Vortex for 5 seconds.
   c. Centrifuge for 1 minute.
   d. Return tubes to the combination tube stand. Make sure that tubes are fully seated in the tube stand.
   e. Without disturbing the pellet, remove and discard supernatant to the A mark on the combination tube stand.
5. Repeat steps 4 a–e to wash PIPs a **second** time. Use 350 µl 0.5X PWB1.
6. Repeat steps 4 a–e to wash PIPs a **third** time. Use 350 µl 0.5X PWB1.

   Process two reactions at a time during the following centrifuge and supernatant removal steps to ensure optimal PIP pelleting.

7. Centrifuge for 1 minute.
8. Keeping the tube at an angle, immediately remove and discard supernatant to the 0.1 ml mark on the tube.
9. Place ***RT*** tubes on ice and proceed immediately to cDNA amplification.

Amplify cDNA

Consumables

- PMM4 (4X PCR Master Mix)
- WTAP (WTA Primer)
- 0.2 ml 8-tube strips and caps

Preparation

1. Prepare the following consumables:
   - PMM4—Pipette to mix, and then centrifuge briefly.
   - WTAP—Pipette to mix, and then centrifuge briefly.
2. Label 8-tube strips as follows:
   - For four reactions, label one 8-tube strip ***WTA***

- For eight reactions, label two 8-tube strips ***WTA***

3. In the post-PCR amplification area, save the following cDNA Amplification program on the thermal cycler:
   - Set the lid temperature to 105°C
   - 95°C for 3 minutes
   - 5 cycles of:
     - 98°C for 15 seconds
     - 69°C for 10 minutes
   - 72°C for 5 minutes
   - Hold at 4°C

   Annealing and extension both occur at 69°C in this profile.

   The specified program parameters are critical for assay performance. Do not modify the PCR cycle number. Altering the program causes unusable results.

Procedure

1. Combine the following volumes to prepare Whole Transcriptome Amplification (WTA) Master Mix:

Reagent	Volume Per Reaction (µl)	4 Reactions (µl)	8 Reactions (µl)
PMM4	33.8	148.7	297.4
WTAP	1.4	6.2	12.3
Total	35.2	154.9	309.7

   These volumes include 10% overage.

2. Vortex to mix, and then centrifuge briefly.
3. Add 35 µl WTA Master Mix to each ***RT*** tube to create the WTA Reaction Mixture.
4. Distribute the WTA Reaction Mixture ***individually*** per reaction as follows.
   a. Pulse vortex the ***RT*** tube for 5 seconds.
   b. Using a single pipette tip, aliquot 68 µl WTA Reaction Mixture from the ***RT*** tube into two wells of the ***WTA*** tube strip. Do not discard the pipette tip.
   c. Centrifuge the ***RT*** tube briefly.
   d. Using the same pipette tip, distribute any remaining WTA Reaction Mixture from the ***RT*** tube into one of the corresponding wells in the ***WTA*** tube strip.

The following thermal cycler steps are performed in the post-PCR workspace.

5. Place ***WTA*** tube strips on the preprogrammed thermal cycler.
6. Run the cDNA Amplification program.

SAFE STOPPING POINT

If you are stopping, leave on the thermal cycler at 4°C or store at 2°C to 8°C overnight.

Condensation might occur during overnight incubation at 4°C. This does not affect assay performance.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

1. Remove the reagents from the box and thaw as follows.

2°C to 8°C Storage

Reagent	Box Name	Instructions
CEB	Illumina Single Cell 3′ RNA Capture, T10, Box 2	Use at room temperature.
IPB2	Illumina Single Cell 3′ RNA Capture, T10, Box 2	Let stand for 30 minutes to bring to room temperature.
PWB1	Illumina Single Cell 3′ RNA Capture, T10, Box 2	Keep on ice.

-25°C to -15°C Storage

Reagent	Box Name	Instructions
PMM4	Illumina Single Cell 3′ RNA Capture, T10, Box 3	Keep on ice.
WTAP	Illumina Single Cell 3′ RNA Capture T10, Box 3	Thaw on ice.

Isolate cDNA from PIPs

Consumables

- CEB (cDNA Extraction Buffer)
- 1.5 ml Safe-Lock PCR Clean tubes (emulsion safe)

Preparation

1. For each reaction, label one new 1.5 ml tube ***PIPs***.

2. For each reaction, label one new 1.5 ml tube ***cDNA***.
3. Prepare CEB as follows.
   a. Invert to mix.

Procedure

1. If you are resuming after a safe stopping point, briefly centrifuge the ***WTA*** tube strips.
2. Add 100 µl CEB to each well of the ***WTA*** tube strips.
3. Recombine the two WTA aliquots per reaction in the ***WTA*** tube strip into one ***PIPs*** tube.
4. Briefly centrifuge the ***WTA*** tube strips.
5. Transfer any remaining volume from the ***WTA*** tube strips to the corresponding ***PIPs*** tube.
6. Pulse vortex the ***PIPs*** tube, and then centrifuge for 1 minute.
7. Without disturbing the pellet, transfer 200 µl supernatant from the ***PIPs*** tube to the corresponding ***cDNA*** tube.
8. Add 200 µl CEB to the ***PIPs*** tube.
9. Pulse vortex the ***PIPs*** tube, and then centrifuge for 1 minute.
10. Without disturbing the pellet, transfer 200 µl supernatant from the ***PIPs*** tube to the corresponding ***cDNA*** tube.
    The total volume of supernatant per reaction is 400 µl.
11. Briefly centrifuge the ***cDNA*** tube, and then examine the bottom of the tubes for PIPs.
12. If residual PIPs are present, transfer the supernatant to a new 1.5 ml tube, and then measure the transferred supernatant volume.
    If the final volume of supernatant is not 400 µl per reaction, adjust the IPB2 volume during step 1 of cDNA clean up.
13. Save the ***PIPs*** tubes and store at -85°C to -65°C for up to 3 months.

> The saved pellets are required for Illumina Single Cell Supplemental Enrichment and Amplification Kit applications. If library preparation fails to produce sequencing-quality libraries, the saved pellets can be used to recover cDNA. Refer to the *Illumina Single Cell 3′ RNA Prep Replay Protocol* for instructions.

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Clean Up cDNA

Consumables

- IPB2 (Illumina Purification Beads 2)
- IDTE Buffer
- Absolute ethanol (EtOH)
- Nuclease-free water (customer provided)
- 0.2 ml 8-tube strips and caps

Preparation

1. Prepare the following consumables:
   - IPB2—Vortex for 30 seconds to resuspend.
   - For each reaction, prepare 2.2 ml fresh 85% EtOH solution from absolute EtOH, which includes 10% overage.
2. Label an 8-tube strip ***cDNA LP***.

Procedure

1. Add 320 µl IPB2 to each ***cDNA*** tube (0.8X ratio).

   If the final volume of supernatant per reaction was not 400 µl during cDNA isolation, adjust the IPB2 volume for a 0.8X ratio of magnetic beads.

2. Pulse vortex for 5 seconds, and then pulse centrifuge to bring the liquid to the bottom of the tube.
3. Incubate at room temperature for 5 minutes.
4. Place tubes on the magnetic stand and wait until the liquid is clear (~5 minutes).
5. Without disturbing the beads, remove and discard supernatant.
6. Wash beads as follows.
   a. Add 1 ml fresh 85% EtOH.
   b. Wait 30 seconds.
   c. Without disturbing the pellet, remove and discard 1 ml EtOH.
7. Repeat steps 6 a–c to wash beads a **second** time.
8. Without disturbing the beads, use a 20 µl pipette to remove residual EtOH.
9. Air-dry until a slight gloss remains on the bead surface (~5 minutes).
   Depending on humidity level, air-drying can take up to 10 minutes.

   Overdrying the beads can result in cracks and decrease elution efficiency.

10. Remove tubes from the magnetic stand.
11. Add 42 µl IDTE Buffer to each tube.
12. Pulse vortex to resuspend, and then pulse centrifuge to bring the liquid to the bottom of the tube.

    If bead pellets are overdried, pipette mix frequently during the following incubation to minimize sample loss.

13. Incubate at room temperature for 5 minutes.
14. Place tubes on the magnetic stand and wait until the mixture is clear (~2 minutes).
15. Keep tubes on the magnetic stand.
16. Transfer 40 µl supernatant from each ***cDNA*** tube to a well in the ***cDNA LP*** tube strip.
17. Immediately place the ***cDNA LP*** tube strip on ice.
    This cDNA is used as input for library preparation.

    Do not discard the beads with the remaining 2 µl supernatant. The supernatant is used for cDNA QC.

18. Keep the remaining 2 µl supernatant in the ***cDNA*** tube on the magnetic stand and proceed to cDNA QC.
19. Store the ***cDNA LP*** tube strip for library preparation at -85°C to -65°C for up to 72 hours.

    Storing cDNA in a high amplicon-contaminated area might affect the quality of your samples. Storing cDNA for longer than 72 hours can result in final library QC failure from degraded cDNA.

Assess cDNA Quality

This step uses the remaining elution volume (2 µl) from the magnetic bead pellet to assess cDNA QC.

Consumables

- PMM4 (4X PCR Master Mix)
- WTAP (WTA Primer)
- PWB1 (Wash Buffer)
- Nuclease-free water (customer provided)
- 0.2 ml 8-tube strip

Preparation

1. Prepare the following consumables:

- 0.5X PWB1—Combine the following volumes. Multiply each of the following volumes by the number of samples.
  - PWB1 (5 µl)
  - Nuclease-free water (5 µl)

  These volumes produce 10 µl 0.5X PWB1 per sample, which includes overage.
- PMM4—Pipette to mix, and then centrifuge briefly.
- WTAP—Pipette to mix, and then centrifuge briefly.

2. Save the following QC Amplification program on the thermal cycler. Use the table to determine the number of PCR cycles depending on sample input type and concentration.
   - Set the lid temperature to 105°C
   - 95°C for 3 minutes
   - X cycles of:
     - 98°C for 15 seconds
     - 69°C for 4 minutes
   - 72°C for 5 minutes
   - Hold at 4°C

   Annealing and extension both occur at 69°C in this profile.

Sample Input	Number of QC PCR Cycles (X)
High RNA sample types (for example, cell lines)	9
Low RNA sample types (for example, primary cells and nuclei)	12

Procedure

1. Keep ***cDNA*** tubes containing 2 µl supernatant on the magnetic stand and add 9 µl nuclease-free water.
2. Without disturbing the pellet, pipette to mix.
3. Incubate at room temperature for 1 minute.
4. Without disturbing the pellet, transfer 10 µl from the ***cDNA*** tube to an 8-tube strip.
5. Place the 8-tube strip on ice.
6. Combine the following volumes to prepare QC Master Mix:

Reagent	Volume Per Reaction (µl)	4 Reactions (µl)	8 Reactions (µl)
PMM4	6.25	27.5	55.0
WTAP	1.0	4.4	8.8
0.5X PWB1	7.75	34.1	68.2
Total	15.0	66.0	132.0

These volumes include 10% overage.

7. Vortex to mix, and then centrifuge briefly.
8. Add 15 µl QC Master Mix to each reaction in the 8-tube strip.
   The total volume per reaction is 25 µl.
9. Pipette to mix 10 times, and then centrifuge briefly.
10. Place the 8-tube strip on the preprogrammed thermal cycler.
11. Run the QC Amplification program.

SAFE STOPPING POINT

If you are stopping, leave on the thermal cycler at 4°C or store samples at 2°C to 8°C overnight.

Condensation might occur during overnight incubation at 4°C. This does not affect assay performance.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

1. Remove the reagents from the box and thaw as follows.

   2°C to 8°C Storage

Reagent	Box Name	Instructions
IPB2	Illumina Single Cell 3′ RNA Capture, T10, Box 2	Let stand for 30 minutes to bring to room temperature.

Clean Up QC Product

Consumables

- IPB2 (Illumina Purification Beads 2)
- IDTE Buffer
- Absolute ethanol (EtOH)

- Nuclease-free water (customer provided)
- 0.2 ml 8-tube strips

Preparation

1. Prepare the following consumables:
   - IPB2—Vortex for 30 seconds to resuspend.
   - For each reaction, prepare 440 µl fresh 85% EtOH solution from absolute EtOH, which includes 10% overage.

Procedure

1. Add 15 µl nuclease-free water to each tube.
   The total volume is 40 µl per sample.
2. Add 32 µl IPB2 to each tube (0.8X ratio).
3. Pipette 15 times to mix, and then pulse centrifuge to bring liquid to the bottom of the tube.
4. Incubate at room temperature for 5 minutes.
5. Place the 8-tube strip on the magnetic stand and wait until the liquid is clear (~5 minutes).
6. Without disturbing the beads, remove and discard supernatant.
7. Wash beads as follows.
   a. Add 200 µl fresh 85% EtOH.
   b. Wait 30 seconds.
   c. Without disturbing the beads, remove and discard supernatant.
8. Repeat steps 7 a–c to wash the beads a **second** time.
9. Without disturbing the beads, use a 20 µl pipette to remove residual EtOH.
10. Air-dry until a slight gloss remains on the bead surface (~2 minutes).
    Depending on humidity level, air-drying can take up to 5 minutes.

    Overdrying the beads can result in cracks and decrease elution efficiency.

11. Remove the 8-tube strip from the magnetic stand.
12. Add 11 µl IDTE Buffer to each tube.
13. Pipette mix to resuspend, and then pulse centrifuge to bring the liquid to the bottom of the tube.

    If bead pellets are overdried, pipette mix frequently during the following incubation to minimize sample loss.

14. Incubate at room temperature for 5 minutes.
15. Place the 8-tube strip on the magnetic stand and wait until the mixture is clear (~2 minutes).

16. Transfer 10 µl supernatant from each tube on the magnetic stand to a well of a new 8-tube strip and save for QC.

Check QC Product

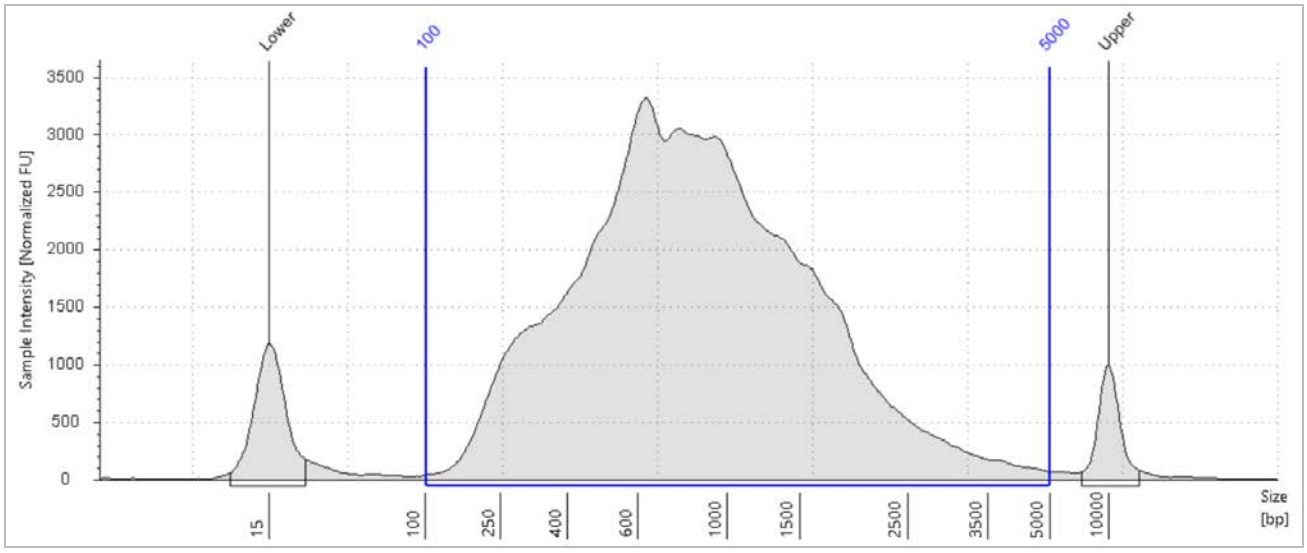
Perform the following to check the quantity and quality of the QC product.

The QC product is stable at -25°C to -15°C for up to four weeks. If necessary, you can submit the QC product with the final libraries when using a core lab for fragment analysis.

1. Quantify 2 µl of each sample using a Qubit 1X dsDNA High Sensitivity Assay kit.
2. Assess average fragment size using an Agilent Bioanalyzer or Tapestation.
   a. If necessary, dilute samples to make sure that they are within the appropriate range for the device.
   b. Load an appropriate volume of the purified library on the fragment analyzer.
   Recommended loading concentration is 1–2 ng/µl on Bioanalyzer or 1–10 ng/µl on Tapestation.
   Loading less than 1 ng/µl may not give accurate results.

Determine average fragment size for cDNA using the fragment analyzer region table (set for 100–5000 bp). Average size can vary depending on cell type and experimental condition.

The following image is a representative cDNA trace for a 17,000 cell human peripheral blood mononuclear cell (PBMC) input using a High Sensitivity D5000 ScreenTape. Expected average size between 100 and 5000 bp is > 500 bp. Proceed to library preparation upon successful cDNA QC.



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Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

1. If you are resuming after a safe stopping point, remove ***cDNA LP*** tube strip from storage and place on ice.

   It is critical to use the ***cDNA LP*** tube strip recovered during the cDNA clean up for library preparation. Using the QC product as input into library preparation results in assay failure.

2. Remove the reagents from the box and thaw as follows.

   2°C to 8°C Storage

Reagent	Box Name	Instructions
IPB2	Illumina Single Cell Library Prep, Box 1	Let stand for 30 minutes to bring to room temperature.
NFW	Illumina Single Cell Library Prep, Box 1	Use at room temperature.

   -25°C to -15°C Storage

Reagent	Box Name	Instructions
PMM4	Illumina Single Cell Library Prep, Box 2	Keep on ice.
LAM	Illumina Single Cell Library Prep, Box 2	Thaw on ice.
LPB	Illumina Single Cell Library Prep, Box 2	Thaw on ice.
LPE	Illumina Single Cell Library Prep, Box 2	Keep on ice.
LPMA	Illumina Single Cell Library Prep, Box 2	Keep on ice.

3. Remove one of the following index adapter options from storage and thaw as follows.

   -25°C to -15°C Storage

Reagent	Box Name	Instructions
Illumina Single Cell Prep UDI Mix Strip	Illumina Single Cell Library Prep, Box 2	Thaw on ice.
Illumina Single Cell Unique Dual Indexes plate	Illumina Single Cell Unique Dual Indexes (96 Indexes, 96 Samples)	Thaw on ice.

Fragmentation, End Repair, & A-Tailing

Consumables

- LPB (Library Prep Buffer)
- LPE (Library Prep Enzymes)

Preparation

1. Prepare LPB as follows.
   a. Vortex to mix, and then centrifuge briefly.
   b. Keep on ice.
2. Prepare LPE as follows.
   a. Pipette to mix, and then centrifuge briefly.
   b. Keep on ice.
3. Save the following cDNA Fragmentation program on the thermal cycler:
   - Set the lid temperature to 105°C
   - Hold at 4°C
   - 30°C for 6 minutes
   - 65°C for 30 minutes
   - Hold at 4°C
4. Run the cDNA Fragmentation program to prechill the thermal cycler block to 4°C and preheat the lid to 105°C.

Procedure

1. Make sure that the ***cDNA LP*** tube strip is on ice. Keep on ice until tubes are placed on the thermal cycler.
2. Combine the following volumes to prepare Library Prep Master Mix:

Reagent	Volume Per Reaction (µl)	4 Reactions (µl)	8 Reactions (µl)
LPB	4.0	18.4	36.8
LPE	6.0	27.6	55.2
Total	10.0	46.0	92.0

   These volumes include 15% overage.
3. Vortex to mix, and then centrifuge briefly.

4. Keeping the ***cDNA LP*** tube strip on ice, add 10 µl Library Prep Master Mix to each well. The total volume is 50 µl per reaction.
5. Pipette 10 times to mix, and then centrifuge briefly.
6. Place the ***cDNA LP*** tube strip on the prechilled thermal cycler.
7. Use the skip step function to run the next steps of the cDNA Fragmentation program.
8. When the thermal cycler program reaches 4°C, immediately remove the ***cDNA LP*** tubes and proceed to the next step.

Ligate Adapters

Consumables

- LAM (Library Adapter Mix)
- LPMA (Library Prep Mix A)
- NFW (Nuclease-Free Water) (kit provided)

Preparation

1. Prepare LPMA as follows:
   a. Pipette slowly 15 times to mix, and then centrifuge briefly.
   b. Keep on ice.

   LPMA is viscous. Pipette slowly and gently to minimize foam and dispense accurate volumes.
2. Prepare LAM as follows.
   a. Vortex to mix.
   b. Centrifuge briefly.
3. Save the following Ligation program on the thermal cycler:
   - 20°C for 20 minutes
   - Make sure that the heated lid is off.

Procedure

1. Combine the following volumes to prepare diluted LAM.

Reagent	Volume Per Reaction (µl)	4 Reactions (µl)	8 Reactions (µl)
LAM	0.75	3.45	6.9
NFW	4.25	19.55	39.1

Reagent	Volume Per Reaction (µl)	4 Reactions (µl)	8 Reactions (µl)
Total	5.0	23.0	46.0

These volumes include 15% overage.

Store dilutions of the LAM for up to one week at -25°C to -15°C.

2. Vortex the diluted LAM, and then centrifuge briefly.
3. Add 5 µl diluted LAM to each well of the ***cDNA LP*** tube strip.
4. Slowly add 20 µl LPMA to each well of the ***cDNA LP*** tube strip.
   The total volume per reaction is 75 µl.
5. Pipette 10 times to mix, and then centrifuge briefly.
6. Place the ***cDNA LP*** tube strip on the thermal cycler.
7. Run the Ligation program.
8. After the thermal cycler program is complete, proceed immediately to the next step.

Clean Up Ligated Fragments

This step uses magnetic beads to purify the adapter-ligated fragments.

Consumables

- IPB2 (Illumina Purification Beads 2)
- NFW (Nuclease-Free Water) (kit provided)
- Absolute ethanol (EtOH)
- 0.2 ml 8-tube strip

Preparation

1. Prepare the following consumables:
   - IPB2—Vortex for 30 seconds to resuspend.
   - For each reaction, prepare 440 µl fresh 85% EtOH solution from absolute EtOH, which includes 10% overage.

Procedure

1. Add 60 µl IPB2 to each well of the ***cDNA LP*** tube strip (0.8X ratio).
2. Pipette 15 times to mix, and then pulse centrifuge to bring liquid to the bottom of the tube.
3. Incubate at room temperature for 5 minutes.

4. Place the ***cDNA LP*** tube strip on the magnetic stand and wait until the liquid is clear (~5 minutes).
5. Without disturbing the beads, remove and discard the supernatant.
6. Wash beads as follows.
   a. Add 200 µl fresh 85% EtOH.
   b. Wait 30 seconds.
   c. Without disturbing the pellet, remove and discard the supernatant.
7. Repeat steps 6 a–c to wash the beads a **second** time.
8. Without disturbing the beads, use a 20 µl pipette to remove residual EtOH.
9. Air-dry until a slight gloss remains on the bead surface (~2 minutes).
   Depending on humidity level, air-drying can take up to 5 minutes.

   Overdrying the beads can result in cracks and decrease elution efficiency.
10. Remove the ***cDNA LP*** tube strip from the magnetic stand.
11. Add 34 µl NFW over the bead pellet in each well.
12. Pipette 10 times to resuspend, and then centrifuge briefly.
13. Incubate at room temperature for 5 minutes.
14. Place the ***cDNA LP*** tube strip on the magnetic stand and wait until the liquid is clear (~2 minutes).
15. Transfer 32.5 µl supernatant from each well of the ***cDNA LP*** tube strip to a new 8-tube strip.

Amplify Library

Consumables

- PMM4 (4X PCR Master Mix)
- One of the following index set options:
  - Illumina Single Cell Prep UDI Mix Strip
  - Illumina Single Cell Unique Dual Indexes plate

  There are eight index mixes provided with this kit to allow for unique dual indexing of eight samples. To pool more than eight libraries together in a sequencing run, use the Illumina Single Cell Unique Dual Indexes plate.

Preparation

1. Prepare the following consumables:
   - PMM4—Pipette 10 times, and then centrifuge briefly.
2. Save the following PCR program on the thermal cycler. Use the table to determine the number of PCR cycles depending on sample input type.

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- Set the lid temperature to 105°C
- 98°C for 45 seconds
- X cycles of:
  - 98°C for 15 seconds
  - 67°C for 30 seconds
  - 69°C for 45 seconds
- 72°C for 1 minute
- Hold at 4°C

PCR Cycle Recommendations Per Input Type

Sample Input	Number of PCR Cycles* (X)
High RNA sample types (for example, cell lines)	10
Low RNA sample types (for example, primary cells)	15

* Decrease by 1–2 cycles for cDNA QC concentrations > 200 ng/µl.

3. Prepare the index strip as follows.
   a. Make sure the hole on the side of the index strip is in the following orientation:



   b. For each reaction, choose and record a unique index mix.

Make sure that no sample index combinations overlap in a multiplexed sequencing run. For more information on pooling, refer to *Index Adapters Pooling Guide*. For more information on indexes, refer to *Illumina Adapter Sequences*.

Procedure

1. Using a 20 µl pipette tip, pierce the foil on each well of the index strips. Discard the pipette tips after piercing the foil.
2. Add the following volumes to each well of the 8-tube strip containing the supernatant, ***in the order listed***:
   - Unique Library Index Mix (5 µl)
   - PMM4 (12.5 µl)

The total volume is 50 µl per reaction.

3. Pipette 10 times to mix, and then centrifuge briefly.
4. Place the 8-tube strip on the preprogrammed thermal cycler.
5. Run the PCR program.

SAFE STOPPING POINT

If you are stopping, leave on the thermal cycler at 4°C or store samples at 2°C to 8°C overnight.

Prepare for Protocol

This preparation is required to perform the protocol steps leading up to the next safe stopping point.

1. Remove the reagent from the box and thaw as follows.

   2°C to 8°C Storage

Reagent	Box Name	Instructions
IPB2	Illumina Single Cell Library Prep, Box 1	Let stand for 30 minutes to bring to room temperature.
NFW	Illumina Single Cell Library Prep, Box 1	Use at room temperature.

Clean Up Library

Consumables

- IPB2 (Illumina Purification Beads 2)
- IDTE Buffer
- Absolute ethanol (EtOH)
- NFW (Nuclease-Free Water) (kit provided)
- 0.2 ml 8-tube strips

Preparation

1. Prepare the following consumables:
   - IPB2—Vortex for 30 seconds to resuspend.
   - For each reaction, prepare 440 µl fresh 85% EtOH solution from absolute EtOH, which includes 10% overage.

Procedure

1. If you are resuming after a safe stopping point, briefly centrifuge the tubes.
2. Add 45 µl NFW to each well of the 8-tube strip.
3. Add 76 µl IPB2 to each well of the 8-tube strip (0.8X ratio).
   The total volume is 171 µl per library.
4. Pipette 15 times to mix, and then pulse centrifuge to bring liquid to the bottom of the tube.
5. Incubate at room temperature for 5 minutes.
6. Place tubes on the magnetic stand and wait until the liquid is clear (~5 minutes).
7. Without disturbing the beads, remove and discard supernatant.
8. Wash beads as follows.
   a. Add 200 µl fresh 85% EtOH.
   b. Wait 30 seconds.
   c. Without disturbing the beads, remove and discard EtOH.
9. Repeat steps 8 a–c to wash the beads a **second** time.
10. Without disturbing the beads, use a 20 µl pipette to remove residual EtOH.
11. Air-dry until a slight gloss remains on the bead surface (~2 minutes).
    Depending on humidity level, air-drying can take up to 5 minutes.

    Overdrying the beads can result in cracks and decrease elution efficiency.
12. Remove tubes from the magnetic stand.
13. Add 21 µl IDTE Buffer to each well of the 8-tube strip.
14. Pipette 10 times to resuspend, and then centrifuge briefly.
15. Incubate at room temperature for 5 minutes.
16. Place tubes on the magnetic stand and wait until the liquid is clear (~2 minutes).
17. Transfer 20 µl supernatant from each well of the 8-tube strip to a new 8-tube strip.
    The supernatant in the new 8-tube strip contains the sequencing-ready purified libraries.

SAFE STOPPING POINT

If you are stopping, seal the tubes and store at -25°C to -15°C for up to 30 days.

Check Library

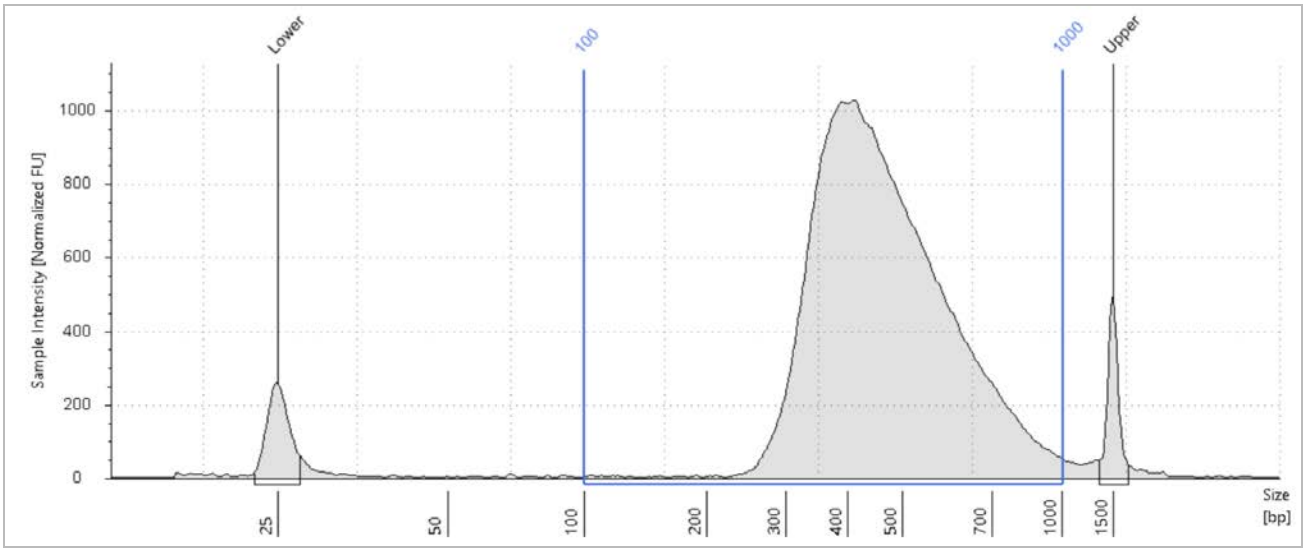
This step checks the concentration and quality of the final libraries.

1. Quantify 2 µl of each sample using a Qubit 1X dsDNA High Sensitivity Assay kit.
2. Assess average fragment size using an Agilent Bioanalyzer or Tapestation.
   a. If necessary, dilute samples to make sure that they are within the appropriate range for the device.

b. Load an appropriate volume of the purified library on the fragment analyzer. Recommended loading concentration is 1–2 ng/µl on Bioanalyzer or 1–10 ng/µl on Tapestation.

Determine average fragment size for GEX and CRISPR libraries using the fragment analyzer region table (set for 100–1000 bp). Average size can vary depending on cell type and experimental condition.

The following image is a representative mRNA library trace for a 17,000 cell human PBMC input on a High Sensitivity D1000 ScreenTape. Expected average size between 100 and 1000 bp is 370–600 bp.

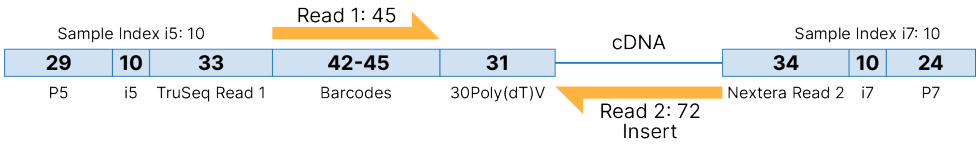


Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

Illumina Single Cell 3′ RNA Prep, T10 kit libraries are composed of standard Illumina paired-end constructs that begin with P5 and end with P7. Read 1 contains the barcode information while Read 2 contains the gene expression information. These libraries are dual-indexed with 10-base i5 and i7 indexes. Read 1 length must be ≥ 45 bases and the recommended Read 2 length ≥ 72 bases.

Illumina Single Cell 3′ RNA Prep libraries can be pooled for sequencing, taking into account the differences in cell number and read depth requirements. Sequencing depth varies based on your application needs, but it is recommended to start with a depth of 20,000 reads per target cell. Samples must have distinct index combinations to avoid failures in sample demultiplexing. Refer to *Index Adapters Pooling Guide* for discussion of appropriate color balance combinations for the selected sequencing platform.



1. Calculate the molarity value of the libraries using the following formula:

$$\frac{\text{concentration in ng/}\mu\text{l}}{660\ \text{g/mol} \times \text{average library size in bp}} \times 10^6 = \text{Molarity (nM)}$$

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)	Recommended PhiX Spike	Denature and Dilute Protocol
NextSeq 500/550	2	1.6	1%	Illumina Single Cell 3′ RNA Prep
NextSeq 1000/2000	2	550	1%	Onboard or XLEAP-SBS Onboard
NovaSeq 6000, standard loading	1	200	1%	Standard
NovaSeq 6000, XP loading	1	70	1%	XP
NovaSeq X Series	2	200	2%	Standard

3. Dilute each library to the starting concentration for your system.
4. Pool equal volumes of each library to create a multiplexed library pool.
5. Follow the denature and dilute instructions for your system in the Denature and Dilute protocol generator.

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Resources & References

The Illumina Single Cell Prep pages on the Illumina support site provide additional resources. These resources include training, compatible products, and other considerations. Always check the support pages for the latest versions.

Revision History

Document	Date	Description of Change
Document # 200063182 v02	October 2026	Updated • Accessories bag contents. • Bead clean up instructions throughout the protocol. • Cell loading volume and total input cells recommendations. • Dry bath programming instructions and preparation steps. • Guidance for CSB resuspension during cell preparation. • Guidance on removing PIPs in Normalize PIP Volume • Guidance on the range for expected average fragment size in Check QC Product and Check Library. • Instructions for adding RNI and the cell or nuclei suspension to the PIPs during capture. • Master mix table volumes. • Mixing guidance after adding QC Master Mix in Assess cDNA Quality. • Nuclei suspension preparation instructions. • Overages for solutions and master mixes throughout protocol. • Prepare for Protocol section tables. • Product content overview information. • Starter Kit contents. • Thawing instructions for CSB, RTEM, LPE, and LPMA. • The required Nuclease Free Water used during library clean up to be kit supplied. • Tips and Techniques. • User-supplied consumables. • Vortexer operating instructions. • WTA reaction mixture distribution instructions.

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Document	Date	Description of Change
Document # 200063182 v02 (cont.)	October 2026	Removed • Cautions that are no longer relevant. • HTML format. • Optional cell straining step during cell preparation. • Pipette stroke details. Added • Combination tube stand to consumables lists for required sections. • Horizontal and vertical tube position vortexer illustrations. • Information on switching to the post-PCR space during cDNA amplification. • Instruction to discard the pipette tips used to pierce the foil of the index strip wells after use in Amplify Library. • Link to the Illumina Single Cell 3' RNA Prep Replay Protocol. • Master mix table for diluted LAM. • Note addressing potential cloudy PAR appearance. • Pictures to critical steps throughout the protocol. • Pipette details when necessary throughout protocol. • Preparation instructions for LAM, RNI, TSO1, and IPB2. • Sequencing depth recommendations per target cell. • Statements around working in the biosafety cabinet. • Steps for mixing and spinning down diluted LAM during adapter ligation before addition to each reaction. • Step to place PIP tubes aside at room temperature before CLE preparation. • Tube labeling throughout the protocol. • Warning addressing the high lid temperature on the dry bath and the risk of burns. Moved • Construct information from Introduction to Dilute Libraries to the Starting Concentration. • Cell loading information from Tips and Techniques to Sample Preparation.

Document	Date	Description of Change
Document # 200063182 v01	August 2025	Added instructions for diluting libraries to starting concentrations and relevant sequencing information. Added alternative dry bath and starter equipment kit options. Corrected volume of 0.5X Wash Buffer to prepare for assessing cDNA quality. Corrected volume of ethanol to prepare for cDNA clean up. Updated vortexer setting guidance. Updated catalog number for user-supplied USA Scientific 0.2 ml PCR 8-tube strips and caps. Updated catalog number for user-supplied Fisher Scientific 0.5 ml PCR Safe-Lock PCR Clean tubes.
Document # 200063182 v00	March 2025	Initial release.

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