Fluent BioSciences

PIPseq™ T20 3' Single Cell RNA Kit v4.0

User Guide

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Introduction

The Fluent BioSciences PIPseq™ T20 3′ Single Cell RNA Kit uses Particle-templated Instant Partitions (PIPs) to combine individual cells or nuclei with barcoded beads to produce sequencing-ready libraries starting from single cell suspensions in 15 hours. These libraries enable 3′ gene expression by profiling 80,000 individual cells or nuclei per kit. 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	20°C for up to 96 hrs
mRNA isolation		
Breaking emulsions	15 min	
Washing PIPs with 1X Washing Buffer	30 min	
cDNA synthesis	2 hr 30 min	4°C overnight
Washing PIPs with 0.5X Washing Buffer	20 min	
cDNA amplification	1 hr 30 min	4°C overnight
Isolate cDNA from PIPs	30 min	
SPRI purification	30 min	-20°C < 2 wks
cDNA QC and Quantification		
Qubit Quantification	5 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	1 hr	-20°C long-term
Post Library Preparation QC		
Qubit Quantification	20 min	
Fragment size analysis	20 min	

Product Applications

The PIPseq T20 3′ Single Cell RNA Kit is designed for gene expression profiling of up to 80,000 cells or nuclei from a wide range of sample types. This kit is configured with 4 individual reactions, in which up to 20,000 cells or nuclei can be profiled per reaction. It is recommended that a minimum of 2 samples are processed at a time. Starting from single cell suspensions users will produce single cell 3′ gene expression libraries compatible with Illumina sequencing. The sequencing data can be processed through Fluent's PIPseeker™ software, a comprehensive analysis solution that provides users with detailed metrics, gene expression profiles, basic cell quality and clustering indicators. The "Getting Started with PIPseeker tutorial" can be downloaded at https://www.fluentbio.com/products/pipseeker-software-for-data-analysis/.

PIPseq Platform Overview

The PIPseq platform consists of four reagent kits (Ambient, 4°C, -20°C and -80°C), a Consumable kit and a one-time Starter kit containing required equipment.

PIPseq T20 3′ Single Cell RNA Kit Product Numbers

Product Name	Catalog Number	Storage
PIPseq T20 3′ Single Cell RNA Ambient Kit v4.0	FBS-SCR-T20-4-V4-1	15°C to 30°C
PIPseq T20 3′ Single Cell RNA 4℃ Kit v3.0 or v4.0	FBS-SCR-T20-4-V3&V4-2	2°C to 8°C
PIPseq T20 3′ Single Cell RNA -20℃ Kit v3.0 or v4.0	FBS-SCR-T20-4-V3&V4-3	-30°C to -15°C
PIPseq T20 3′ Single Cell RNA -80℃ Kit v4.0	FBS-SCR-T20-4-V4-4	-90°C to -75°C
PIPseq T20 3′ Single Cell Consumable Kit v3.0 or v4.0	FBS-SCR-T20-4-V3&V4-6	15°C to 30°C
PIPseq T20 3′ Single Cell Starter Equipment Kit	FBS-SCR-T20-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 brand of plastic consumables have been validated to ensure stability of PIP emulsions. Substituting these materials may adversely affect performance.

PIPseq T20 3′ Single Cell Starter Equipment Kit

Component Name	Part Number	Units
PIPseq rotating vortex assembly for 1.5 mL tubes	FB0002100	1
Rotating vortex base assembly	FB0003847	2
PIPseq vortex mixer	FB0002373	1
PIPseq Dry bath with heated lid	FBS-SCR-PDB	1
PIPseq Dry bath 1.5 mL block	FB0002498	1
2.5 mm Allen Key Wrench	FB0001723	1
US Power Supplies for Vortex Mixer	FB0002353	1
US Power Supplies for PIPseq Dry bath	FB0002363	1
PIPseq 4-tube stand, blue, for 1.5 mL tubes	FB0002590	2
User Manual for PIPseq Dry bath	FB0002664	1
User manual for PIPseq Vortex Mixer	FB0002745	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 T20 3′ Single Cell RNA Consumables Kit

Component Name	Part Number	Units
Centrifuge Tube Filter	FB0001100	1 Bag of 8
1.5 mL Safe-Lock PCR Clean tubes, Eppendorf 022363212	FB0001870	1 Bag of 4
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	4 Strips of 8
3 mL syringe	FB0002625	4
1 mL syringe	FB0002892	4
G22 blunt bottom syringe needle	FB0002623	8

PIPseq T20 3′ Single Cell RNA Reagent Kit Components

Component Name	Part Number	Kit	Storage	Units
T20 PIPs	FB0003914	PIPseq T20 3′ Single Cell RNA -80°C Kit v4.0	-90°C to -75°C	4
Cell Suspension Buffer	FB0002440	PIPseq T20 3′ Single Cell RNA -80°C Kit v4.0	-90°C to -75°C	4
Partitioning Reagent	FB0003123	PIPseq T20 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	1
CLB3	FB0003910	PIPseq T20 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	4
Breaking Buffer	FB0003128	PIPseq T20 3′ Single Cell RNA 4°C Kit v4.0	2°C to 8°C	1
Washing Buffer	FB0003139	PIPseq T20 3′ Single Cell RNA 4°C Kit v4.0	2°C to 8°C	1
De-Partitioning Reagent	FB0002516	PIPseq T20 3′ Single Cell RNA Ambient Kit	15°C to 30°C	1
RT Enzyme Mix	FB0002206	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
RT Additive Mix	FB0002205	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
TSO	FB0003140	PIPseq T20 3′ Single Cell RNA -80°C Kit v4.0	-90°C to -75°C	4
WTA Buffer Mix	FB0002208	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
WTA Primer	FB0002006	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
SPRI Beads	FB0002215	PIPseq T20 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	1
Nuclease-free water	FB0002216	PIPseq T20 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	1
IDTE pH 8.0	FB0002684	PIPseq T20 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	1
Library P7 Indices	FB0001626-1627, FB0001629-FB0001633, FB0002092	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1 each
Library P5 Indices	FB0001915-1918, FB0001666-1669	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1 each
Resuspension Buffer	FB0002210	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Prep Buffer	FB0002211	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Prep Enzymes	FB0002212	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Prep Mix A	FB0002213	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Adapter Mix	FB0001605	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Prep Mix B	FB0002214	PIPseq T20 3′ Single Cell RNA -20°C Kit v4.0	-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
BioAnalyzer High Sensitivity DNA kit or Tapestation HS-D5000 for cDNA and HS-D1000 for NGS library	Agilent; 5067-4627, 5067-5593
100% Ethanol, molecular biology grade	General Laboratory supplier

Required Third Party Consumables

Item	Supplier
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	
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 centrifuge tubes, sterile	General laboratory supplier, recommend Corning # 430790
Qubit Assay Tubes	Thermo Fisher; Cat # Q32856
PCR-clean tubes, strip tubes, 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 / 1.5 mL Magnetic Separation Combo Rack	Permagen, MSR1224B or equivalent from alternative supplier
Benchtop centrifuge with swinging bucket rotor, suitable for 5 mL, 15 mL tubes	General laboratory supplier, recommend ST40
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
Laboratory balance	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 before stopping 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
Thermocycler	General laboratory supplier

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 40,000 cells into each PIPseq reaction, resulting in recovery of > 20,000 cells and a multiplet rate of < 8%. The optimal input cell concentration is 4,000 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 and may not be representative of expected results with other sample types.

# Cells loaded	# Cells recovered	Multiplet rate
20,000	>10,000	4%
40,000	>20,000	7%
60,000	>30,000	13%

PIPseq T20 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

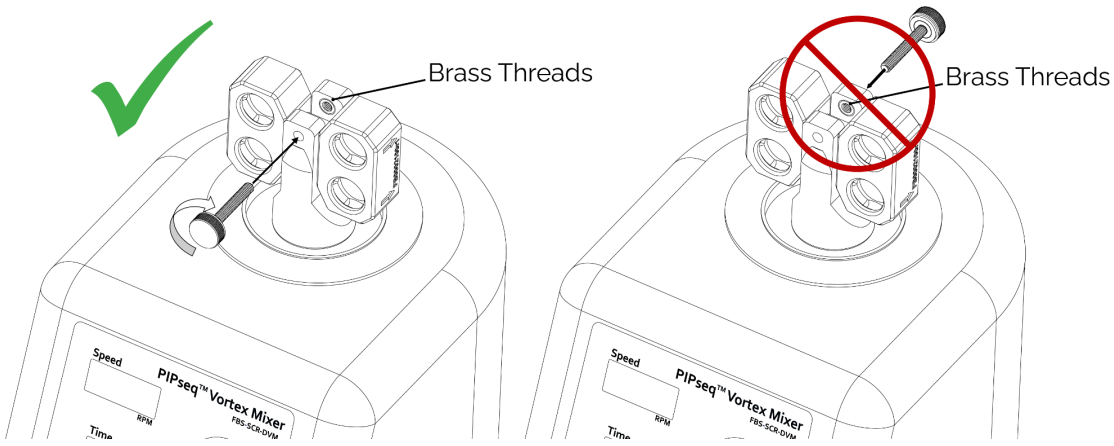
Upon receiving the PIPseq T20 3' Single Cell RNA kits, remove the -80℃ box with TSO aliquots (FB0003140), Cell Suspension Buffer (FB0002440), and PIP reactions (FB0003914) from the dry ice shipping container and store in a -80°C freezer.

Prior to each experiment, thaw one aliquot of Cell Suspension Buffer per cell preparation and dilute the 1X Washing Buffer 1:1 with nuclease-free water to yield 4 mL 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 1.5 mL 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).

PIPseq Dry Bath Lid Settings and Control

The PIPseq Dry Bath lid temperature should be adjusted and controlled to maintain proper temperature control separate from the heating block temperature. There are two primary modes to control the temperature of the lid:

Mode 1: *Specific temperature control.* This mode enables the lid to be held at a specific temperature. For example, the user requires the temperature to be held at 105°C. This mode is used for nuclei lysis.

Mode 2: *Offset temperature control to heat block.* This mode enables the user to set the lid temperature to be a specific number of degrees greater than the block temperature, which can vary according to the temperature profile that is selected. For example, a setting of "+5" will set the lid temperature 5°C greater than the block temperature. Thus, when the block is set at 37°C the lid will be set at 42°C. This mode is used exclusively for cell lysis.

To change the lid temperature and lid heating mode:

1. Determine if the lid temperature control is on or off by checking the button on the bottom-right corner of the screen. The button will display "LidOff" if the lid is ON or "LidOn" if the lid is OFF. If the lid button is set to "LidOn", press the button to turn ON the lid heating feature.
2. Press the "Edit" button located in the upper right corner of the screen.
3. Press the "Lid Mode" button located at the bottom left of the screen to switch between Mode 1 and Mode 2.
    a. To confirm which mode is set, refer to the "Lid Temperature" window on the screen. If the window displays a number with no characters (e.g. 105) then Mode 1 is enabled. If the window displays a plus sign (+) followed by a number (e.g. +5) then Mode 2 is enabled.
4. To change the value used in Mode 1 or Mode 2, press the "Lid" window located near the top-left portion of the screen.
5. A new window will appear to set the value. Set the value and press the "Enter" button.
6. Press the "Save/Return" button located at the top-right corner of the screen to save the value.

Note: The lid heating mode is independent of the set programs. This mode applies across all programs, regardless of thermal profiles set.

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 cells or aggregates to ensure the highest quality data. See the appropriate section for sample preparation instructions, for cells or nuclei.

Note: For sample types known to have high RNase content (e.g. pancreatic cells, monocytes), it is recommended that users consider adding RNase inhibitors into the PIP reaction (see Capture and Lysis, optional step).

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 steps 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 pelletting.

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 of cold Cell Suspension Buffer and gently mix 10-15 times until the 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 4000 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 to the Cell Capture and Lysis section.

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 13 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 2-4 samples. Thaw the desired number of PIP tubes, one for each sample that will be processed.

WORKFLOW TIP: Begin preheating the PIPseq Dry bath and lid to the appropriate temperature depending on sample input type (refer to table in step 13). Set the time to hold for this step as users will skip to the next step once they reach step 13 of this section.

2. Thaw the PIP tubes at room temperature for 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. Mix the cell or nuclei suspension 10 times with a **wide-bore** P200 tip set to 80% of the cell suspension volume.

4. Add exactly 10 µL of prepared cells or nuclei (40,000 total) directly into the PIPs while avoiding the creation of air bubbles. Ensure cells are dispensed within the PIPs and not just on the surface of the PIPs. If performing multiple reactions, add cells to all of them sequentially and then proceed to the next step.

> **Optional Step:** If working with sample types that have high RNase content users may add RNase Inhibitor into the cell:PIP mixture before moving onto step 5. Be advised that it is preferred that users prepare higher concentration cell input to ensure the total cell volume + RNAse inhibitor volume remains at 10 µL.

5. Mix the cell:PIP mixture 10 times using a **standard bore**, low-retention P200 tip at 180 µL stroke while moving the tip throughout the mixture starting from the bottom, moving up through the mixture, and returning to the bottom. Take care not to create bubbles, especially in the first six mixing strokes. For this step, we recommend using a P200 multichannel (in a staggered position).

   Troubleshooting: If foaming occurs, users may centrifuge the PIP tubes for ~ 5 seconds to remove the bubbles. Repeat the mixing in step 5 while avoiding excessive foaming.

Figure 1. (circled in green) Cells and PIPs mixture floating on top of excess Partitioning Reagent

6. Add 1000 µL Partitioning Reagent, along the side wall of the tube, to the cell:PIP mixture (Figure 1) using a P1000 pipette. Do not use serological pipettes.
7. Ensure the tube caps are tightly sealed and place the tubes in the rotating vortex adapter in the horizontal configuration (Figure 2A). Ensure the tubes are fully depressed into the adapter. Vortex at 3000 RPM for 15 seconds.
8. Rotate the vortex adapter into the vertical configuration (Figure 2B). Vortex vertically at 3000 RPM for 2 minutes.

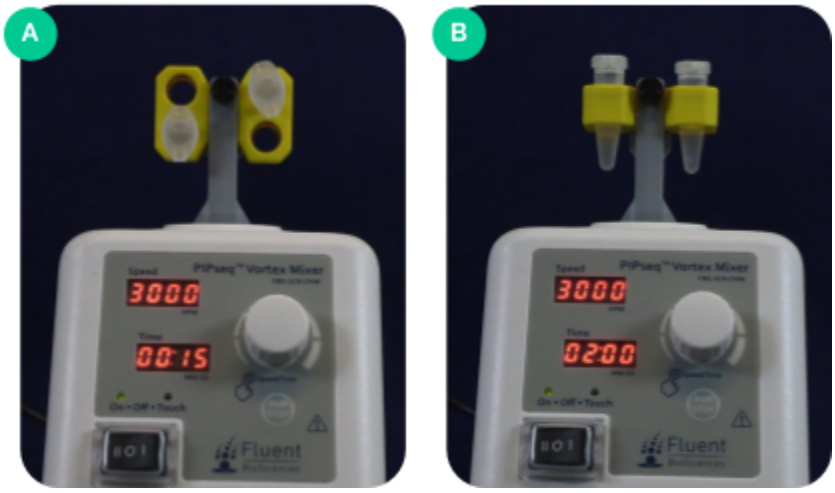


Figure 2. The rotating vortex adaptor shown in the horizontal configuration **(A)** and the vertical configuration **(B)**.

9. Remove the PIP tubes from the vortex adapter and place them into the 1.5 mL stand (FB0002590). Let the emulsion stabilize for 30 seconds and then proceed to the next step. The reaction will first appear cloudy then the PIP emulsion will separate and two phases can be observed (Figure 3).

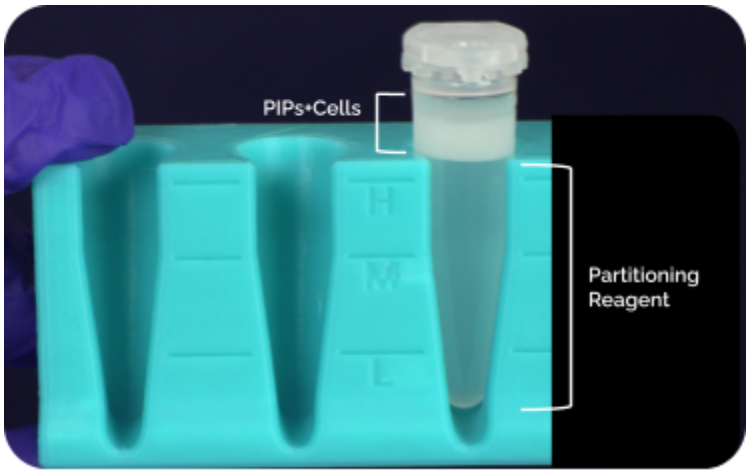


Figure 3. Emulsified PIPs and cells are seen floating in the top phase while the excess Partitioning Reagent in the bottom phase.

10. Slowly transfer Partitioning Reagent (bottom) out of each tube of PIPs using a 3 mL syringe attached with a G22 blunt tip syringe needle (Figure 4A) until the bottom of the PIPS+cells emulsion is aligned with the low ("L") volume marker of the stand (Figure 4C). Do this by slowly moving the tip through the emulsion to the bottom of the tube, wait for five seconds, and then aspirate out the bottom phase (Figure 4B). Be careful not to aspirate out any emulsion.

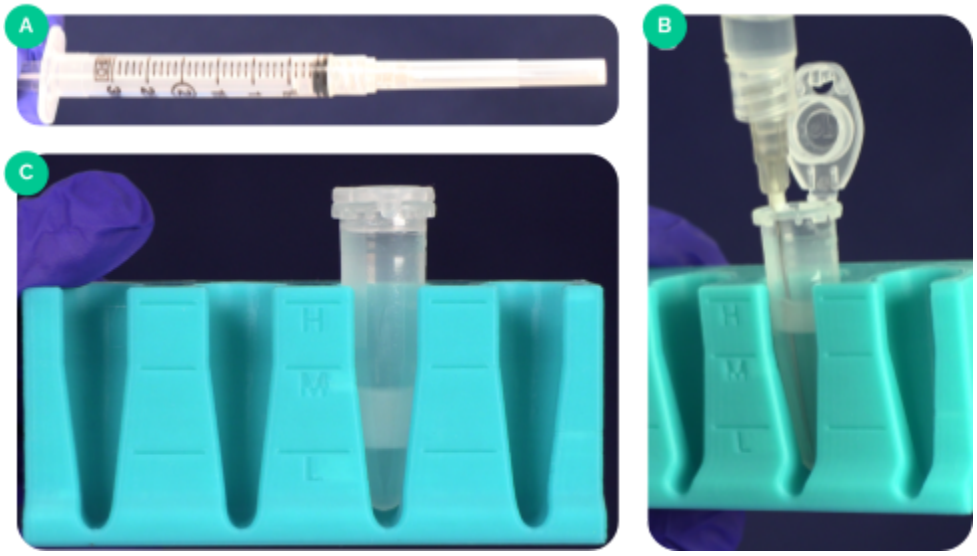


Figure 4. Use a 3 mL syringe with a G22 blunt bottom needle shown in **(A)** to remove excess Partitioning Reagent from the bottom phase as shown in **(B)**. Remove excess until the PIPs layer sits at the "Low" or "L" bar of the blue tube rack as shown in **(C)**.

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

11. Prepare one Chemical Lysis Emulsion per reaction by adding 630 µL of Partitioning Reagent to the provided CLB3 (FB0003910) tube containing chemical lysis buffer. Vortex for 10 seconds to generate the emulsion and immediately use a P1000 low-retention tip to add the whole volume of the Chemical Lysis Emulsion (~840 µL) on top of each PIP emulsion in the PIP tubes. Use one tube of Chemical Lysis Emulsion per sample (Figure 5A).

Figure 5. Chemical Lysis Emulsion after addition of Partitioning Reagent and vortexing (A). Sample tube after addition of Chemical Lysis Emulsion and inverting (B).

12. Mix the combined emulsions gently by inversion 10 times. It should look cloudy (Figure 5B).

13. Verify the PIPseq Dry bath (Figure 6) is preheated to the appropriate temperature according to the sample type in use (see table below) with the lid temperature set to +5°C for cells or 105°C for

nuclei. Refer to the *PIPseq Dry Bath Lid Settings and Control section* of this user guide for instructions on how to program the desired heated lid setting. 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.

Ensure the volume of the emulsion is below the top of the thermal block to ensure consistent heating.

Figure 6. The PIPseq™ Dry bath to be used for the lysis incubation step.

Cells		
Lid Temp	**+5 °C**	
Preheating step	25 °C	hold (“0000”)
After preheating complete and samples inserted, skip to next step		
	25 °C	15 min
	37 °C	45 min
	25 °C	10 min
	20-25 °C	hold

Nuclei		
Lid Temp	**105 °C**	
Preheating step	66 °C	hold (“0000”)
After preheating complete and samples inserted, skip to next step		
	66 °C	45 min
	25 °C	10 min
	20-25 °C	hold

14. **WORKFLOW TIP**: During incubation, complete step 1 of the Breaking emulsions section, step 1 of the cDNA synthesis section, and step 1 of the Washing PIPs in 1X Washing Buffer section.

15. After incubation is complete, the samples can be held at 20°C for up to 96 hours before proceeding to mRNA isolation. **Do not freeze**! Alternatively, you can move forward to mRNA isolation after

the 25°C incubation. Note: Some condensation may appear on the tubes, which is not a concern. DO NOT centrifuge the emulsion. If liquid remains on the tube cap, invert the sample 3 times.

mRNA Isolation

Breaking Emulsions

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

⚠ **WARNING: Do not centrifuge the emulsion.**

2. Place the PIP tubes into the 1.5 mL stand (FB0002590). Remove and discard any remaining partitioning reagent using a 1 mL syringe attached with a G22 blunt bottom syringe needle. Place the tip through the emulsion slowly until it touches the bottom of the tube, wait for five seconds, and then aspirate out the bottom phase until the top of the emulsion is level with the middle volume marker ("M") on the stand (Figure 7). **Save this syringe and needle for step 6 and label with the sample name.**

⚠ 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.

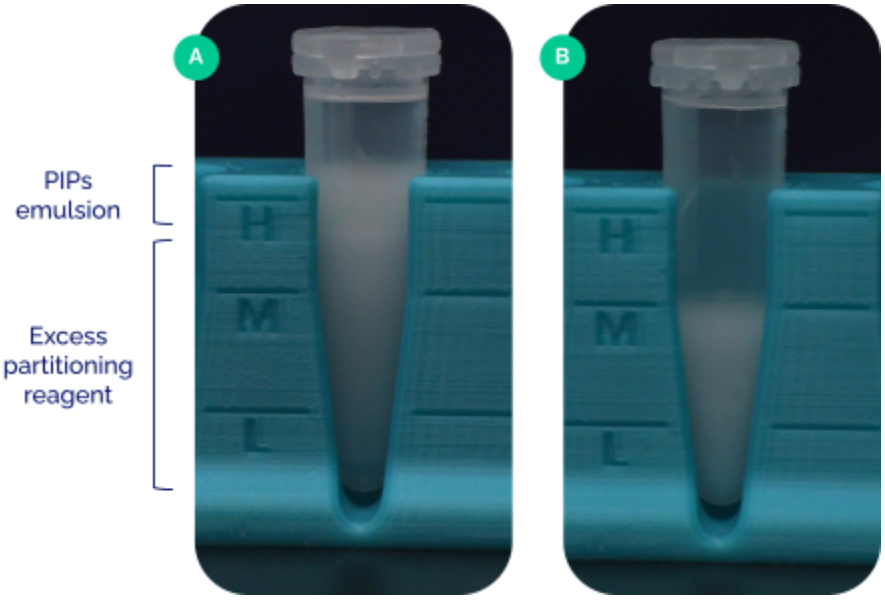


Figure 7: PIP tubes after removing them from the Dry bath (A) and after discarding most of the remaining partitioning fluid (B).

3. Add 750 µL of Breaking Buffer to each sample. Then, add 200 µL of De-partitioning reagent to each sample, along the side wall of the tube (Figure 8A).

4. Securely close the tube, then fully invert the tube ten times to break the emulsion.

⚠ Critical: **DO NOT** vortex the tube during breaking.

5. Centrifuge 5-10 seconds on a benchtop minifuge. 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, Figure 6B). If red precipitate is detected, be sure to remove it along with the bottom phase during step 6.

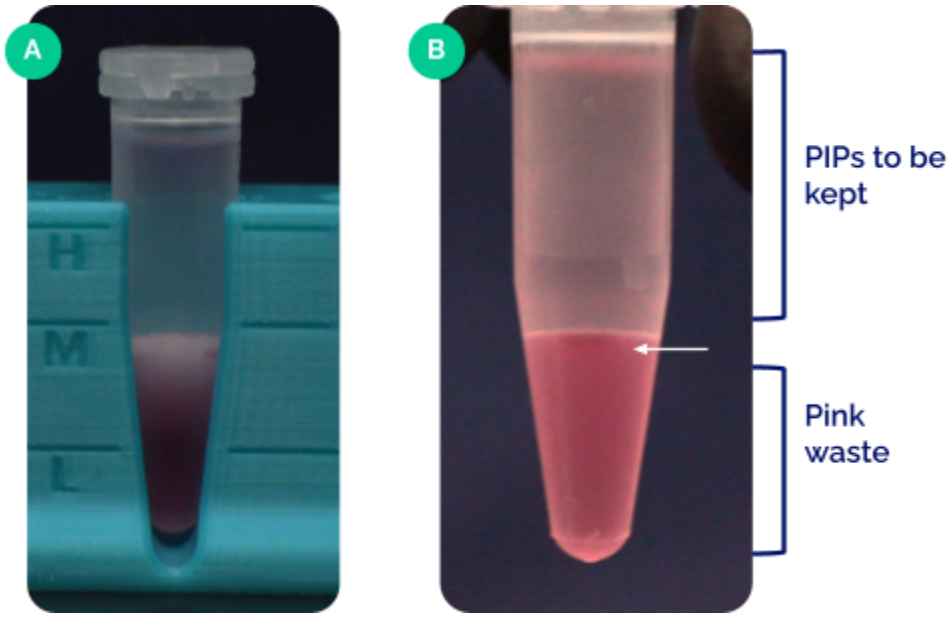


Figure 8. PIPs after addition of breaking buffer and de-partitioning reagent (A) and after inverting and spinning down (B). The PIPs are now suspended in the top phase. Take note of the red droplet that must be removed at the interphase (white arrow).

6. Remove the pink-colored bottom phase (250-300 µL) from each tube of PIPs using a 1 mL syringe attached with a G22 blunt bottom syringe needle. If a red droplet is observed in between the two layers, aspirate to remove it first, then proceed to removal of the remaining pink bottom phase.
7. Centrifuge for < 2 seconds on a benchtop minifuge. Begin timing when the minifuge reaches top speed.
8. Remove any remaining pink-colored bottom phase with a P20 pipette tip from each tube of PIPs. 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.**

9. Keep the tubes on ice.

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

Washing PIPs with 1x Washing Buffer

1. Make one aliquot per sample of 12 mL of Washing Buffer in a 15 mL centrifuge tube and label it Wash 1. Store on ice until use.

2. Using a P1000 low retention tip, transfer the PIPs into chilled Wash 1.

WORKFLOW TIP: If there are visible droplets inside the P1000 tip, wash the pipette tip inside the 15 mL tube by aspirating up and down at least three times.

3. Briefly centrifuge the 1.5 mL tube on a benchtop minifuge after the first transfer to collect all the PIPs at the bottom of the tube. Aspirate remaining PIPs from the 1.5 mL tube and dispense rinsate into the 15 mL tube containing the transferred PIPs (Wash 1).

This rinsing procedure is important to ensure PIPs are not left behind in the pipette tip or the 1.5 mL tube.

4. Gently mix each tube by tapping the bottom to disperse the pellet and invert 10 times. Centrifuge the tubes for 2 minutes at 750 x g (use ~70-80% of the maximum braking speed) on a centrifuge with a swinging bucket rotor (e.g. ST40) to pack the PIPs (Figure 9).

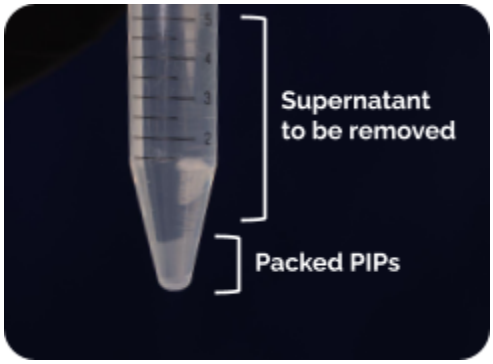


Figure 9. Packed PIPs after centrifuging

5. Remove supernatant until ~ 1 mL Washing Buffer remains **WITHOUT** disturbing PIPs pellet.

WORKFLOW TIP: For all supernatant removal steps it is convenient to set up an aspirator to reduce the overall time required during the workflow. Ensure a clean tip is used for each aspiration.

Warning: Refrain from aspirating below the 0.5 mL mark on the tube to avoid loss of PIPs during washing. Aspirate slowly and carefully while keeping the tip at the top of the liquid level; don't disturb the pellet.

6. Add 12 mL of Washing Buffer from the stock bottle directly into the Wash 1 tube containing packed PIPs and repeat steps 4-5 to complete the second wash.
7. Add 12 mL of Washing Buffer from the stock bottle directly into the tube containing packed PIPs and repeat steps 4-5 to complete the third and final wash.
8. In steps 9-13, a mixture of PIPs and supernatant will be prepared such that the final volume of the mixture is 250 µL. **It is critical to perform the following normalization steps to ensure accurate concentrations of reagents in the cDNA synthesis reaction.**
9. Pre-weigh one clean 1.5 mL Safe-Lock Eppendorf tube per sample and record tared mass in chart below.

Warning: Use the Safe-lock Eppendorf tubes provided in the kit for this step. Do not substitute any other tube type.

10. Transfer the ~ 1 mL PIP mixture into the pre-tared 1.5 mL tube using a P1000 low retention tip.
11. Weigh and record the total mass of each tube with PIPs, then convert it to volume (assuming density=1g/mL). Using the calculator below calculate the aspiration volume necessary to normalize each mixture volume to 250 µL. Note: Each empty tube is identical to within a few mg (the tared

tube mass is generally between 1000 mg and 1010 mg) so a single measurement of the blank tube is sufficient to use for all tubes.

12. Centrifuge for ~30 seconds on a benchtop minifuge.

Warning: Allow the minifuge to slow to a complete stop before removing the tube.

13. Aspirate and discard the designated volume of the Washing buffer.

WORKFLOW TIP: If you are touching the PIP layer as you remove the designated volume, spin down the tubes in a fixed angle minifuge for 30 seconds to clear out more supernatant volume.

Warning: Do not remove aqueous below the level of the PIPs (Figure 10).

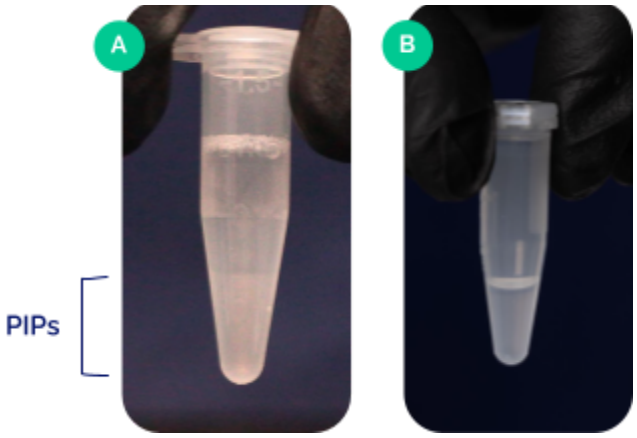


Figure 10. Packed PIPs in a 1.5 mL tube after centrifuging (A) and after volume normalization (B).

Sample ID	
1.5 mL tube mass (mg)	Tube mass
Total mass (mg)	Tube mass + PIPs
Solution volume (µL)	Total mass - Tube mass = PIP mass / 1= PIP volume
Wash buffer to remove (µL)	PIP volume - 250 µL

14. Store samples on ice until ready to proceed to cDNA synthesis.

cDNA Synthesis

1. **WORKFLOW TIP:** Prepare the cDNA synthesis reaction on ice as indicated in the table below for a 250 µL reaction. The TSO must be thawed on ice after removal from -80°C storage. Note that fill volumes provide sufficient reagents for 5.5 total reactions so it is recommended not to exceed 0.5X 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.
2. **When ready for RT, add the RT Enzyme Mix to the mastermix** and mix well by pipette mixing.

Reagent	Volume Per Reaction (µL)
RT Additive Mix	215
TSO	20
RT Enzyme Mix	15
Total	**250**

3. Add 250 µL of the mastermix to each 1.5 mL tube of PIPs and mix well by flicking or tapping the tube to break up the pellet and inverting the tube 20 times. Briefly centrifuge the tube on a benchtop minifuge.
4. Perform cDNA synthesis with the following protocol in the PIPseq dry bath with the heated lid set to 105°C (refer to the Mode 1 protocol described in the *PIPseq Dry Bath Lid Settings and Control section* for instructions on how to program this heated lid setting).

25 °C	30 minutes
42 °C	90 minutes
85 °C	10 minutes
4 °C	Hold

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

Washing PIPs with 0.5x Washing Buffer

1. Centrifuge the 1.5 mL tubes for 30 seconds on a benchtop minifuge.
2. Aspirate and discard 300 µL of supernatant without disturbing the PIPs pellet.
3. Add 1 mL 0.5X Washing Buffer to each PIP tube.
4. Vortex each PIP tube for 5 seconds.

5. Centrifuge for ~30 seconds on a benchtop minifuge.

   Warning: Allow the minifuge to slow to a complete stop before removing the tubes.

6. Aspirate and discard 0.8 mL of the supernatant **WITHOUT** disturbing the PIPs pellet.

7. Repeat steps 3-6 two more times using 0.8 mL of 0.5X Washing Buffer for the addition and removal steps.

8. Weigh and record the total mass of each tube with PIPs to convert it to volume (assuming density = 1g/mL). Using the calculator below, calculate the aspiration volume necessary to normalize each mixture volume to 250 µL.

Sample ID	
1.5 mL tube mass (mg)	Tube mass
Total mass (mg)	Tube mass + PIPs
Solution volume (µL)	Total mass - Tube mass = PIP mass / 1= PIP volume
Wash buffer to remove (µL)	PIP volume - 250 µL

9. Centrifuge for 30 seconds on the benchtop microfuge.

10. Remove the designated amount of supernatant to yield a 250 µL total volume.

WORKFLOW TIP: Users may do the supernatant removal in two steps. First remove the > 100 µL volume by rotating the tube to aspirate along the side-wall away from the PIPs (Figure 11). Second, repeat step 10 and then remove the remaining supernatant in the same manner.

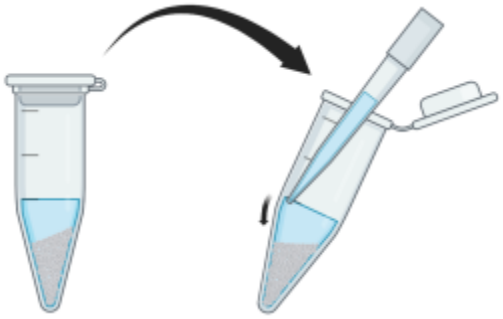


Figure 11. Tip the tube and aspirate supernatant along the side wall to avoid accidental removal of PIPs.

11. Proceed promptly to cDNA amplification.

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	250
WTA primer	2.5
Total	**252.5**

2. Add 250 µL of WTA mastermix into each RT product mix.
3. Mix by vortexing briefly (~ 5 sec).
4. Distribute the WTA reaction mixture into 8 x 62.5 µL aliquots. Label appropriately so samples can be recombined on a per sample basis after cDNA amplification.

Note: For the last aliquot, briefly centrifuge the 1.5 mL tubes on a benchtop minifuge and transfer all residual volume to the Greiner Bio-One 8-tube strip (FB0002076).

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.

5. Run the program below with the thermocycler 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)
<20,000	<10,000	12	16
20,000-40,000	10,000-20,000	12	16
40,000-60,000	20,000-30,000	11	15

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

Isolate cDNA from PIPs

The remaining steps in the protocol will be performed using plasticware from a general laboratory supplier. These supplies are not included in the kit, with the exception of the centrifuge filters which are provided. There are multiple options for the 0.2 mL sample tubes used in the remaining steps, depending on your preference. These include 0.2 mL individual PCR tubes, strip tubes, or a PCR reaction plate (requires a plate-compatible magnetic bead rack).

1. Add 25 uL of IDTE to each WTA reaction.
2. Pool all WTA products from the same sample onto one centrifuge filter per sample.
3. Centrifuge filter column for 6 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.

4. Transfer the flow-through into new 1.5 mL tubes.
5. Measure the volume of each sample using a P1000.
6. Add enough IDTE to each sample to achieve a total volume of 600 µL per sample.

SPRI Purification

1. Make fresh 85% (v/v) ethanol.
2. Thoroughly vortex bottle of SPRI beads to mix.
3. For 600 µL reaction volumes, add 480 µL SPRI . This is a 0.8x ratio of SPRI beads.
4. Vortex for 10 seconds and briefly centrifuge on a benchtop minifuge (~2 seconds).
5. Incubate for 5 minutes at ambient temperature.
6. Place the 1.5 mL 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 1 mL 85% ethanol for 30 seconds each. Do not disturb the SPRI beads.
9. Remove final traces of ethanol with a P20 or P200 pipette, taking care 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 or P200 pipette being careful not to disturb the SPRI beads.
11. Remove tubes from the magnetic rack and add 52 µL IDTE. Mix the IDTE and SPRI beads by vortexing for 10 seconds, 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. Briefly centrifuge on a benchtop minifuge (~ 2 seconds).
13. Incubate for 5 minutes at room temperature.

14. Place tubes into the magnetic rack and bind to the magnet for 2 minutes.

15. Remove and **SAVE** 50 µL of supernatant in a new PCR tube strip. Do not disturb the SPRI beads.

16. Amplified cDNA may be stored at -20°C up to 2 weeks

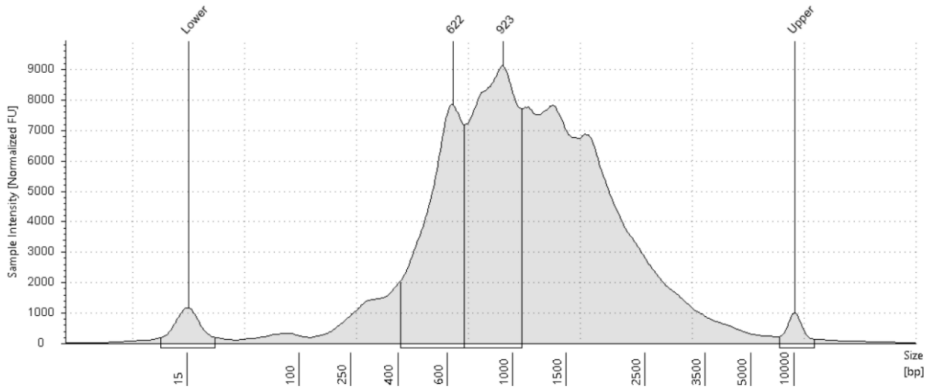
cDNA QC and Quantification

Qubit Quantification

1. Using a Qubit High Sensitivity kit, quantify 2 µL 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 40,000 cell input human/mouse cell mixture (HEK 293T/NIH 3T3) using a high sensitivity D5000 ScreenTape.

Library Preparation

Fragmentation, End Repair & A-Tailing

WORKFLOW TIP: It is recommended to carry forward no more than 50% of the total cDNA yield into library preparation. The complexity of this library will be comparable to one generated using a higher proportion (>50%) of the cDNA.

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 µL of 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.

3. Vortex the Library Prep Buffer for 5 seconds, and briefly centrifuge to collect all liquid at the bottom of the tube.
4. Pipette mix the Library Prep Enzymes 15 times at the 30 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.

5. 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.

6. 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**

7. 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.**

8. Set up the thermocycler with the program below, with the heated lid set to 105°C.
9. 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

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

Adapter Ligation

1. Prepare a dilution of the Library Adapter Mix (100 µM) to a final concentration of 3 µM or 15 µM according to the desired input cDNA, as described in the table below.

Input cDNA (ng)	Library Adapter Mix Per Sample (µL)	Nuclease-free water Per Sample (µL)	Total Volume Per Sample (µL)
1 - 10	0.15	4.85	5
11-500	0.75	4.25	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.

2. Remove the samples from the thermocycler immediately upon return to 4°C.
3. 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.
4. Pipette mix the Library Prep Mix A 15 times at the 100 µL stroke to homogenize (do NOT vortex) and place on ice.
5. 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.

⚠
6. 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.
7. 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 µL of each cleaned library, add the following *individually*, in the order in which they appear in the table below.

Reagent	Volume Per Sample (µL)
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 µL stroke, and briefly centrifuge to collect all liquid at the bottom of the tubes.

Note: The total number of PCR cycles should be optimized using a fixed proportion (e.g. 50%) of the total cDNA yield quantified during cDNA QC and Quantification. The below table presents recommendations based upon input into library preparation, which are starting points for optimization.

DNA input into library preparation (ng)	Recommended cycles
401-500	6
101-400	7
51-100	8
11-50	10
1-10	12

4. Place samples in the thermocycler and run the program below, with the heated lid set to 105°C:

Temperature (°C)	Time	Cycles
98	45 sec	1x
98	15 sec	See table above
67	30 sec	
69	45 sec	
72	1 min	1x
4	Hold	Hold

5. 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 µL by addition of approximately 35 µL nuclease-free water.
3. For 85 µL reaction volumes, add 51 µL SPRI. This is a 0.6x ratio of SPRI beads. Adjust as necessary if reaction volume is not 85 µL.

4. Pipette up and down 15 times at the 115 µL 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 µL SPRI beads to the supernatant solution. This is a 0.8x ratio relative to the original PCR reaction volume.

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

10. Pipette up and down 15 times at the 115 µL 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 µL of 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 of µL IDTE. Mix the IDTE and SPRI beads by pipetting up and down 10 times at the 20 µL stroke.
18. Incubate for 5 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 µL of supernatant in a new PCR tube strip. This sample can be stored at -20°C long-term.

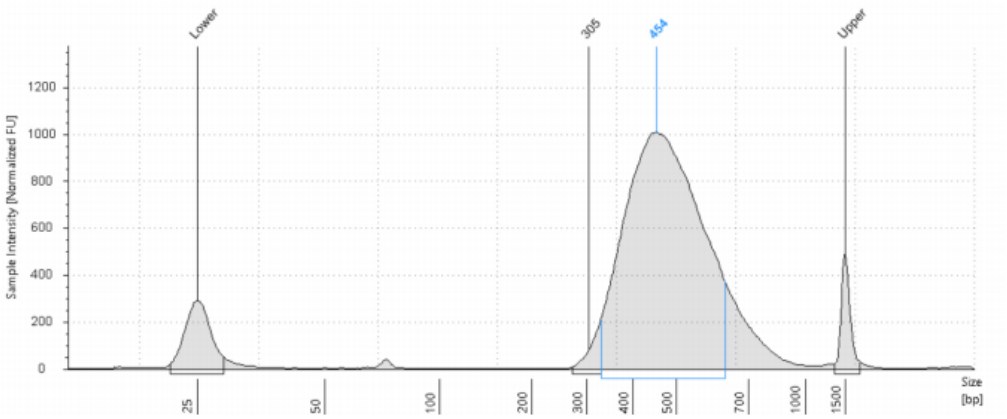
Post Library Preparation QC

Qubit Quantification

1. Using a Qubit High Sensitivity kit, quantify 2 µL 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.

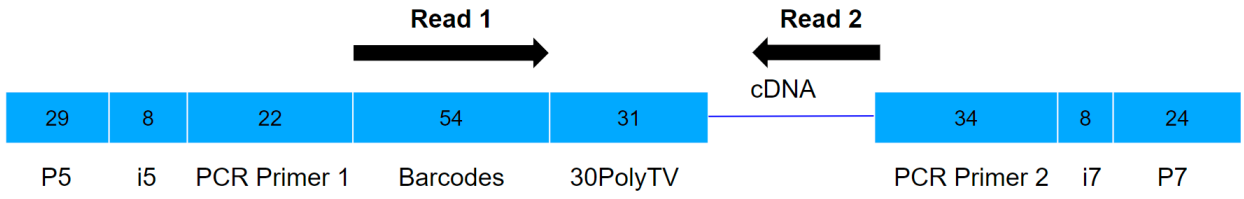


Representative library trace for a 1000 cell capture human/mouse cell mixture (HEK 293T/NIH 3T3) on a High Sensitivity D1000 ScreenTape (above).

Sequencing

PIPseq T20 3′ Single Cell gene expression 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 8-base i5 and i7 indexes. Read 1 length must be ≥ 54 bases and the recommended read 2 length ≥ 67 bases. It is recommended to maximize the use of the flow cell with longer reads when possible.

Library Structure



Recommended Sequencing Depth

Sequencing depth will vary based on your application needs but it is recommended to start with a depth of 20,000 reads per input cell. After sequencing, users may evaluate the Sequencing Saturation metric output from PIPseeker software to optimize sequencing depth for specific sample types. Users are referred to the FB0002787 PIPseeker User Guide for more information.

Sequencer Compatibility

The sequencing instruments below have been verified as compatible with PIPseq by Fluent BioSciences. Some variation in assay performance may be experienced on distinct sequencing platforms.

- MiSeq
- NextSeq 550
- NextSeq 2000
- NovaSeq 6000

Long read sequencing technologies can be used for sequencing PIPseq libraries however Fluent does not currently provide support for these applications.

Pooling for Illumina Sequencers

PIPseq 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. 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 1% PhiX to be added to the loaded library pools.

Informatics Analysis

Fluent provides the PIPseeker™ software for analysis of single cell RNA data obtained with Fluent BioSciences' PIPseq 3′ Single Cell RNA (scRNA-seq) Kits. PIPseeker offers a comprehensive analysis solution that provides the user with detailed metrics, gene expression profiles, basic cell quality and clustering indicators, and cell type annotation for some sample types.. The outputs of PIPseeker can then be used for subsequent, specialized tertiary analysis streams. PIPseeker also supports specialized applications like measuring surface protein levels using antibody-derived tags (ADTs) and cell hashing using hashtag oligonucleotides (HTOs).
Visit *www.fluentbio.com/products/pipseeker-software-for-data-analysis/* to download the latest PIPseeker v2.0 software.

PIPseq v4.0 chemistry kits are only compatible with PIPseeker v2.x analysis software. Analysis of single cell data generated using PIPseq v4.0 3' Single Cell RNA Kits using PIPseeker 1.x analysis software will result in compromised performance.

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	CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX**GTCTCG TGGGCTCGGAGATGTGTATAAGAGACAG
FB0001915-1918, FB0001666-1669	Library P5 Index	AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**A CACTCTTTCCCTACACGACGC
FB0002006	WTA Primer	Forward: CTCTTTCCCTACACGACGCTC Reverse: AAGCAGTGGTATCAACGCAGAGT
FB0003140	TSO	AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG
FB0001605	Library Adapter Mix	Ligation primer: /5Phos/CTGTCTCTTATACACATCTCCGAGCC Ligation adapter: TATAAGAGACAGT

Abbreviations

PIPs	Particle-templated Instant Partitions
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
dsDNA	Double-Strand DNA
cDNA	Complementary DNA
TSO	Template Switch Oligo
WTA	Whole Transcriptome Amplification
RPM	Revolutions Per Minute

Document Revision Summary

Doc ID: **FB0002130**
Revision: **8.3**
Revision date: June 2023

General Changes:

- N/A

Specific Changes:

- Updated library structure figure

Legal Notices

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