



PIPseq™ T2 3' Single Cell RNA Kit v3.0

User Guide

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Introduction

The Fluent BioSciences PIPseq™ T2 3' Single Cell RNA Kit v3.0 uses pre-templated instant partitions to combine individual cells with barcoded beads to produce sequencing-ready libraries starting from single cell suspensions. These libraries enable 3' gene expression profiling of up to 2000 individual cells (captured) per sample. This protocol can be completed in a convenient 2-day workflow.

Protocol Timing

Step	Duration	Stopping point
Reagent Preparation	~30 min	
General Cell Preparation Dependent on cell type	~1-1.5 hrs	
Cell capture and lysis	1 hr 40 min	4°C overnight or 0°C up to 72 hours
mRNA isolation		
Breaking emulsions	30 min	
Washing PIPs	1 hr	
cDNA synthesis	2 hr 30 min	4°C overnight
Washing PIPs	15 min	
cDNA amplification	2 hours	4°C overnight
Isolate cDNA from PIPs		
SPRI purification	1 hr 30 min	-20°C < 2 wks
cDNA QC and Quantification		
Qubit Quantification	20 min	
Fragment size analysis	20 min	
Library preparation		
Fragmentation, End Repair & A-tailing	55 min	
Adapter Ligation	25 min	
Post Ligation Cleanup	20 min	
Sample Index PCR	1 hr	4°C overnight
Post Sample Index PCR Cleanup	20 min	-20°C long-term
Post Library Preparation QC		
Qubit Quantification	20 min	
Fragment size analysis	20 min	

Recommended Use

The recommended application for this kit is 8 reactions in which 2000 mammalian cells are individually captured for creation of single cell 3' gene expression libraries to be sequenced on an Illumina platform. It is recommended that a minimum of 2 samples are processed at a time.

PIPseq Platform Overview

The PIPseq T2 3' Single Cell RNA Kit v3.0 consists of five reagent kits (Ambient, 4°C, -20°C Box 1 and Box 2, and -80°C), a consumables kit, and a Starter equipment kit containing required equipment.

PIPseq T2 3' Single Cell RNA Kit Product Numbers

Product Name	Catalog Number	Storage
PIPseq T2 3' Single Cell RNA Ambient Kit v3.0	FBS-SCR-T2-8-V3-1	15°C to 30°C
PIPseq T2 3' Single Cell RNA 4°C Kit v3.0	FBS-SCR-T2-8-V3-2	2°C to 8°C
PIPseq T2 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	FBS-SCR-T2-8-V3-3	-30°C to -15°C
PIPseq T2 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	FBS-SCR-T2-8-V3-4	-30°C to -15°C
PIPseq T2 3' Single Cell RNA -80°C Kit v3.0	FBS-SCR-T2-8-V3-5	-90°C to -75°C
PIPseq T2 3' Single Cell RNA Consumables Kit v3.0	FBS-SCR-T2-8-V3-6	15°C to 30°C
PIPseq T2 3' Single Cell Starter Equipment Kit v3.0	FBS-SCR-T2-V3-STKIT	15°C to 30°C



Warning: The items in the Starter kit below are highly recommended for the PIPseq 3' Single Cell RNA workflow. The specific brands of plastic consumables have been validated to ensure stability of PIP emulsions. Substituting these materials may adversely affect performance.

PIPseq-T2 3' Single Cell RNA Starter Equipment Kit

Component Name	Part Number	Units
PIPseq vortex mixer	FB0002373	1
PIPseq rotating vortexer adapter for 0.5 mL tubes	FB0002084	1
PIPseq rotating vortexer adapter base	FB0002101	2
Platefuge microcentrifuge	FB0002555	1
PIPseq Dry bath with heated lid	FB0001963	1
US Power Supplies for Vortex Mixer	FB0002353	1
US Power Supplies for Platefuge microcentrifuge	FB0002358	1
US Power Supplies for PIPseq Dry bath	FB0002363	1
2.5 mm Allen Key Wrench	FB0001723	1
PIPseq guide rack, red	FB0001549	2
PIPseq 4-tube stand, blue, for 1.5 mL tubes	FB0002590	2
Platefuge adapter	FB0002082	2
PIPseq 8-tube stand for 0.2 mL tubes	FB0001024	1
PIPseq 4-tube stand for 0.5 mL tubes	FB0002687	2
PIPseq dry bath with heated lid User Manual	FB0002664	1
2.5 mm Allen key wrench, silver	FB0002666	1
PIPseq dry bath Stylus	FB0002667	1
Replacement tips for PIPseq dry bath Stylus	FB0002668	1
5x20 mm 5A 250V Fast blow fuse for PIPseq dry bath	FB0002669	1
Dry block removal tool, white	FB0002670	1

PIPseq-T2 3' Single Cell RNA Consumables Kit

Component Name	Part Number	Units
Centrifuge Tube Filter	FB0003696	1 Bag of 16
1.5 mL Snap Cap Low Retention microcentrifuge tubes, ThermoFisher 3451	FB0001859	1 Bag of 16
0.2 mL PCR 8-tube strip without Cap, Greiner Bio-One, 673210	FB0002076	4 Strips of 8
PCR 8-Cap strips, domed cap, Greiner Bio-One, 373270	FB0001055	8 Strips of 8

PIPseq T2 3' Single Cell RNA Reagent Kit Components

Component Name	Part Number	Kit	Storage	Units
T2 PIPs	FB0003297	PIPseq T2 3' Single Cell RNA -80°C Kit v3.0	-90°C to -75°C	8
Cell Suspension Buffer	FB0002440	PIPseq T2 3' Single Cell RNA -80°C Kit v3.0	-90°C to -75°C	8
Partitioning Reagent	FB0001550	PIPseq T2 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	1
CLB2	FB0003722	PIPseq T2 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	8
Breaking Buffer	FB0003127	PIPseq T2 3' Single Cell RNA 4°C Kit v3.0	2°C to 8°C	1
Washing Buffer	FB0003138	PIPseq T2 3' Single Cell RNA 4°C Kit v3.0	2°C to 8°C	1
De-Partitioning Reagent	FB0002376	PIPseq T2 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	1
RT Enzyme Mix	FB0001971	PIPseq T2 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
RT Additive Mix	FB0001965	PIPseq T2 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
TSO	FB0001042	PIPseq T2 3' Single Cell RNA -80°C Kit v3.0	-90°C to -75°C	4
WTA Buffer Mix	FB0001043	PIPseq T2 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
WTA Primer	FB0002006	PIPseq T2 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
SPRI Beads	FB0001533	PIPseq T2 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	1
Nuclease-free water	FB0001077	PIPseq T2 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	1
IDTE pH 8	FB0001076	PIPseq T2 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	1
Library P7 Indices	FB0001626-1627, FB0001629-1633, FB0002092	PIPseq T2 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1 each
Library P5 Indices	FB0001915-1918, FB0001666-1669	PIPseq T2 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1 each
Resuspension Buffer with Tween	FB0001826	PIPseq T2 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
Library Prep Buffer	FB0001602	PIPseq T2 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	-30°C to -15°C	1
Library Prep Enzymes	FB0001603	PIPseq T2 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	-30°C to -15°C	1
Library Prep Mix A	FB0001604	PIPseq T2 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	-30°C to -15°C	1
Library Adapter Mix	FB0001605	PIPseq T2 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
Library Prep Mix B	FB0001606	PIPseq T2 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	-30°C to -15°C	1

Reagent, Equipment, and Consumable Requirements

Required Third Party Reagents

Reagent	Supplier
Cell Counting Materials using laboratory's preferred method (e.g. AO/PI)	Preferred Vendor
Nuclease-free water	Thermo Fisher; Cat # AM9937 or preferred laboratory supplier
Qubit 1X dsDNA High Sensitivity Assay Kit	Thermo Fisher; Cat #Q33231
High Sensitivity DNA kit for BioAnalyzer or TapeStation	Agilent; 5067-4627, 5067-5593
100% Ethanol, molecular biology grade	General Laboratory supplier

Recommended Third Party Consumables

Item	Supplier
<i>*NOTE* Sterile, low retention tips are required for this protocol. Multiple suppliers may be used however only the below listed suppliers have been tested in this protocol*</i>	
Sterile Tips 20 µL, filtered, low retention	Rainin; Cat # 30389226 Filtrous; Cat # PTF-LS-0020 VWR;76322-528
Sterile Tips 200 µL, filtered, low retention and Sterile Tips 200 µL, filtered, low retention, wide-bore	Rainin; Cat # 30389240, 30389241 Filtrous; Cat # PTF-LS-0200 VWR; Cat #76322-150
Sterile Tips 1000 µL, filtered, low retention and Sterile Tips 1000 µL, filtered, low retention, wide-bore	Rainin; Cat # 30389213, 30389218 Filtrous; Cat # PTF-LS-1000 VWR; Cat #76322-154
15 mL conical tube, sterile and nuclease-free	General laboratory supplier
Qubit Assay Tubes	Thermo Fisher; Cat # Q32856
PCR-clean tubes, strips, or plates for library preparation. *NOTE* Specific consumables may be required for certain steps, see warnings in protocol.	General laboratory supplier

Required Third Party Equipment

Description	Supplier
0.2 ml Magnetic Separation Rack	Permagen, MSRLV08 or equivalent from alternative supplier
Ice bucket or cold blocks, suitable for 0.2mL and 0.5mL PCR tubes and 1.5mL microcentrifuge tubes	General laboratory supplier
Hemocytometer or automated cell counter	General laboratory supplier
Micropipettes, 1µL-1000µL capabilities	General laboratory supplier
Qubit Fluorometer	Thermo Fisher
Bioanalyzer 2100 or TapeStation 4200	Agilent
Benchtop microcentrifuge 2000xg, suitable for 1.5 mL tubes and 0.5 mL tubes. *NOTE* The microcentrifuge should decelerate <u>before stopping</u> in order to prevent beads from returning to the solution*	USA Scientific; Cat # 2631-0006
Inserts for benchtop microcentrifuge that allow compatibility with 0.5 mL tubes	Provided accessory with USA Scientific 2631-0006

For some reagents and consumables, multiples may be required for pre-PCR and post-PCR use. Dedicated items and workspaces should be separated for pre-PCR and post-PCR processes to avoid carryover contamination.

Best Practices

Notes for working with RNA

Before executing this protocol, become familiar with working with RNA. The following section provides general guidelines for working with RNA, but the guidelines below are not all-inclusive.

- Due to the ubiquitous presence of RNases, RNA is susceptible to degradation. RNases are robust nucleases specific to RNA. Unlike DNases, they are not easily denatured or inactivated.
- The most common external RNase contamination comes from the skin. Therefore, any item that will be used for RNA work should be not handled without gloves to maintain nuclease-free surfaces. Wear a clean laboratory coat or gown to prevent shedding of skin or hair in and around the RNA work surface.
- Dust and bacteria are also sources of RNases. Keep surfaces and items free of dust and work with RNA away from bacterial processes.

- Refrigerator and freezer handles should be treated as RNase-free surfaces and should only be opened with gloved hands.
- Maintain reagents, pipettes, and a work space specifically for working with RNA. Wipe down pipettes and the working space regularly with a laboratory alcohol cleaning solution (70% alcohol). Periodically (once a week to once a month), clean surfaces with a 10% sodium hypochlorite solution. Alternatively, commercial solutions are available to inactivate RNases.
- Only use consumables and reagents that are nuclease-free and dedicated for RNA use. Reagents should be thoroughly thawed and aliquoted. In case of contamination, an aliquot can be discarded without compromising the entire reagent stock.

Cell loading

This protocol describes addition of 5,000 cells into the PIPseq assay, resulting in recovery of 2,000 cells and a multiplet rate of < 5%. The optimal input cell concentration is 1,000 cells per microliter. Alternative cell loading concentrations are allowed, as long as input volume is maintained, and increased cell loading will impact the observed multiplet rate according to the table below. Note that these data were obtained from species mixture experiments with mammalian cell lines.

# Cells loaded	# Cells recovered	Multiplet rate
50	~10	0%
100	~35	0%
500	~120	0%
2,500	~1400	~4.5%
5,000	~2000	~5%

PIPseq T2 Protocol

The PIPseq protocol generally takes about 2 days from cell addition to a sequencing-ready library, with multiple stopping points that are noted.

Reagent Preparation

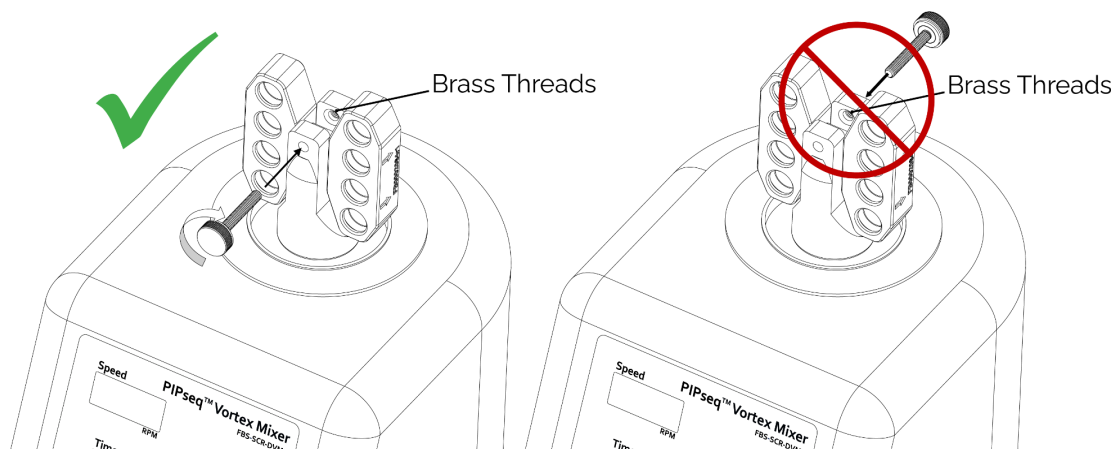
Upon receiving the PIPseq T2 3' Single Cell RNA kits, remove the -80°C components TSO (FB0001042), Cell Suspension Buffer (FB0002440), and PIP reactions (FB0003297) from the dry ice shipping container and store in a -80°C freezer.

Prior to each experiment, thaw one aliquot of Cell Suspension buffer at room temperature for each cell preparation and dilute the 1X Washing Buffer 1:1 with nuclease-free water to yield 500 μL of 0.5X Washing Buffer for each PIP reaction. Ensure the 1X Washing Buffer and 0.5X working stock are kept on ice during preparation and while in use. Please note that the 1X Washing Buffer is specifically for use prior to reverse transcription and the 0.5X Washing Buffer is for use subsequent to reverse transcription.

Equipment Preparation

IMPORTANT NOTE: Users will be required to alter the configuration of the rotating vortex adapter from horizontal to vertical during the PIPseq protocol. It is recommended that users practice altering the configuration of the adapter to ensure the process can be completed in 30 seconds prior to starting this protocol.

The vortex adapter head (yellow) is attached to the vortex base (gray) with a thumbscrew. The thumbscrew should be fastened from the front of the vortex mixer (see below). For further information on proper installation, review FB0003717 PIPseq Vortex Adapter Installation Guide.



The thumbscrew should be just tight enough such that the fixture does not rotate during vortexing, but not too tight such that changing from horizontal to vertical orientation takes significant effort. The tightness of the thumbscrew may need to be periodically adjusted. Users should ensure that the tube block is affixed securely within the PIPseq dry bath prior to beginning this protocol. For every sample, assemble a syringe and a needle (provided).

Sample Preparation

The PIPseq single-cell protocol requires a suspension of viable single cells or nuclei as input which may be derived from cell culture, dissociated tissues, cell sorting or other isolation methods. Users should minimize the presence of dead or aggregated cells to ensure the highest quality data. See the appropriate section for sample preparation instructions, for cells or nuclei.

Cell Preparation

This general cell preparation section describes a generic protocol for washing and resuspending cells starting from cryopreserved vials which is applicable to mammalian cell lines but may not be generalizable to all cell types (consult FB0001843 for more information on preparing cells for PIPseq). Preparation of single cell suspensions from tissues or fragile cell types may require additional dissociation or cell handling which are not described here. Users may substitute their own cell preparation protocol, however the final cell dilution step must be made using the Fluent Cell Suspension Buffer. Usage of wide-bore pipette tips is recommended to minimize cell damage.

1. Obtain a vial of the cryopreserved cells of interest from liquid nitrogen storage. If starting from fresh cells, skip to step 6.
2. Submerge the cryopreserved cell vial and an aliquot of Cell Suspension Buffer in a water bath set to 37°C ensuring that most of the tube is under the water line without being fully submerged.
3. After 1-1.5 minutes of thawing, check the cryopreserved cell vial. When there is only a moderate ice chunk remaining (60-70% thawed), remove the vial from the water bath. Decontaminate the outside of the vial with alcohol and move it into the biosafety cabinet. The remaining ice will thaw over the next 30-60 seconds at room temperature.
4. Use a **wide bore** P1000 or 2mL serological pipette to transfer the cell suspension to a 15 mL conical tube. Gently pipette up and down to collect all cell contents from the tube.
5. **SLOWLY** add 9 mL of warmed thawing media to the cell suspension in the 15 mL conical tube and mix by inversion three to five times. The initial 2 mL should be added over **at least** 30 seconds with the remaining media added at an increasing pace.
6. Centrifuge cells at 200 x g for 5 min to pellet cells.



The cell pellet may form on the side or the bottom of the tube depending on the rotor type. Swinging bucket rotors are recommended for pelleting. Centrifugation speed

depends on cell type and size. Generally, up to 300g x 5min can be used safely for smaller cell types. The goal is to form a loosely packed pellet with little to no cells in the supernatant. To this end, when working with new cell types it is important to examine the supernatant and monitor the difficulty of resuspension (such as pellet packed too tight) until you find the minimum speed and time required for optimal pelleting.

7. Aspirate as much of the supernatant as possible without disturbing the cell pellet.
8. Add 1 mL pre-warmed Cell Suspension Buffer, gently mix 5 times with a P1000 **wide-bore** low retention pipette tip. Place the remaining Cell Suspension Buffer on ice to cool.
9. Centrifuge cells at 200 x g for 3 min to pellet cells. Aspirate as much of the supernatant as possible without disturbing the cell pellet.
10. Using a **standard** bore low retention pipette tip, add 200-400 µL cold Cell Suspension Buffer and gently mix 10-15 times until cells are completely resuspended. If clumps are visible, increase the amount of force used during pipetting. The goal is to use the minimum force necessary to create a homogeneous cell suspension.

OPTIONAL STEPS: Users may choose to skip use of the tip strainer to minimize volume and cell concentration losses that can occur during filtration.

- Using a **wide-bore** low retention pipette tip, withdraw 200 µL of the cell suspension and add a 40 micron FlowMi tip strainer onto the end of the tip.
- Gently dispense the cell suspension through the tip strainer into a fresh 1.5 mL tube. **Do not "blow out" while pushing the cell suspension through the strainer as this will push unwanted debris through it and back into the cell suspension.**

11. Determine cell concentration using the laboratory's preferred method (e.g. trypan blue staining on an automated cell counter).

Before proceeding, cells should be >90% viable. If cells have low viability, spin and count again to remove as much necrotic material as possible.

12. Prepare a cell suspension in Cell Suspension Buffer at a concentration of 1000 live cells per microliter using a **wide-bore** low retention pipette tip.
13. Once the target concentration is achieved, place the cells on ice.
14. Proceed with the Fluent PIPseq T2 Cell Capture and Lysis protocol.

Nuclei Preparation

Fluent recommends that users prepare nuclei suspensions using the PIPseq Nuclei Isolation Kit (FBS-SCR-NUC4) to ensure consistent, high quality nuclei across a diversity of tissues. See FB0003716 PIPseq Nuclei Isolation Kit User Guide for instructions.

IMPORTANT NOTE: The lysis temperature for nuclei is 66°C while the lysis temperature for cells is 37°C. At step 14 of Capture and Lysis, be sure to use the appropriate lysis temperature according to the sample type of interest.

Capture and Lysis

1. Users may choose to process 2-8 samples. Thaw the desired number of PIP tubes, one for each sample that will be processed.



WORKFLOW TIP: Begin preheating the PIPseq Dry bath to the appropriate temperature depending on sample input type with the lid temperature set to 105°C (green dot will appear next to this temperature when the heated lid is on). Set the time to hold for this step as users will skip to the next step once they reach step 15 of this section.

2. Thaw the PIP tubes on ice for at least 15 minutes. After the PIP tubes have fully thawed, centrifuge them for ~5 seconds on a benchtop minifuge to remove air bubbles, then place them on ice.
3. Place PIP tubes in the PIPseq 4-tube stand (FB0002687) to aid visual confirmation of effective mixing.
4. Mix the cell or nuclei suspension 10 times with a **wide-bore** P200 tip set to 80% of the cell suspension volume.
5. Add exactly 5 μ L of prepared cells or nuclei (5000 total) directly into the PIPs while avoiding the creation of air bubbles. Ensure cells are dispensed within the PIP layer and not just on the surface of the PIPs. When preparing multiple samples, add cells to all samples before proceeding to the next step.



6. Mix the cell:PIP mixture 10 times with a P200 pipette using a **standard bore**, low-retention P200 tip at 32 μ L stroke while moving the tip throughout the mixture starting from the bottom, moving up through the mixture, and returning to the bottom (Figure 1). Take care not to create bubbles, especially in the first six mixing strokes.



Troubleshooting: If bubbles are entrained during the first few mixing strokes, centrifuge the PIP tubes for ~ 5 seconds to remove the bubbles. Repeat the mixing in step 6 while avoiding the creation of bubbles.

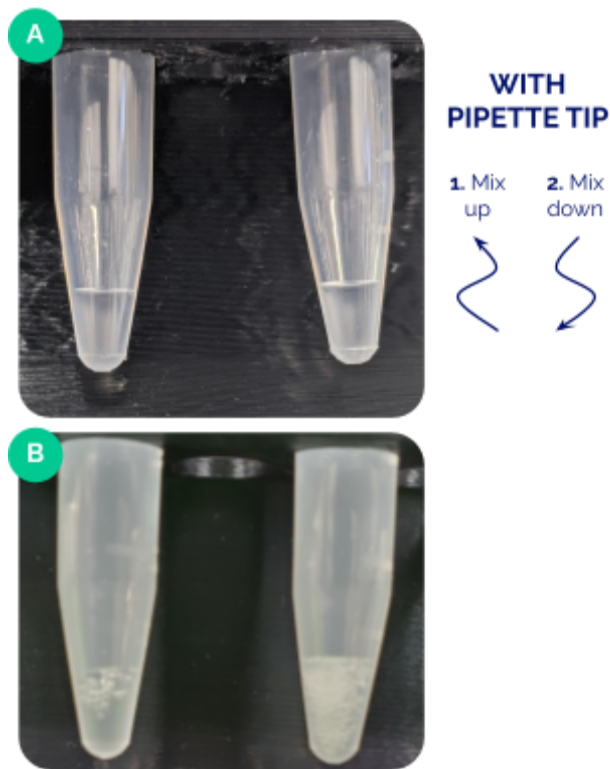


Figure 1. (A) PIPs following cell mixing with no apparent bubbles. (B) PIPs following cell mixing with an acceptable level of bubbles (left) or an unacceptable level of bubbles (right). Follow troubleshooting suggestions if an unacceptable level of bubbles are produced.

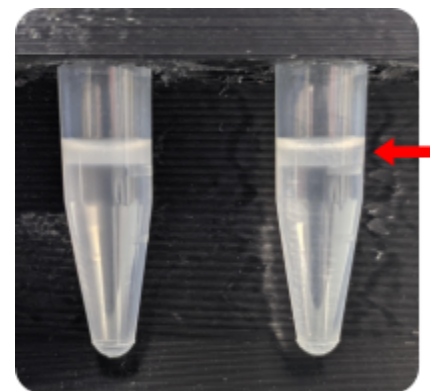


Figure 2. PIPs following addition of emulsification reagent. Take notice of the PIPs floating on top of the oil (red arrow).

7. Add 280 μ L Partitioning Reagent, along the side wall of the tube, to the cell:PIP mixture (Figure 2).
8. Ensure the tube caps are tightly sealed and place the tubes into the rotating vortex adapter in the horizontal configuration (Figure 3A). Ensure the tubes are fully depressed into the adapter. Vortex at 3000 RPM for 15 seconds.
9. Rotate the vortex adapter into the vertical configuration (Figure 3B). Vortex vertically at 3000 RPM for 2 minutes.



Figure 3. The rotating vortex adaptor shown in the horizontal configuration (A) and the vertical configuration (B).

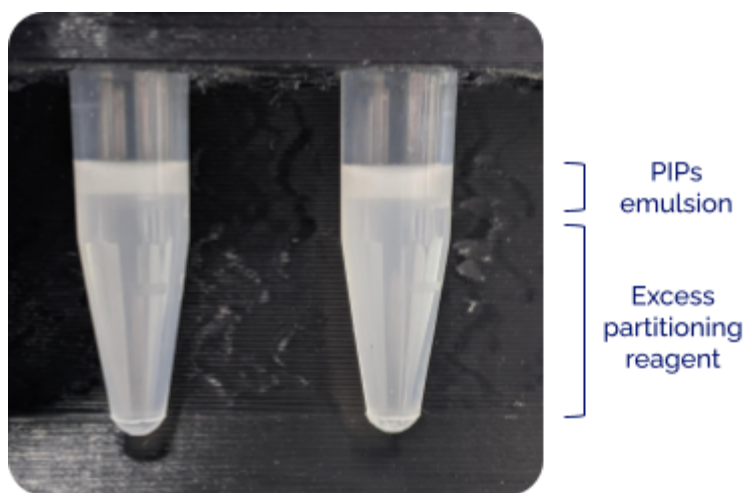


Figure 4. PIPs following emulsification

10. Remove the emulsion from the adapter for visual confirmation of the emulsion quality. The emulsion should be a homogenous white color (Figure 4). Proceed promptly to the next step.
11. Place a low-retention P200 pipette tip toward the bottom of the tube, wait for five seconds, and then aspirate out 115 μL x 2 of the Partitioning Reagent in the bottom phase. Be careful not to aspirate out any emulsion (Figure 5).



Tips for pipetting through an emulsion: Be cognizant of emulsion sticking tightly to the end of the pipette tip. It is usually helpful to wipe any tip that has passed through an emulsion on the side of the tube on the way out.

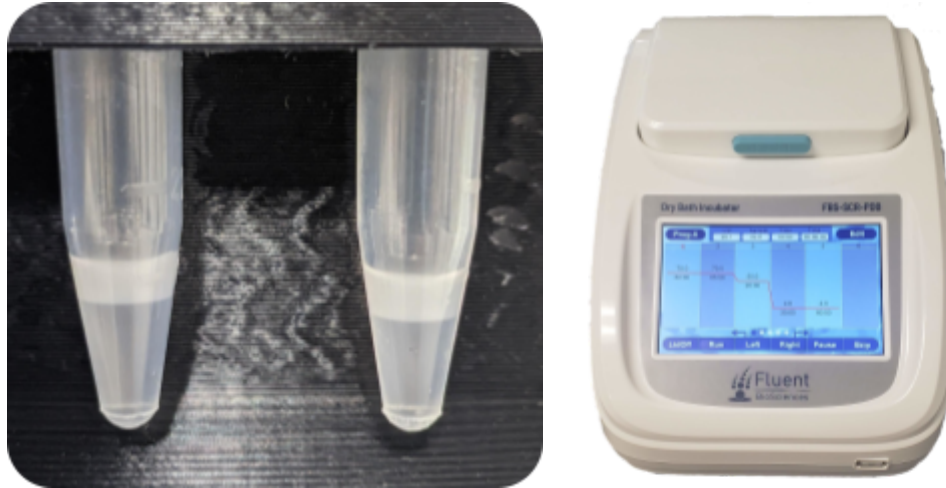


Figure 5. PIPs following removal of bottom phase (left). The PIPseq™ Dry bath (right) to be used for the lysis incubation step.

12. Prepare one Chemical Lysis Emulsion per reaction by adding 120 μ L of Partitioning Reagent to one of the provided CLB2 tubes (FB0003722). Vortex for 10 seconds to generate the emulsion and immediately add the whole volume of the Chemical Lysis Emulsion (160 μ L) on top of the PIP emulsion. Use one tube of Chemical Lysis Emulsion per sample.
13. Mix the combined emulsions gently by inversion 10 times.
Ensure the volume of the emulsion is below the top of the thermal block to ensure consistent heating.
14. Verify the PIPseq Dry bath (Figure 5) is preheated to the appropriate temperature according to the sample type in use (see table below) with the lid temperature set to 105°C. After at least 10 minutes of preheating is complete, insert samples and then skip to the next step to begin the lysis incubation as indicated in the table below.



Figure 5. The PIPseq™ Dry bath to be used for the lysis incubation step.

Cells		
Preheating step	37 °C	hold ("0000")
<i>After preheating complete and samples inserted, skip to next step</i>		
	37 °C	45 min
	4 °C	10 min
	4 °C	hold

Nuclei		
Preheating step	66 °C	hold ("0000")
<i>After preheating complete and samples inserted, skip to next step</i>		
	66 °C	45 min
	4 °C	10 min
	4 °C	hold

- 15. WORKFLOW TIP:** Complete steps 1-2 of the Breaking Emulsions section and step 1 of the cDNA synthesis section during incubation if you plan to proceed.
- 16.** After incubation is complete you can hold the samples at 4°C overnight or 0°C for up to 72 hours before proceeding to mRNA isolation. **Do not freeze!** Alternatively, you can move forward to mRNA isolation after the 4°C incubation. **Note: Some condensation may appear on the tubes, which is not a concern.**



mRNA isolation

Breaking Emulsions

1. Take the Breaking Buffer out of 4°C storage and let it warm for 10 minutes at room temperature.



WARNING: Do not centrifuge the emulsion. If liquid is observed on the cap, users may choose to invert 3 times.

2. Prepare aliquots of 1 mL chilled 1X Washing Buffer into one low retention 1.5 mL microcentrifuge tube (ThermoFisher Cat #3451) per sample and keep these aliquots on ice. **Do not substitute alternative brands of 1.5 mL tubes at this step.**
3. Place the emulsified PIP samples in the FB0002687 **blue** PIPseq 4-tube stand.
4. Discard most of the remaining partitioning fluid by aspirating up to ~140 μ L from the bottom of each emulsion.



Move slowly and do not induce mixing of the PIPs into the partitioning fluid layer. It is acceptable to leave a few microliters of partitioning fluid in order to avoid aspirating the emulsified PIPs. See tips for pipetting through an emulsion above.

5. Add 200 μ L of Breaking Buffer at room temperature on top of the emulsion without disturbing the emulsion.
6. Add 40 μ L of De-Partitioning Reagent on top of the emulsion (Figure 6).



Figure 6. PIPs right after addition of Breaking Buffer and before adding De-Partitioning Reagent.

7. Securely close the tubes and wait 1 min for breaking to complete.



Critical: DO NOT vortex the tube during breaking.

8. Centrifuge briefly (< 2 seconds) on a minifuge using compatible 0.5 mL inserts. Begin timing when the minifuge reaches top speed.

Critical: Ensure the emulsion is completely broken by visually confirming there is a clear interface between the bottom phase and the aqueous layer containing the PIPs (top). If opaque regions are still visible, wait an additional 30 seconds and repeat brief centrifugation. If red precipitate is detected, be sure to remove it along with the bottom phase during step 9.



9. Discard the bottom phase by aspirating 2 x 20 μ L from the bottom of the tube (Figure 7).
10. Repeat step 8 and then aspirate any remaining bottom phase. Move the pipette tip in very slow circular motions at the bottom of the tube to ensure you aspirate all of the bottom phase.



Critical: Ensure all of the bottom phase is removed. It is acceptable to remove a small amount of the clear aqueous to ensure complete removal.

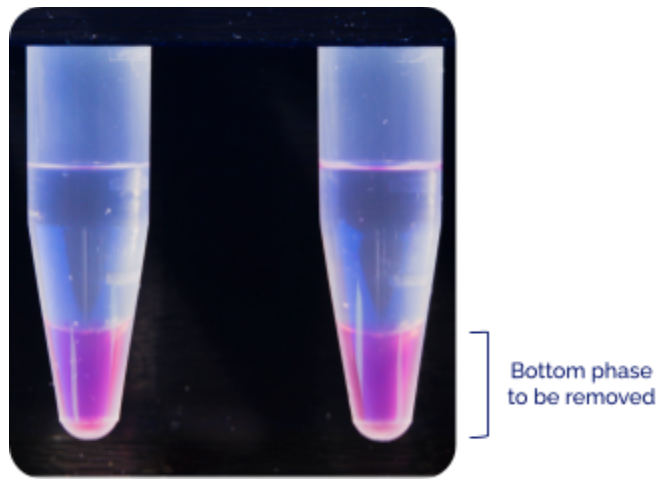


Figure 7. PIPs after addition of De-Partitioning Reagent and centrifugation.



11. Keep the tubes on ice or on a compatible cold block.

Note: Do not allow the PIP tubes to freeze. This will negatively impact assay performance.

Washing PIPs with 1x Washing Buffer

1. With a pipette set to 180 μ L, transfer the PIPs into 1 mL of chilled, pre-aliquoted 1X Washing Buffer on ice or in a compatible cold block. Transfer with the same pipette tip **twice** to make sure all of the PIPs are transferred. **Recommendation: Don't eject the pipette tips.**
2. Centrifuge the 0.5 mL PCR tube previously containing PIPs for 5 seconds and using the same pipette tip used to transfer in the previous step, aspirate any remaining PIPs and dispense rinsate into the 1.5 mL tube containing the transferred PIPs.

This second aspiration is done to ensure PIPs are not left behind in the pipette tip or the 0.5 mL tube. If residue liquids are observed inside the pipette tip, users can rinse the pipette tips one more time in the 1.5 mL wash buffer tube and dispense rinsate into the 1.5 mL tube containing the transferred PIPs.



3. Place the 1.5 mL tubes of washed PIPs into the 1.5 mL stand (FB0002590). Hold the stand horizontally on a flat-head vortex mixer for ~ 3 seconds to ensure that no PIPs are stuck at the bottom of the tube.
4. Ensure the FB0002082 adapters are securely placed into the Platefuge and properly balanced. Slide the 1.5 mL stand (FB0002590) with the PIP tubes into the adapter.
5. Spin the tubes for one minute on the platefuge microcentrifuge as shown in Figure 8.

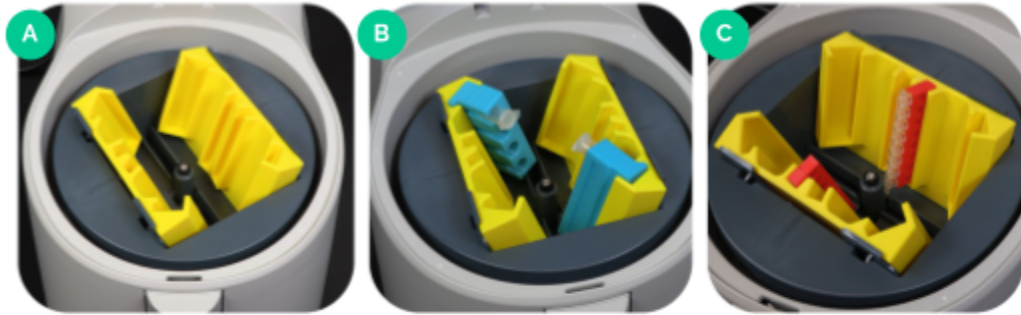


Figure 8. Load the yellow adaptors into the platefuge as shown in (A). For washes with 1X wash buffer pre-RT, load your tubes in the blue tube racks, and the tube racks into the yellow adaptors as shown in (B). For 0.5X washes post-RT and pre-WTA on page 22, load the washes in the red guide-wire racks as shown in (C).



IMPORTANT: Allow the centrifuge to slowly come to a stop. Abrupt halting will result in loss of PIPs. After centrifugation the PIP pellet will appear angled but will not reach above the 0.1 mL mark on the 1.5 mL tube.

- Aspirate and discard the aqueous volume above the 0.1 mL mark on the outside of the 1.5 mL tube **WITHOUT** disturbing PIPs pellet (Figure 9).

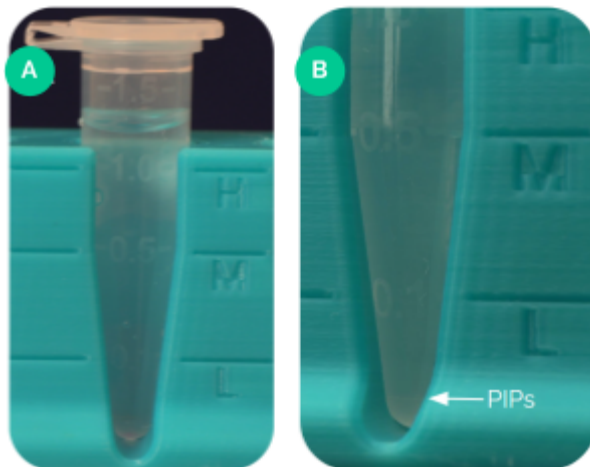


Figure 9. PIPs are difficult to visualize in (A) before centrifugation. PIPs may be seen in the bottom of the 1.5 mL tube during washes (white arrow) in (B).



Warning: Refrain from placing the tip below the 0.1 mL mark on the tube during aspiration to avoid loss of PIPs during washing. Aspirate slowly and carefully; don't disturb the pellet.

- Add 1.0 mL Washing buffer to PIPs and repeat steps 3-6 to complete the second wash of the PIPs.
- Add 1.0 mL Washing buffer to PIPs and repeat steps 3-6 to complete the third wash of the PIPs.
- Add 1.0 mL Washing buffer to PIPs and repeat steps 3-6 to complete the fourth and final wash of the PIPs.

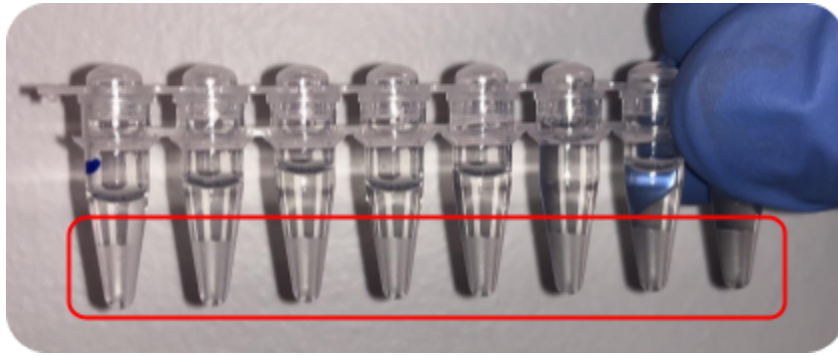


Figure 10. PIPs packed in 0.2 mL strip tubes following centrifugation with PlateFuge.

10. Move the entire volume of the ~ 100 μ L PIP mixture into one new 0.2 mL Greiner Bio-One PCR tube per sample (FB0002076). Seal the strip securely with the 8-strip cap (FB0001055). Place the strip into the FB0001549 **red** PIPseq guide rack, and slide the rack containing the strip into the FB0002082 adapter (ensure that the platefuge is properly balanced before spinning). Spin for ~30 seconds on a benchtop plate microfuge with a swing-out rotor (Figure 8).



Note: The swing-out rotor of the Platefuge enables the PIP/aqueous interface to remain flat instead of angled. **Allow the centrifuge to slowly come to a stop. Abrupt halting will result in loss of PIPs.**

11. Keeping the strip in the red PIPseq guide rack, inspect the supernatant level across all samples. If variable volumes are observed across samples, remove the strip from the rack and ensure that all partitioning fluid has been removed from the bottom of each tube. If any fluid remains, aspirate and discard it prior to reinsertion into the guide rack.



Critical: Ensure the strip is securely inserted into the guide rack. Improper insertion may result in PIP loss.

12. **Remove the supernatant above the PIPs to the level indicated by the guide wire.**



Warning: Do not remove aqueous below the visible level indicator. The base of the meniscus should be hidden behind the guide wire (Figure 11).

13. Remove the 0.2 mL strip from the **red** PIPseq guide rack and place on a cold block or ice until the next step.

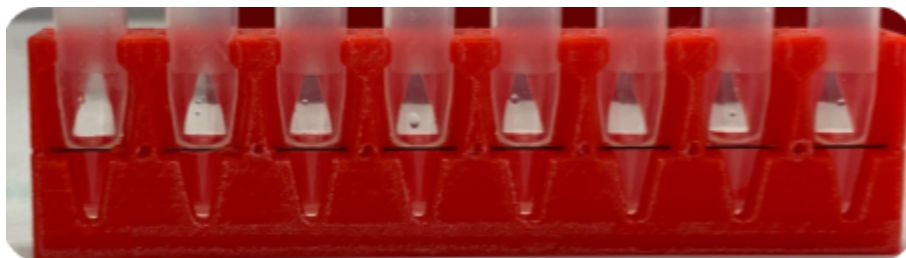


Figure 11. PIP tubes in the guide rack where the volume has been reduced to the level of the wire. Note that the base of the meniscus is hidden behind the wire.

cDNA synthesis



- WORKFLOW TIP:** Prepare the cDNA synthesis reaction on ice as indicated in the table below for a 39 μL reaction. The TSO must be thawed on ice after removal from -80°C storage. Remember to prepare the working stock dilutions of 0.5X Washing Buffer prior to the start of this step (see Reagent Preparation above). Note that fill volumes provide sufficient reagents for 9.5 total reactions so it is recommended not to exceed 0.5 reaction overage when creating a mastermix. Users may prepare this mastermix **without the RT Enzyme Mix** ahead of time (e.g. while cells are in lysis) and place it on ice.
- When ready for RT, add the RT Enzyme Mix to the mastermix** and mix well by pipetting 10 times.

Reagent	Volume Per Reaction (μL)
RT Additive Mix	33.5
TSO	3.1
RT Enzyme Mix	2.4
Total	39.0

- Add 39 μL of the mastermix to each 0.2 mL PCR tube of PIPs. Place the PIP strip into the FB0001024 PIPseq 8-tube stand.
- Vortex the sample at 3000 rpm with the reverse transcription components for 5 seconds by placing the stand on a flat head vortex mixer. To avoid accidental uncapping and sample loss, be sure to maintain pressure on the tube caps with your hand while vortexing.

Warning: Ensure the PIP pellet is dispersed during vortexing.



- Place the PCR strip into the **red** PIPseq guide rack, and slide the rack containing the strip into the FB0002082 adapter (ensure that the platefuge is properly balanced before spinning). Briefly spin down any droplets on the platefuge, taking care that the beads do not form a pellet due to the spin (~2 seconds).
- Perform cDNA synthesis with the following protocol in a thermal cycler with heated lid set to 105°C .

25 $^{\circ}\text{C}$	30 minutes
42 $^{\circ}\text{C}$	90 minutes
85 $^{\circ}\text{C}$	10 minutes
4 $^{\circ}\text{C}$	Hold



The samples can be stored overnight at 4°C in the thermocycler or the fridge before proceeding to the next step.

Washing PIPs with 0.5x Washing Buffer

1. Place the strip into the **red** PIPseq guide rack, and slide the rack containing the strip into the FB0002082 adapter (ensure that the platefuge is properly balanced before spinning). Spin for 1 min on a benchtop plate microfuge with a swing-out rotor.
2. Keeping the strip in the red PIPseq guide rack, remove and discard the strip cap. Carefully remove the supernatant above the PIPs to the level indicated by the guide wire. Make sure not to disturb the PIP pellet. Replace the strip cap with a new 8-tube strip cap (FB0001055) and place the samples on ice.
3. Add 170 μL 0.5X Washing Buffer to each PIP tube.
4. Seal the strip caps and place the PIP tubes into the PIPseq 8-tube stand. Vortex mix the PIP tubes at 3000 rpm for 5 seconds by placing the stand on a flat head vortex mixer. To avoid accidental uncapping and sample loss, be sure to maintain pressure on the tube caps with your hand while vortexing. Do not pipette mix.
5. Place the strip into the **red** PIPseq guide rack, and slide the rack containing the strip into the FB0002082 adapter (ensure that the platefuge is properly balanced before spinning). Spin for 1 min on the benchtop plate microfuge.
6. Aspirate and discard 150 μL supernatant.
7. Add 150 μL 0.5X Washing Buffer to each PIP tube and repeat steps 4-6 to complete the second wash of the PIPs.
8. Add 150 μL 0.5X Washing Buffer to each PIP tube and repeat steps 4-5 to complete the third wash of the PIPs. During this final aspiration, remove the supernatant above the PIPs to the level indicated by the guide wire. Proceed promptly to cDNA amplification.

Note: If the PIPs are disturbed during these steps, spin for 1 min on the benchtop plate microfuge before proceeding to aspirate.

cDNA amplification

1. Prepare whole transcriptome amplification (WTA) mastermix as described in the table below and mix briefly by vortexing.

Reagent	Volume Per Reaction (μL)
WTA buffer mix	39.0
WTA primer	0.39
Total	39.39

2. Add 39.0 μL of WTA mastermix into each RT product mix.

- Place the PIP strip into the PIPseq 8-tube stand.
- Vortex mix the samples for 5 seconds by placing the stand on a flat head vortex mixer. To avoid accidental uncapping and sample loss, be sure to maintain pressure on the tube caps with your hand while vortexing.
- Place the strips into the **red** PIPseq guide racks, and slide the guide racks into the FB0002082 adapter. Spin down each strip tube on the platefuge for ~ 2 seconds.



WORKFLOW TIP: The cycle number below is the recommended starting point for PCR cycle number optimization. The optimal cycle number is a trade-off between generating sufficient mass for library preparation and minimization of PCR amplification artifacts. If more cells than recommended are added to this protocol the number of amplification cycles should be reduced accordingly.

- Run the program below with the heated lid set to 105°C.

Temperature (°C)	Time	Cycle Number
95	3 min	x1
98	15 sec	See table below
69	4 min 20 sec	
72	5 min	x1
4	hold	

Cells Loaded	Targeted cell capture	Recommended Cycles - high RNA samples (e.g. cell lines, cancer cells)	Recommended Cycles - low RNA samples (e.g. primary cells, nuclei)
<500	<200	16	20
500-1000	200-400	14	18
1000-5000	400-2000	12	17
>5000	>2000	12	16



The samples can be stored overnight at 4°C in the thermocycler or the fridge before proceeding to the next step.

Isolate cDNA from PIPs

- Add 40 uL IDTE to each WTA reaction and add the mixture to one centrifuge filter per sample.
- Centrifuge filter column for 5 min at 13,000 x g and **keep the flow-through**.



WORKFLOW TIP: If the WTA volume appears to clog the filter, briefly pipette mix the liquid remaining on top of the filter and repeat step 3.

- Transfer the flow-through into new 0.2 mL PCR tubes.
- Measure the volume of each sample using a P200.
- Add enough IDTE to each sample to achieve a total volume of 120 µL per sample.

SPRI Purification

1. Make fresh 85% ethanol (e.g. 4.25 ml ethanol, 0.75 ml nuclease-free water).
2. Thoroughly vortex bottle of SPRI beads to mix.
3. For 120 μ L reaction volumes, add 72 μ L SPRI. This is a 0.6x ratio of SPRI beads.
4. Pipette up and down 10 times at the 200 μ L stroke and incubate for 5 minutes at ambient temperature.
5. If necessary, do a final quick spin down of the SPRI beads.
6. Place the 0.2 mL PCR tubes in the magnetic stand and bind to the magnet for 5 minutes.
7. Discard the supernatant being careful not to touch the SPRI beads.
8. Carefully wash twice with 200 μ L 85% ethanol for 30 seconds each. Do not disturb the SPRI beads.
9. Remove final traces of ethanol with P20 pipette, careful not to disturb the SPRI beads.
10. Air dry for 5 minutes with the top open to remove any residual ethanol, taking care not to overdry. The SPRI beads should still look glossy, not cracked. Remove any remaining traces of ethanol with a P20 pipette being careful not to disturb the SPRI beads.
11. Remove tubes from the magnetic rack and add 22 μ L IDTE. Mix the IDTE and SPRI beads by pipetting up and down 10 times at the 22 μ L stroke, ensuring that the bead pellet is fully resuspended in the IDTE.



Workflow Tip: Wash the IDTE over the SPRI pellet on the side of the tube until the pellet is fully washed off the tube, then pipette up and down 10 times.

12. Incubate for 5 minutes at room temperature.
13. If necessary, do a quick centrifuge of the SPRI beads.
14. Place tubes into the magnetic rack and bind to the magnet for 5 minutes.
15. Remove and **SAVE** 20 μ L of supernatant in a new PCR tube strip. Do not disturb the SPRI beads.



Amplified cDNA may be stored at -20°C < 2 weeks.

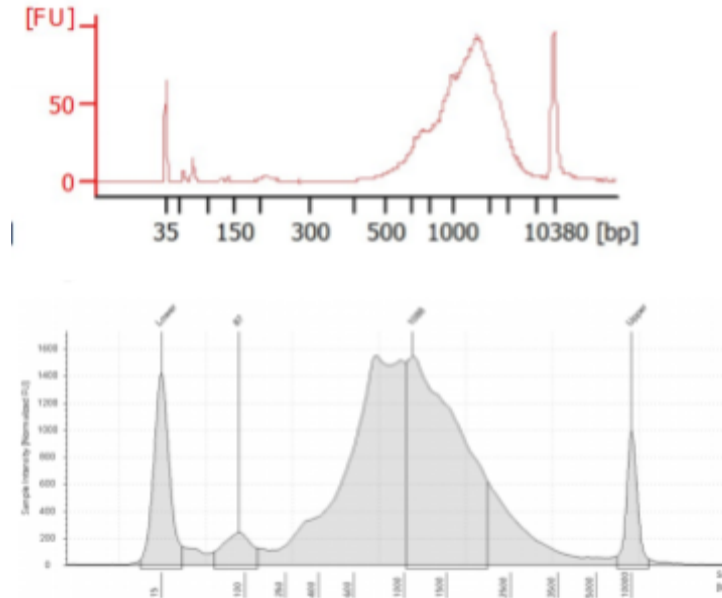
cDNA QC and Quantification

Qubit Quantification

1. Using a Qubit High Sensitivity kit, quantify 2 μ L of each sample according to the manufacturer's instructions.

Fragment Analysis

1. Users may use an Agilent BioAnalyzer or TapeStation. Dilute samples if necessary to ensure they are within the appropriate range of the device.
2. Load an appropriate volume of the purified library on the fragment analyzer and follow the manufacturer's instructions.



Representative cDNA traces for a 1000 cell input human/mouse cell mixture (HEK 293T/NIH 3T3) using an Agilent High Sensitivity DNA (top) and a high sensitivity D5000 ScreenTape (bottom).

Library preparation

Fragmentation, End Repair & A-Tailing

1. Remove all kit components from the freezer and thaw or equilibrate on ice.
2. Prepare 10-500 ng of input cDNA in a total volume of 40 μ L nuclease-free water in a PCR tube and place on ice.

Note: If a user cannot input 10 ng cDNA into library prep it is highly recommended to use a new sample and increase the number of PCR cycles used during cDNA amplification.

- Vortex the Library Prep Buffer for 5 seconds, and briefly centrifuge to collect all liquid at the bottom of the tube.
- Pipette mix the Library Prep Enzymes 15 times at the 40 μL stroke to homogenize, and briefly centrifuge to collect all liquid at the bottom of the tube.

Note: The user may *briefly pulse-vortex the enzyme on a benchtop vortex to ensure complete mixing.*

- Create a Library master mix of the Library Prep Buffer and Enzymes according to the table below. Homogenize by vortexing at a moderate intensity (avoid bubbling) for 5 seconds.

Reagent	Volume Per Sample (μL)
Library Prep Buffer	4
Library Prep Enzymes	6
Total	10

IMPORTANT: Add 0.5x reaction overage when calculating volumes for the master mix to account for standard pipetting error. The kit includes 9,5 total reactions.

- Add 10 μL of the Library master mix from step 5 to each sample, resulting in a total volume of 50 μL per reaction as shown in the table below.

Reagent	Volume Per Sample (μL)
cDNA	40
Library Master Mix	10
Total	50

- Vortex the samples at a moderate intensity for 5-10 seconds to homogenize (avoid bubbling), and briefly centrifuge to collect all liquid at the bottom of the tubes.



Note: Complete mixing is crucial; you may alternatively pipette mix 10 times using a 25 μL stroke.

- Set up the thermocycler with the program below, with the heated lid set to 105°C.
- Place samples in the thermocycler **during the initial 4°C hold** once the block and lid have reached the designated temperatures. Use the 'skip step' function on the thermocycler to start the 30°C step.

Temperature (°C)	Time
4	HOLD

30	8 min
65	30 min
4	HOLD



- Workflow tip: During this incubation prepare the Library Adapter Mix dilution as shown in the Adapter Ligation step 1 and place on ice.
- Proceed immediately to Adapter Ligation after the program has finished and the samples have returned to 4°C.

Adapter Ligation

- Prepare a dilution of the Library Adapter Mix (100 µM) to a final concentration of 3 µM in 5 µL for 1-10 ng DNA input according to the total number of samples for adapter ligation. If DNA input 11-500 ng is desired the Library Adapter Mix must be diluted to a final concentration of 15 µM in 5 µL. The below table illustrates volumes per reaction for DNA input 1-10 ng.

Library Adapter Mix Per Sample (µL)	Nuclease-free water Per Sample (µL)	Total Volume Per Sample (µL)
0.15	4.85	5

IMPORTANT: Add 0.5x reaction overage when calculating volumes for the dilutions to account for standard pipetting error. The kit includes a total of 15 µL of Library Adapter Mix, so plan accordingly to ensure you do not run out of reagents.



NOTE: Store dilutions of the Library Adapter Mix for less than 1 week at -20°C.

- Remove the samples from the thermocycler immediately upon return to 4°C.
- Add 5 µL of appropriately diluted Library Adapter Mix to each reaction, mix thoroughly by brief vortexing or pipetting followed by a brief centrifugation to collect any droplets.
- Pipette mix the Library Prep Mix A 15 times at the 100 µL stroke to homogenize (do NOT vortex) and place on ice.
- Add 20 µL of Library Prep Mix A to each sample yielding a total reaction volume per sample of 75 µL.

Note: Library Prep Mix A is very viscous, when pipetting make sure to do so slowly and allow time for the mix to be drawn into the pipette tip before adding to the samples to ensure the proper volumes are dispensed.



- Mix the reactions well by pipetting up and down 10 times at the 40 µL stroke, and briefly centrifuge to collect all liquid at the bottom of the tubes.
- Place the mixed sample reactions in the thermocycler and run the program below, with the heated lid OFF.

Temperature (°C)	Time
20	15 min

8. Proceed immediately to the Post Ligation Cleanup.

Post Ligation Cleanup

1. Freshly prepare at least 400 μL of an 85% ethanol solution for each reaction.



Resuspend the SPRI beads by vortexing thoroughly to ensure a homogeneous mixture.

2. Remove the ligation reaction from the thermocycler.
3. To each sample (still in the PCR strip), add 60 μL (0.8X) of resuspended SPRI beads, and mix thoroughly by pipetting up and down at the 70 μL stroke 10 times.
4. Incubate the library-bead mixtures at room temperature for 5 min.
5. Place the PCR strip into the magnetic rack and allow it to bind to the magnet for 5 minutes, until the solution is clear and all beads have collected on the tube wall.
6. Remove the supernatant from each tube (~135 μL), being careful to not disturb the beads.
7. Carefully wash twice with 200 μL of 85% ethanol for 30 seconds each. Do not disturb the SPRI beads.
8. Remove final traces of ethanol with P20 pipette, careful not to disturb the SPRI beads.
9. Air dry for 5 minutes with the top open, taking care not to overdry.



Note: The SPRI beads should still look glossy, not cracked.

10. Remove tubes from the magnetic rack and add 21 μL nuclease-free water. Mix the water and SPRI beads by pipetting up and down 10 times at the 21 μL stroke, ensuring that the bead pellet is fully resuspended in the water.



Workflow Tip: Wash the water over the SPRI pellet on the side of the tube until the pellet is fully washed off the tube, then pipette up and down 10 times.

11. Incubate tubes at room temperature for 5 min.
12. Place tubes into the magnetic rack and bind to the magnet for 5 min, or until the solution is clear.
13. Carefully transfer 20 μL of each library-containing supernatant to a new 0.2 mL PCR tube strip.

Sample Index PCR



Before starting the Sample Index PCR, choose the appropriate sample index sets to ensure that no sample index combinations overlap in a multiplexed sequencing run (See Illumina Sequencing section for recommendations). There are 8 i7 and 8 i5 indexes provided with this kit to allow for unique dual indexing of 8 samples. Note that each index contains sufficient reagent volume for 3 individual reactions.

1. Thaw Library Prep Mix B on ice. Once thawed, flick the tubes several times, pipette mix 10 times, and then briefly centrifuge to collect (do NOT vortex).
2. To the 20 μ L of each cleaned library, add the following *individually*, in the order in which they appear in the table below.

Reagent	Volume Per Sample (μ L)
Cleaned library DNA	20
Library P7 Index 70X	2.5
Library P5 Index 50X	2.5
Library Prep Mix B	25
Total	50

3. Mix the reactions by pipetting up and down 10 times at the 25 μ L stroke, and briefly centrifuge to collect all liquid at the bottom of the tubes.
4. Place samples in the thermocycler and run the program below, with the heated lid set to 105°C:

Temperature ($^{\circ}$ C)	Time	Cycles
98	45 sec	1x
98	15 sec	12x
67	30 sec	
69	45 sec	
72	1 min	1x
4	Hold	Hold



Samples may be stored at 4°C overnight prior to proceeding to Post Sample Index PCR Cleanup.

Post Sample Index PCR Cleanup — Double-Sided Size Selection

1. Make fresh 85% ethanol.



Resuspend the SPRI beads by vortexing thoroughly to ensure a homogeneous mixture.

2. Dilute the PCR reaction to 85 μ L by addition of approximately 35 μ L nuclease-free water.
3. For 85 μ L reaction volumes, add 51 μ L SPRI. This is a 0.6x ratio of SPRI beads. Adjust as necessary if reaction volume is not 85 μ L.
4. Pipette up and down 15 times at the 115 μ L stroke and incubate for at least 7 minutes at room temperature.
5. If necessary, do a final quick spin down of the beads.
6. Place tubes onto the magnetic rack and bind for 5 minutes.
7. Prepare new PCR tubes, one for each sample, in order to save the supernatant.
8. SAVE the supernatant and transfer it into the newly prepared PCR tubes.
9. Thoroughly vortex bottle of SPRI beads and then add 17 μ L SPRI beads to the supernatant solution. This is a 0.8x ratio relative to the original PCR reaction volume.

$$\frac{\text{Total volume of SPRI added to sample (steps 3+9)}}{\text{Original PCR reaction volume}} = \frac{51 \mu\text{L} + 17 \mu\text{L}}{85 \mu\text{L}} = 0.8X$$

10. Pipette up and down 15 times at the 115 μ L stroke and incubate for at least 5 minutes at room temperature.
11. If necessary, do a final quick spin down of the beads.
12. Place tubes onto the magnetic rack and bind for 5 minutes.
13. Discard the supernatant. Be careful not to disturb the SPRI beads.
14. Carefully wash twice with 200 μ L 85% ethanol for 30 seconds each. Do not disturb the SPRI beads.
15. Remove final traces of ethanol with P20 pipette being careful not to disturb the SPRI beads.
16. Air dry for 5 minutes with the top open to remove any residual ethanol, taking care not to overdry the SPRI beads. **Note: The SPRI beads should still look glossy, not cracked.**



17. Remove tube from the magnetic rack and add 21 μ L IDTE. Mix the IDTE and SPRI beads by pipetting up and down 10 times at the 20 μ L stroke.
18. Incubate for 4 minutes at room temperature.
19. If necessary, do a quick spin down of the SPRI beads
20. Place tubes onto the magnetic rack and bind to the magnet for 5 minutes.



21. Remove and **SAVE** 20 μ L of supernatant in a new PCR tube strip. This sample can be stored at -20°C long-term.

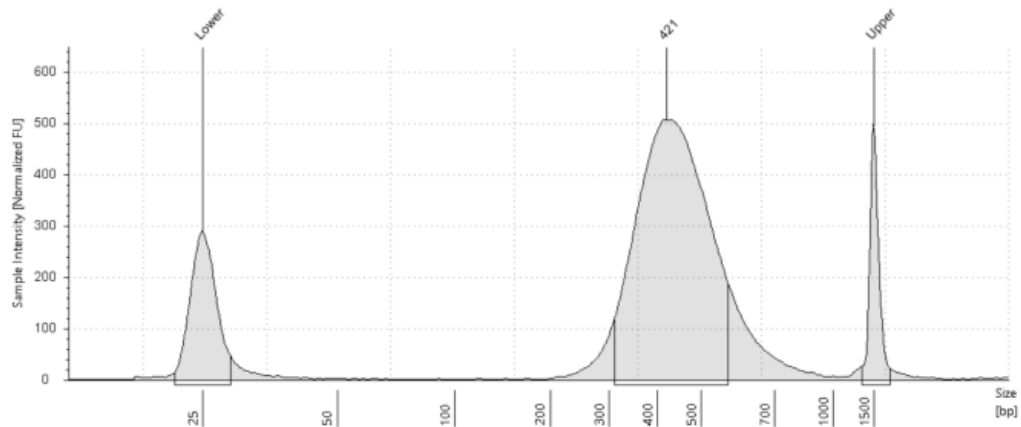
Post Library Preparation QC

Qubit Quantification

1. Using a Qubit High Sensitivity kit, quantify 2 μ L of each sample according to the manufacturer's instructions.

Fragment Analysis

1. Users may use an Agilent BioAnalyzer or TapeStation. Dilute samples if necessary to ensure they are within the appropriate range of the device.
2. Load an appropriate volume of the purified library on the fragment analyzer and follow the manufacturer's instructions.



Representative library trace for a 1000 cell capture human/mouse cell mixture (HEK 293T/NIH 3T3) on a High Sensitivity D1000 ScreenTape (above).

ILLUMINA SEQUENCING

PIPseq T2 3' Single Cell gene expression libraries are composed of standard Illumina paired-end constructs that begin with P5 and end with P7. These libraries are dual-indexed with 8-base i5 and i7 indexes. Read 1 length must be ≥ 51 bases and the recommended read 2 length ≥ 70 bases. Sequencing depth will vary based on your application needs but it is recommended to start with a depth of 20,000 reads per cell.

The PIPseq T2 3' Single Cell gene expression libraries may be pooled for sequencing, taking into account the differences in cell number and read depth requirements. Samples must have distinct index combinations to avoid failures in sample demultiplexing. Refer to Illumina documentation for discussion of appropriate color balance combinations for the selected sequencing platform.

Valid combinations for a 4-sample pool	
Library i7 index	Library i5 index
701, 702, 704, 705	At least two unique color balanced indexes (e.g. 503,504)
703, 705, 706, 701	At least two unique color balanced indexes (e.g. 503,504)

Once quantified and normalized, the PIPseq 3' gene expression libraries should be diluted and prepared as recommended in Illumina documentation for the selected Illumina sequencing platforms. PIPseq libraries require a minimum of 15% PhiX to be added to the loaded library pools. A simplified pooling scheme for the Illumina NextSeq2000 platform is described below.

1. Dilute the individual sample libraries with Resuspension Buffer (RSB) with Tween to 4 nM in a 6 μ L final volume.
2. Mix 5 μ L of diluted DNA from each sample library together in a 1.5 mL tube to create the sample library pool.
3. Prepare 24 μ L of a 2 nM dilution of the sample library pool.
4. Prepare 10 μ L of a 2 nM dilution using the 10 nM PhiX stock provided by Illumina.
5. Prepare a 15% PhiX final pool by combining 3.6 μ L of the 2 nM PhiX dilution with 20.4 μ L of the 2 nM library pool.
6. Dilute this final pool to 550 pM which is the final loading concentration for the Illumina NextSeq 2000 platform.
7. Load 20 μ L of this final pool onto the sequencing cartridge.

PIPseq has been verified to be compatible with the following Illumina platforms: NextSeq 500/550, the NextSeq2000 and the NovaSeq.

PIPseq 3' Index Sequences

Library i7 index	i7 sequence (for sample sheet)	i7 adapter sequence	Library i5 index	i5 adapter sequence
701	TAAGGCGA	TCGCCTTA	501	TAGATCGC
702	CGTACTAG	CTAGTACG	502	CTCTCTAT
709	GCTACGCT	AGCGTAGC	503	TATCCTCT
704	TCCTGAGC	GCTCAGGA	504	AGAGTAGA
705	GGACTCCT	AGGAGTCC	505	GTAAGGAG
706	TAGGCATG	CATGCCTA	506	ACTGCATA
707	CTCTCTAC	GTAGAGAG	507	AAGGAGTA
708	CAGAGAGG	CCTCTCTG	508	CTAAGCCT

Oligonucleotide Sequences

Part Number	Name	Sequence (5' - 3')
FB0001626-1627, FB0001629-1633, FB0002092	Library P7 Index	CAAGCAGAAGACGGCATAACGAGATXXXXXXXXXXGTCTCGT GGGCTCGGAGATGTGTATAAGAGACAG
FB0001915-1918, FB0001666-1669	Library P5 Index	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXXXAC ACTCTTCCCTACACGACGC
FB0002006	WTA Primer	Forward: CTCTTCCCTACACGACGCTC Reverse: AAGCAGTGGTATCAACGCAGAGT
FB0003140	TSO	AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG
FB0001605	Library Adapter Mix	Ligation primer: /5Phos/CTGTCTTTATACACATCTCCGAGCC Ligation adapter: TATAAGAGACAGT

Abbreviations

PIPs	Pre-templated Instant Partitions
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
dsDNA	Double-Strand DNA
cDNA	Complementary DNA
RTPCR	Reverse Transcription Polymerase Chain Reaction
TSO	Template Switch Oligo
WTA	Whole Transcriptome Amplification
RPM	Revolutions Per Minute

Document Revision Summary

Doc ID: FB0001026

Revision: 11.3

Revision date: December 2022

Specific Changes:

- Revamped "Equipment Preparation" section to provide clearer instructions.
- Clearer instructions in "Washing PIPs with 1X Wash Buffer" section.
- Revised cycling recommendations in "cDNA Amplification" section.

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