Fluent BioSciences

PIPseq™ T2 3' Single Cell RNA Kit v4.0

User Guide

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Table of Contents

Introduction

The Fluent BioSciences PIPseq™ T2 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 16,000 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	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	

Product Applications

The PIPseq T2 3′ Single Cell RNA Kit is designed for gene expression profiling of up to 16,000 cells or nuclei from a wide range of sample types. This kit is configured with 8 individual reactions, in which up to 2,000 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℃, -20℃ and -80℃), a Consumable kit, and a one-time Starter 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 v4.0	FBS-SCR-T2-8-V4-1	15°C to 30°C
PIPseq T2 3′ Single Cell RNA 4°C Kit v3.0 or v4.0	FBS-SCR-T2-8-V3&V4-2	2°C to 8°C
PIPseq T2 3′ Single Cell RNA -20°C Kit v3.0 or v4.0	FBS-SCR-T2-8-V3&V4-3	-30°C to -15°C
PIPseq T2 3′ Single Cell RNA -80°C Kit v4.0	FBS-SCR-T2-8-V4-4	-90°C to -75°C
PIPseq T2 3′ Single Cell RNA Consumables Kit v3.0 or v4.0	FBS-SCR-T2-8-V3&V4-6	15°C to 30°C
PIPseq T2 3′ Single Cell Starter Equipment Kit	FBS-SCR-T2-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 vortex assembly for 0.5 mL tubes	FB0002083	1
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	FB0001100	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	FB0003913	PIPseq T2 3′ Single Cell RNA -80°C Kit v4.0	**-90°C to -75°C**	8
Cell Suspension Buffer	FB0002440	PIPseq T2 3′ Single Cell RNA -80°C Kit v4.0	**-90°C to -75°C**	8
Partitioning Reagent	FB0001550	PIPseq T2 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	1
CLB3	FB0003909	PIPseq T2 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	8
Breaking Buffer	FB0003127	PIPseq T2 3′ Single Cell RNA 4°C Kit v4.0	2°C to 8°C	1
Washing Buffer	FB0003138	PIPseq T2 3′ Single Cell RNA 4°C Kit v4.0	2°C to 8°C	1
De-Partitioning Reagent	FB0002376	PIPseq T2 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	1
RT Enzyme Mix	FB0001971	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
RT Additive Mix	FB0001965	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
TSO	FB0001042	PIPseq T2 3′ Single Cell RNA -80°C Kit v4.0	**-90°C to -75°C**	4
WTA Buffer Mix	FB0001043	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
WTA Primer	FB0002006	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
SPRI Beads	FB0001533	PIPseq T2 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	1
Nuclease-free water	FB0001077	PIPseq T2 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	1
IDTE pH 8	FB0001076	PIPseq T2 3′ Single Cell RNA Ambient Kit v4.0	15°C to 30°C	1
Library P7 Indices	FB0001626-1627, FB0001629-1633, FB0002092	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1 each
Library P5 Indices	FB0001915-1918, FB0001666 -1669	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1 each
Resuspension Buffer with Tween	FB0001826	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Prep Buffer	FB0001602	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Prep Enzymes	FB0001603	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Prep Mix A	FB0001604	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Adapter Mix	FB0001605	PIPseq T2 3′ Single Cell RNA -20°C Kit v4.0	-30°C to -15°C	1
Library Prep Mix B	FB0001606	PIPseq T2 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 conical tube, sterile and nuclease-free	General laboratory supplier
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 Magnetic Separation Rack	Permagen, MSRLV08 or MSR1224B (for T2 and T20 kit compatibility) or equivalent from alternative supplier
Ice bucket or cold blocks, suitable for 0.2 mL and 0.5 mL PCR tubes and 1.5 mL microcentrifuge tubes	General laboratory supplier
Hemocytometer or automated cell counter	General laboratory supplier
Micropipettes, 1 µL-1000 µL 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 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 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 and may not be representative of expected results with other sample types.

# 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

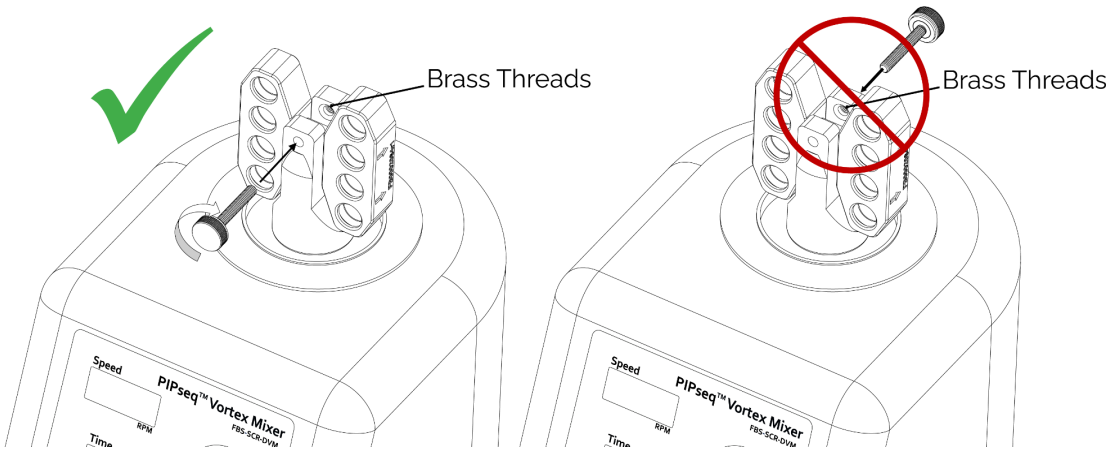
Upon receiving the PIPseq T2 3' Single Cell RNA kits, remove the -80°C components TSO (FB0001042), Cell Suspension Buffer (FB0002440), and PIP reactions (FB0003913) from the dry ice shipping container and store in a -80°C freezer.

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).

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 or aggregated cells 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 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 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 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 14 of Capture and Lysis, be sure to use the appropriate lysis temperature according to the sample type of interest.

Capture and Lysis

1. Thaw one PIP tube for each sample to be processed. Users may choose to process 2-8 samples.

   WORKFLOW TIP: Begin preheating the PIPseq Dry bath and lid to the appropriate temperature depending on sample input type (refer to table in step 14). Set the time to hold for this step as users will skip to the next step once they reach step 14 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 µL of prepared cells or nuclei (5000 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 are advised to add 1 µL of RNase Inhibitor (20 U/µL) into the cell:PIP mixture before moving onto step 6.

6. Mix the cell:PIP mixture 10 times with a P200 pipette using a **standard bore**, low-retention P200 tip at 32 µL 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 formed 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.

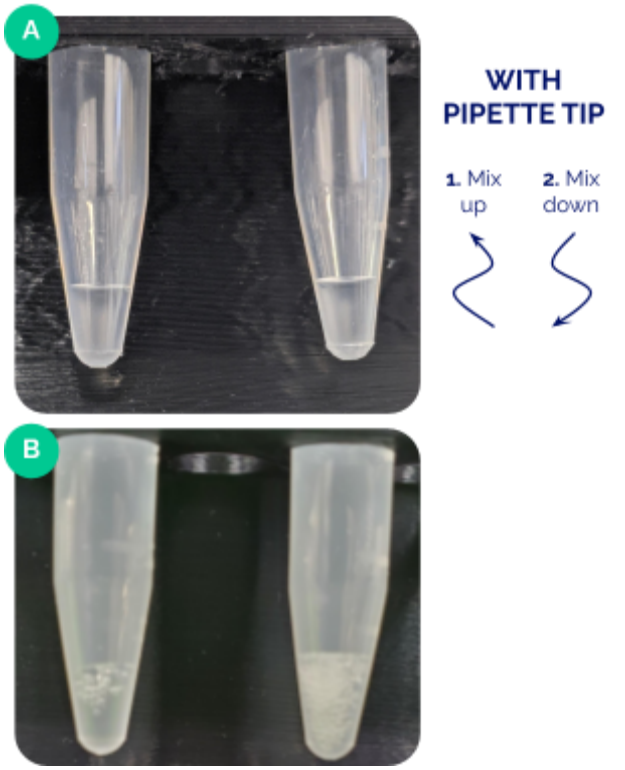


Figure 1. (A) PIPs following cell mixing with no apparent bubbles. **(B)** PIPs following cell mixing with an acceptable level of bubbles (left) or an unacceptable level of bubbles (right). Follow troubleshooting suggestions if an unacceptable level of bubbles are produced.

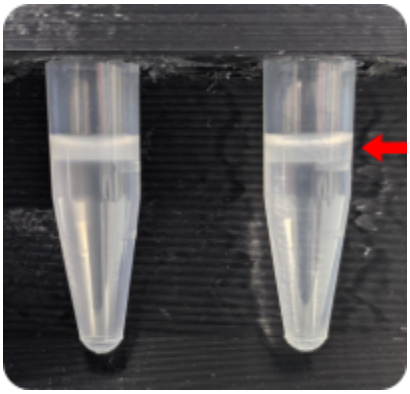


Figure 2. PIPs following addition of emulsification reagent. Take notice of the PIPs floating on top of the oil (red arrow). If the PIPs remain lodged at the bottom of the tubes, users still may proceed to step 8.

7. Add 280 µL 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.

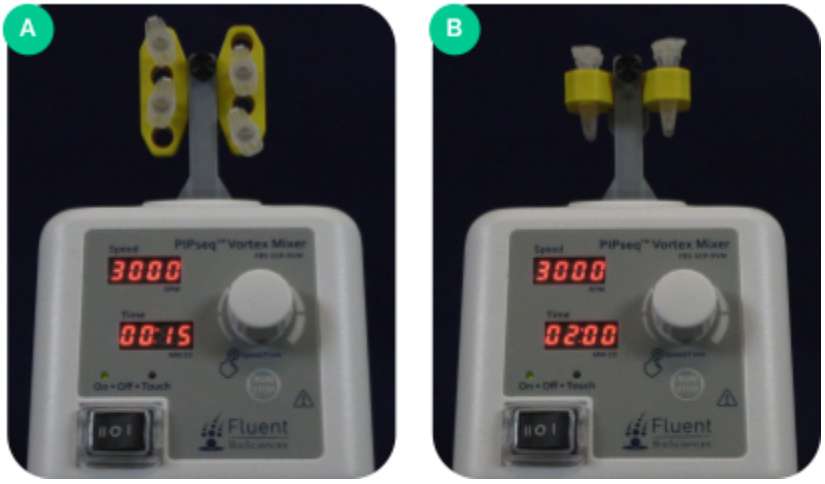


Figure 3. The rotating vortex adaptor shown in the horizontal configuration **(A)** and the vertical configuration **(B)**.

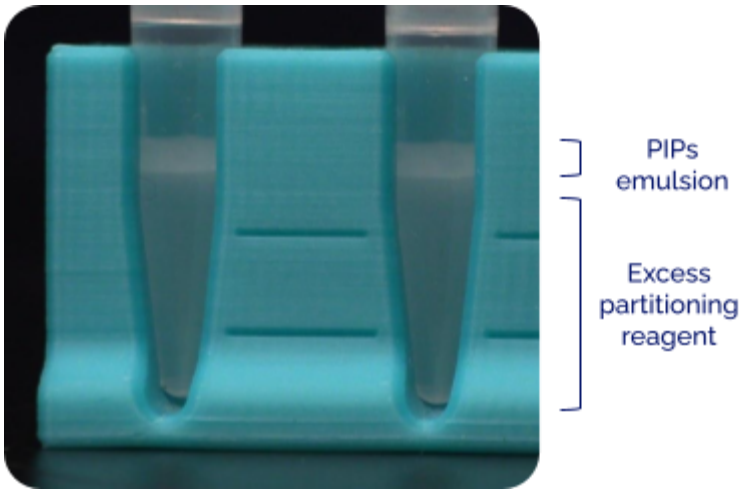


Figure 4. PIPS following emulsification.

10. Let the emulsion stabilize for 30 seconds and then remove the emulsion from the adapter for visual confirmation of the emulsion quality. The emulsion should be a homogenous white color (Figure 4).

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 5A).

⚠ Tips for pipetting through an emulsion: Be cognizant of emulsion sticking tightly to the end of the pipette tip. It is usually helpful to scrape the tip that has passed through an emulsion on the inner rim of the tube a few times to prevent the loss of PIPs by keeping them in the tube.

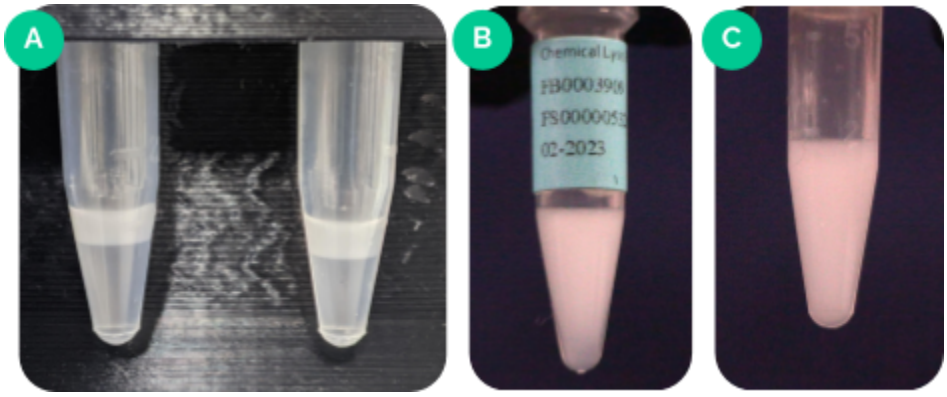


Figure 5. PIPs following removal of bottom phase (A). Chemical Lysis Emulsion after addition of Partitioning Reagent and vortexing (B). Sample tube after addition of Chemical Lysis Emulsion and inverting (C).

12. Prepare one Chemical Lysis Emulsion per reaction by adding 120 µL of Partitioning Reagent to the provided CLB3 (FB0003909) tube containing chemical lysis buffer. Vortex for 10 seconds to generate the emulsion (Figure 5B) and immediately use a P200 low-retention tip to add the whole volume of the Chemical Lysis Emulsion (~160 µL) on top of each PIP emulsion in the PIP tubes (Figure 5C). Use one tube of Chemical Lysis Emulsion per sample.

13. Mix the combined emulsions gently by inversion 10 times.

14. 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 in the dry bath 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

15. **WORKFLOW TIP**: Complete steps 1-2 of the Breaking Emulsions section and step 1 of the cDNA synthesis section.

16. 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.
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. Retrieve the PIP tubes from the dry bath and place the emulsified PIP samples in the **blue** PIPseq 4-tube stand (FB0002687).

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

4. Discard most of the remaining partitioning fluid by aspirating up to ~130 µL from the bottom of each emulsion. It may be necessary to complete a second aspiration using a P20 pipette set to a low volume to ensure only a few µL of partitioning fluid remains.

⚠ 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.

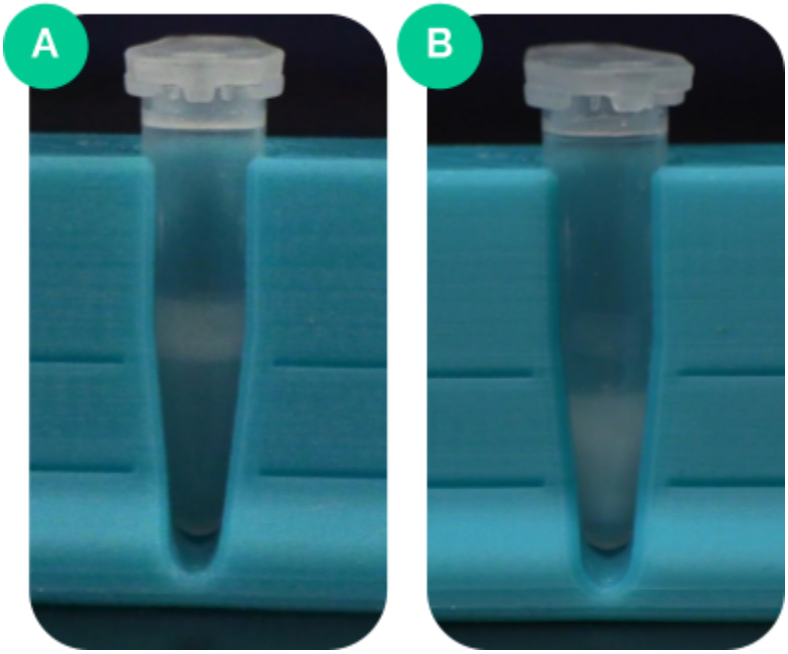


Figure 7: PIP tubes after removing them from the Dry bath (A) and after discarding most of the remaining partitioning fluid (B).

5. Add 200 µL of Breaking Buffer at room temperature on top of the emulsion without disturbing the emulsion.
6. Add 40 µL of De-Partitioning Reagent on top of the emulsion (Figure 8A).

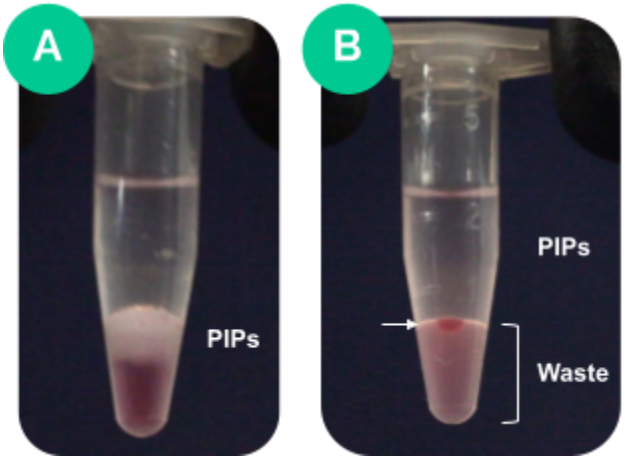


Figure 8. PIPs after addition of breaking buffer and de-partitioning reagent (A) and after inverting and spinning down (B). The PIPs are now suspended in the top phase. Take note of the red droplet that must be removed at the interphase (white arrow).

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

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

8. Centrifuge 5-10 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 a red droplet is detected at the interphase, be sure to remove it along with the bottom phase during step 9.

9. Discard the bottom phase by aspirating 70 µL from the bottom of the tube. 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 (Figure 8B).

10. Repeat step 8 and then aspirate any remaining bottom phase with a P20 pipette. 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.**

11. 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. With a pipette set to 180 µL, 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. **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 platefuge adapters (FB0002082) 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 9.

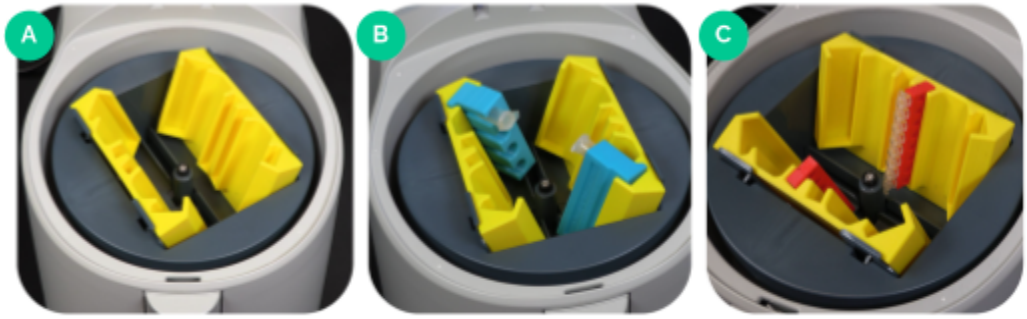


Figure 9. Load the yellow platefuge adapters into the platefuge as shown in **(A)**. For washes with 1X wash buffer pre-RT, load your tubes in the blue tube racks, and the tube racks into the yellow adaptors as shown in **(B)**. For 0.5X washes post-RT and pre-WTA on page 22, load the washes in the red guide-wire racks as shown in **(C)**.

⚠ **IMPORTANT: Allow the platefuge 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 which aligns with the "L" marker on the 1.5 mL stand.**

6. Aspirate and discard the aqueous volume above the 0.1 mL mark on the outside of the 1.5 mL tube ("L" marker on the stand) **WITHOUT** disturbing PIPs pellet (Figure 10).

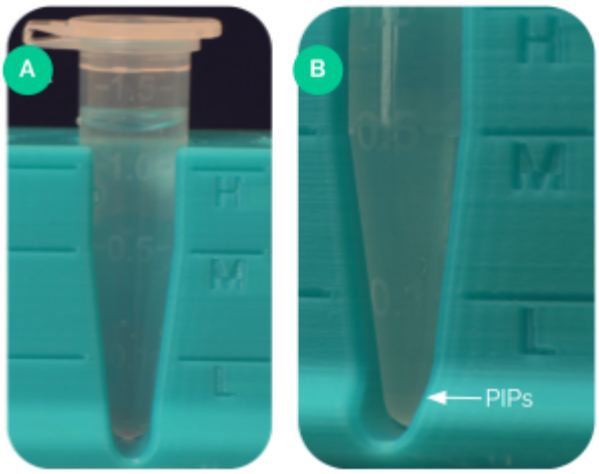


Figure 10. PIPs are difficult to visualize in **(A)** before centrifugation. PIPs may be seen in the bottom of the 1.5 mL tube during washes (white arrow) in **(B)**.

⚠ **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.**

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

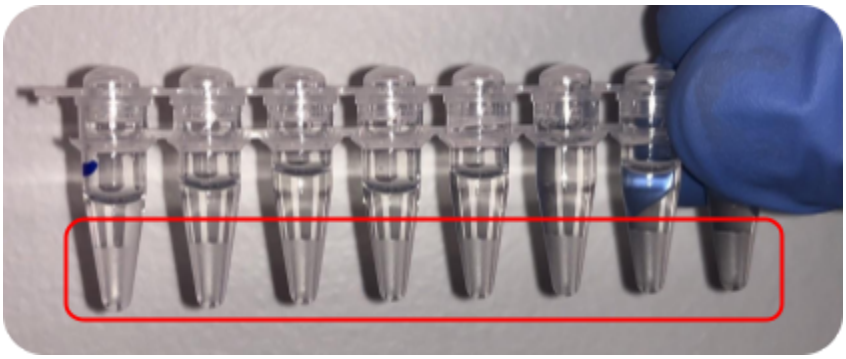


Figure 11. PIPs packed in 0.2 mL strip tubes following centrifugation with PlateFuge.

10. Transfer the entire volume of the ~ 100 µL PIP mixture per sample into each tube of a Greiner Bio-One 8-tube strip (FB0002076) . Seal the strip securely with the 8-strip cap (FB0001055). Place the strip into the **red** PIPseq guide rack (FB0001549), and slide the rack containing the strip into the platefuge adapter. Ensure that the platefuge is properly balanced before spinning. Spin for ~30 seconds (Figure 9C).

⚠ Note: The swing-out rotor of the platefuge enables the PIP/aqueous interface to remain flat instead of angled (Figure 11). **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 12).**

13. Remove the 8-tube strip from the **red** PIPseq guide rack and place on a cold block or ice until the next step.

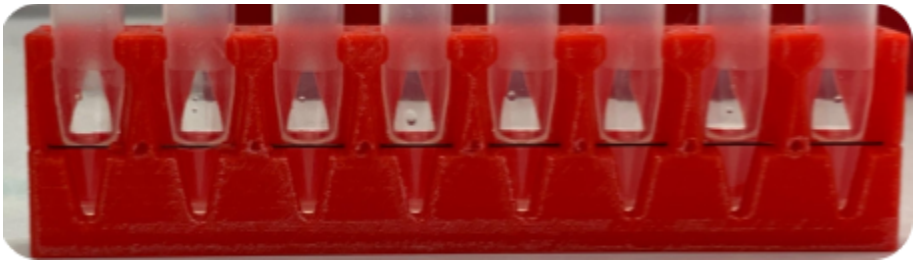


Figure 12. PIP tubes in the guide rack where the volume has been reduced to the level of the wire (~39 uL). Note that the base of the meniscus is hidden behind the wire.

cDNA synthesis

1. **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℃ storage. Remember to prepare the working stock dilutions of 0.5X Washing Buffer prior to the start of this step (see Reagent Preparation section at the start of the protocol). Note that fill volumes provide sufficient reagents for 9.5 total reactions so it is recommended not to exceed 10% pipetting 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 pipetting 10 times.

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

3. Add 39 µL of the mastermix to each PIP sample in the 8-tube strip. Place the PIP strip into the PIPseq 8-tube stand (FB0001024).
4. 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.
5. Place the 8-tube strip into the **red** PIPseq guide rack, and slide the rack containing the strip into the platefuge 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).
6. Perform cDNA synthesis with the following protocol in a thermocycler with heated lid set to 105°C (set thermocycler reaction volume to 78 µL).

25 °C	30 minutes
42 °C	90 minutes
85 °C	10 minutes
4 °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 8-tube strip into the **red** PIPseq guide rack, and slide the rack containing the strip into the platefuge adapter. Ensure that the platefuge is properly balanced before spinning. Spin for 1 min.
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 platefuge adapter. Ensure that the platefuge is properly balanced before spinning. Spin for 1 min.
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 in the platefuge 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 (~39 uL).
3. Place the PIP strip into the PIPseq 8-tube stand.
4. 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.
5. Place the strips into the **red** PIPseq guide racks, and slide the guide racks into the platefuge 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.

6. Run the program below on a thermocycler with the heated lid set to 105°C (set thermocycler reaction volume to 78 µL).

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

STOP

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

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 30 uL IDTE to each WTA reaction and add the mixture to one centrifuge filter per sample.
2. 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 2.

3. Transfer the flow-through into new 0.2 mL sample tubes.
4. Measure the volume of each sample using a P200.
5. Add enough IDTE to each sample to achieve a total volume of 100 µL per sample.

SPRI Purification

1. Make fresh 85% (v/v) ethanol.
2. Thoroughly vortex bottle of SPRI beads to mix.
3. For 100 µL reaction volumes, add 80 µL SPRI. This is a 0.8x ratio of SPRI beads.
4. Pipette up and down 10 times at the 170 µL stroke.
5. Incubate for 5 minutes at ambient temperature.
6. If necessary, do a final quick spin down of the SPRI beads.
7. Place the 0.2 mL sample tubes in the magnetic stand and allow the beads to bind to the magnet for 5 minutes.
8. Discard the supernatant being careful not to touch the SPRI beads.
9. Carefully add 200 uL of 85% ethanol to the PCR tubes on the magnetic stand without disturbing the beads.
10. Incubate for 30 seconds.
11. Carefully discard the 200 uL 85% ethanol wash supernatant.
12. Repeat step 9-11 to complete a second wash.
13. Remove final traces of ethanol with P20 pipette, be careful not to disturb the SPRI beads.
14. 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.
15. Remove the sample tubes from the magnetic rack and add 22 µL IDTE. Mix the IDTE and SPRI beads by pipetting up and down 10 times at the 22 µL 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.

16. Incubate for 5 minutes at room temperature.
17. Briefly centrifuge on a benchtop minifuge (~ 2 seconds).
18. Place the sample tubes into the magnetic rack and allow the beads to bind to the magnet for 5 minutes.
19. Remove and **SAVE** 20 µL of supernatant in new 0.2 mL sample tubes. Do not disturb the SPRI beads.

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

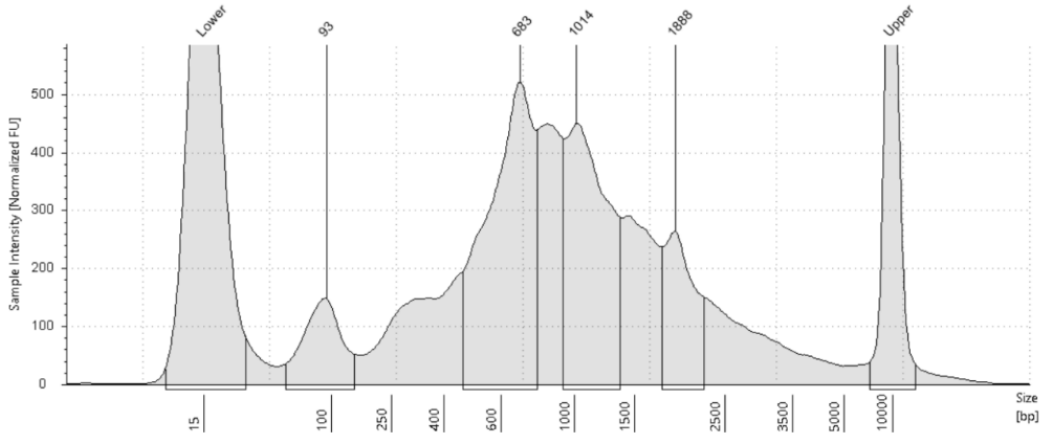
cDNA QC and Quantification

Qubit Quantification

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

Fragment Analysis

1. Users may use an Agilent BioAnalyzer High Sensitivity DNA kit or TapeStation High Sensitivity D5000 ScreenTapes. 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 5,000 cell input of human peripheral blood mononuclear cells (PBMCs) using a high sensitivity D5000 ScreenTape.

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 µL 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 no more than 10% overage when calculating volumes for the master mix to account for standard pipetting error (e.g., for 2 reactions multiply each reagent by 2.2). 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 no more than 10% 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 0.2 mL sample tubes), add 60 µL (0.8X) of resuspended SPRI beads, and mix thoroughly by pipetting up and down at the 70µL stroke 10 times.
4. Incubate the library-bead mixtures at room temperature for 5 min.
5. Place the sample tubes into the magnetic rack and allow the beads 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 a 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 the sample 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 the sample tubes at room temperature for 5 min.
12. Place the sample tubes into the magnetic rack and allow the beads to bind to the magnet for 5 min, or until the solution is clear.
13. Carefully transfer 20 µL of each library-containing supernatant into new 0.2 mL sample tubes.

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 the sample tubes onto the magnetic rack and allow the beads to bind for 5 minutes.

7. Prepare new 0.2 mL sample tubes in order to save the supernatants.
8. SAVE the supernatant and transfer it into the newly prepared sample 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 the sample tubes onto the magnetic rack and allow the beads to bind for 5 minutes.
13. Discard the supernatant. Be careful not to disturb the SPRI beads.
14. Carefully wash twice with 200 µL 85% ethanol for 30 seconds each. Do not disturb the SPRI beads.
15. Remove final traces of ethanol with a 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 the sample tubes from the magnetic rack and add 21 µL IDTE. Mix the IDTE and SPRI beads by pipetting up and down 10 times at the 20 µL stroke.
18. Incubate for 4 minutes at room temperature.
19. If necessary, do a quick spin down of the SPRI beads
20. Place the sample tubes onto the magnetic rack and allow the beads to bind to the magnet for 5 minutes.
21. Remove and **SAVE** 20 µL of the library-containing supernatant into new sample tubes. The samples can be stored at -20°C long-term.

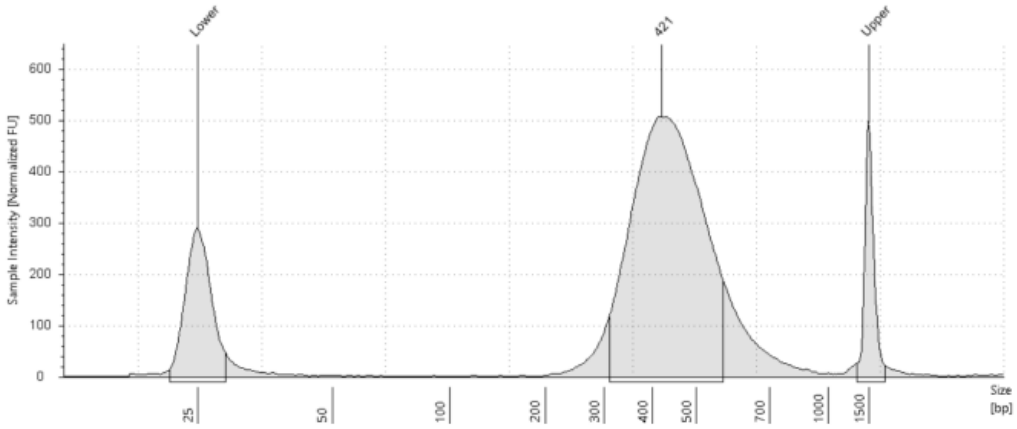
Post Library Preparation QC

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 High Sensitivity kit or TapeStation High Sensitivity D1000 ScreenTapes. 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.

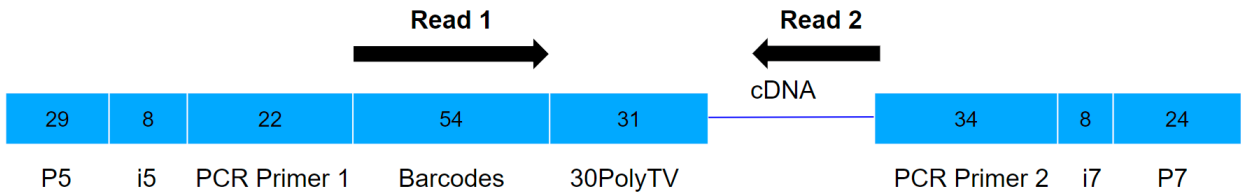


Representative library trace for a 1000 cell capture human/mouse cell mixture (HEK 293T/NIH 3T3) on a High Sensitivity D1000 ScreenTape (above).

Sequencing

PIPseq T2 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**GTCTC GTGGGCTCGGAGATGTGTATAAGAGACAG
FB0001915-1918, FB0001666-1669	Library P5 Index	AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXX**A CACTCTTTCCCTACACGACGC
FB0002006	WTA Primer	Forward: CTCTTTCCCTACACGACGCTC Reverse: AAGCAGTGGTATCAACGCAGAGT
FB0001042	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: **FB0001026**
Revision: **12.3**
Revision date: June 2023

General Changes:

- N/A

Specific Changes:

- Updated library structure figure on page 36

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