Fluent BioSciences

PIPseq™ T100 3' Single Cell RNA Kit v3.0

User Guide

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Table of Contents

Introduction

The Fluent BioSciences PIPseq™ T100 3' Single Cell RNA Kit uses pre-templated instant partitions to combine individual cells with barcoded beads to produce sequencing-ready libraries starting from single cell suspensions in 15 hours. These libraries enable 3' gene expression by profiling 100K individual cells per sample. This protocol can be completed in a convenient 2-day workflow.

Protocol Timing

Step	Duration	Stopping point
Reagent Preparation	~30 min	
General Cell Preparation Dependent on cell type	~1-1.5 hrs	
Cell capture and lysis	1 hr 40 min	4˚C overnight or 0˚C up to 72 hours
mRNA isolation		
Breaking emulsions	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	2 hours	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	

Recommended Use

The recommended application for this kit is 2 reactions in which 200K mammalian cells are individually captured for creation of single cell 3' gene expression libraries to be sequenced on an Illumina platform. It is recommended that a minimum of 2 samples are processed at a time.

PIPseq Platform Overview

The PIPseq platform consists of five reagent kits (Ambient, 4°C, -20°C Box 1, -20°C Box 2, and -80°C), a Consumable Kit, and a one-time Starter Kit containing the required equipment.

PIPseq T100 3' Single Cell RNA Kit Product Numbers

Product Name	Catalog Number	Storage
PIPseq T100 3' Single Cell RNA Ambient Kit v3.0	FBS-SCR-T100-2-V3-1	15°C to 30°C
PIPseq T100 3' Single Cell RNA 4°C Kit v3.0	FBS-SCR-T100-2-V3-2	2°C to 8°C
PIPseq T100 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	FBS-SCR-T100-2-V3-3	-30°C to -15°C
PIPseq T100 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	FBS-SCR-T100-2-V3-4	-30°C to -15°C
PIPseq T100 3' Single Cell RNA -80°C Kit v3.0	FBS-SCR-T100-2-V3-5	-90°C to -75°C
PIPseq T100 3' Single Cell Consumable Kit v3.0	FBS-SCR-T100-2-V3-6	15°C to 30°C
PIPseq T100 3' Single Cell Starter Equipment Kit v3.0	FBS-SCR-T100-V3-STKIT	15°C to 30°C

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

PIPseq T100 3' Single Cell RNA Starter Equipment Kit

Component Name	Part Number	Units
PIPseq rotating vortex adapter for 5 mL tubes	FB0003848	1
PIPseq rotating vortex adapter base	FB0003847	2
PIPseq vortex mixer	FB0002373	1
PIPseq Dry bath with heated lid	FB0001963	1
PIPseq Dry bath 5 mL block	FB0002675	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 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 T100 3' Single Cell RNA Consumables Kit

Component Name	Part Number	Units
Centrifuge Tube Filter	FB0003696	1 Bag of 16
0.2 mL PCR 8-tube strip without Cap, Greiner Bio-One, 673210	FB0002076	10 strips of 8
PCR 8-Cap strips, domed cap, Greiner Bio-One, 373270	FB0001055	10 strips of 8
5 mL tubes	FB0001565	4
3 mL syringe	FB0002625	6
1 mL syringe	FB0002892	4
G22 blunt bottom syringe needle	FB0002623	10

PIPseq T100 3' Single Cell RNA Reagent Kit Components

Component Name	Part Number	Kit	Storage	Units
T100 PIPs	FB0003299	PIPseq T100 3' Single Cell RNA -80°C Kit v3.0	-90°C to -75°C	2
Cell Suspension Buffer	FB0002440	PIPseq T100 3' Single Cell RNA -80°C Kit v3.0	-90°C to -75°C	2
Partitioning Reagent	FB0003123	PIPseq T100 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	1
Breaking Buffer	FB0003129	PIPseq T100 3' Single Cell RNA 4°C Kit v3.0	2°C to 8°C	1
CLB2	FB0003719	PIPseq T100 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	2
Washing Buffer	FB0003074	PIPseq T100 3' Single Cell RNA 4°C Kit v3.0	2°C to 8°C	1
De-Partitioning Reagent	FB0003072	PIPseq T100 3' Single Cell RNA Ambient Kit	15°C to 30°C	1
RT Enzyme Mix	FB0003080	PIPseq T100 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
RT Additive Mix	FB0003076	PIPseq T100 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
TSO	FB0003078	PIPseq T100 3' Single Cell RNA -80°C Kit v3.0	-90°C to -75°C	2
WTA Buffer Mix	FB0003082	PIPseq T100 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
WTA Primer	FB0003084	PIPseq T100 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
SPRI Beads	FB0003088	PIPseq T100 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	1
Nuclease-free water	FB0003090	PIPseq T100 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	1
IDTE pH 8.0	FB0003086	PIPseq T100 3' Single Cell RNA Ambient Kit v3.0	15°C to 30°C	1
Library P7 Indices	FB0001626-1627, FB0001629-FB0001633, FB0002092	PIPseq T100 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1 each
Library P5 Indices	FB0001915-1918, FB0001666-1669	PIPseq T100 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1 each
Resuspension Buffer	FB0003100	PIPseq T100 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
Library Prep Buffer	FB0001602	PIPseq T100 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	-30°C to -15°C	1
Library Prep Enzymes	FB0001603	PIPseq T100 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	-30°C to -15°C	1
Library Prep Mix A	FB0001604	PIPseq T100 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	-30°C to -15°C	1
Library Adapter Mix	FB0001605	PIPseq T100 3' Single Cell RNA -20°C Box 1 of 2 Kit v3.0	-30°C to -15°C	1
Library Prep Mix B	FB0001606	PIPseq T100 3' Single Cell RNA -20°C Box 2 of 2 Kit v3.0	-30°C to -15°C	1

Reagent, Equipment, and Consumable Requirements

Required Third Party Reagents

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

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
Qubit Assay Tubes	Thermo Fisher; Cat # Q32856
15 mL centrifuge tubes, sterile	General laboratory supplier, recommend Corning # 430790
PCR-clean tubes, strips, or plates for library preparation. *NOTE* Specific consumables may be required for certain steps, see warnings in protocol.	General laboratory supplier

Required Third Party Equipment

Description	Supplier
3 x 15 ml / 50 ml Centrifuge Magnetic Separation Combo Rack	Permagen, MSR1550 or MSR6X15
Magnetic separation rack suitable for PCR tubes	General laboratory supplier
Centrifuge tube rack for 5/15 mL tubes	General laboratory 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 1.5 mL microcentrifuge tubes	General laboratory supplier
Hemocytometer or automated cell counter	General laboratory supplier
Micropipettes, 1µL-1000µL capabilities	General laboratory supplier
Qubit Fluorometer	Thermo Fisher
Bioanalyzer 2100 or TapeStation 4200	Agilent
Benchtop microcentrifuge 2000xg, suitable for 1.5 mL tubes and 0.5 mL tubes. ***NOTE* The microcentrifuge should decelerate 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

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 the addition of 200,000 cells into the PIPseq assay, resulting in the recovery of 100,000 cells and a multiplet rate of < 8%. The optimal input cell concentration is 5,000 cells per microliter. Alternative cell loading concentrations are allowed, as long as input volume is maintained, and increased cell loading will increase the observed multiplet rate.

# Cells loaded	# Cells recovered	Multiplet rate
200,000	~100,000	3%

PIPseq T100 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

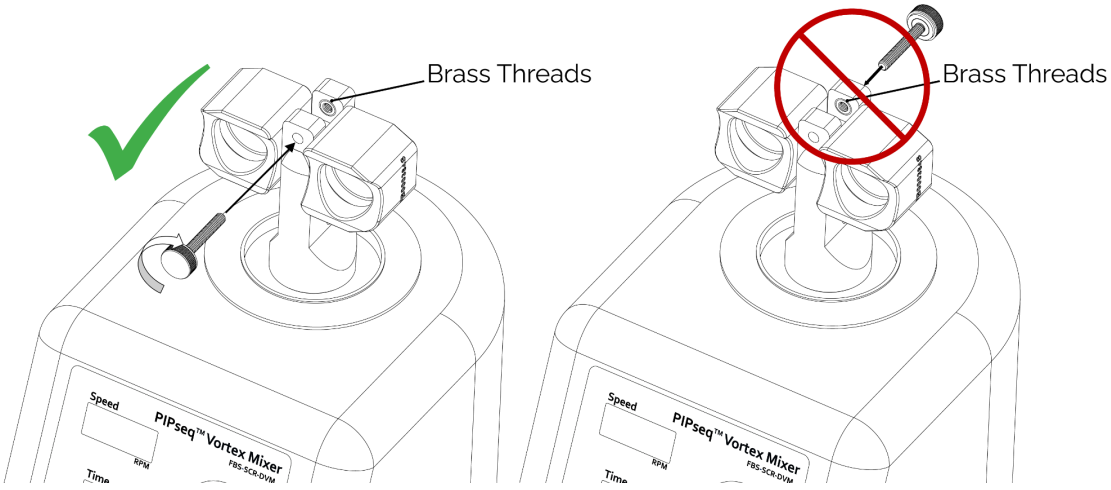
Upon receiving the PIPseq T100 3' Single Cell RNA kits, remove the -80°C box with TSO aliquots (FB0003078), Cell Suspension Buffer (FB0002440), and PIP reactions (FB0003299) from the dry ice shipping container and store in a -80°C freezer.

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 12 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 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).

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.

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 5,000 live cells per microliter using a **wide-bore** low retention pipette tip.
13. Once the target concentration is achieved, place the cells on ice.
14. Proceed with the Fluent PIPseq T100 Capture and Lysis protocol.

Nuclei Preparation

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

IMPORTANT NOTE: The lysis temperature for nuclei is 66°C while the lysis temperature for cells is 37°C. At step 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 1-2 samples. Thaw the desired number of PIP tubes, one for each sample that will be processed.

WORKFLOW TIP: Begin preheating the PIPseq Dry bath to 37˚C with the heated lid turned off. 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 at room temperature for 15 minutes. After the PIP tubes have fully thawed, centrifuge them for 5 seconds 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 40 µL of prepared cells or nuclei (200,000 total) directly into the PIPs while avoiding the creation of air bubbles. If performing multiple reactions, add cells to all of them, no need to mix between additions.

5. Mix the cell:PIP mixture 10 times using a **standard bore**, low-retention P1000 tip at 650 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. On the last stroke, dispense and then wait for PIPs to collect in the tip and expunge.

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

6. Add 4000 µL Partitioning Reagent to the cell:PIP mixture (Figure 1) via multiple dispenses of a P1000 pipette. Do not use serological pipettes.

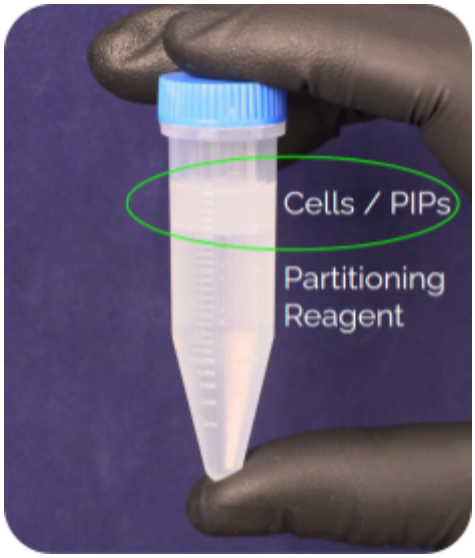


Figure 1. (circled in green) Cells and PIPs mixture floating on top of excess Partitioning Reagent

7. Ensure the screw caps are tightly sealed and place the tubes in the rotating vortex adapter. Rotate the vortex adapter so that the tubes are in a horizontal orientation (Figure 2A). Ensure the tubes are fully depressed into the adapter. Vortex at 3000 RPM for 20 seconds.

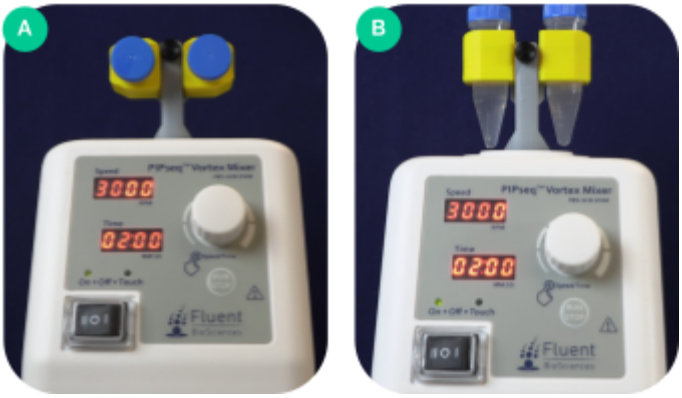


Figure 2. The rotating vortex adaptor shown in the horizontal configuration **(A)** and the vertical configuration **(B)**.

8. Rotate the vortex adapter so that the tubes are in a vertical orientation (Figure 2B). Vortex at 3000 RPM for 2 minutes.
9. Remove the tubes from the vortex adapter and place them into a 5/15 mL centrifuge tube rack. Let emulsion stabilize for 30 seconds and then proceed to the next step (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 bottom syringe needle (Figure 4A) by slowly placing the tip through the emulsion to the bottom of the tube. Wait for five seconds before aspirating out the bottom phase (Figure 4B). Repeat the aspiration until the PIPs layer is aligned with the 1.5 mL volume marker on the 5 mL tube (Figure 4C). Be careful not to aspirate out any emulsion.

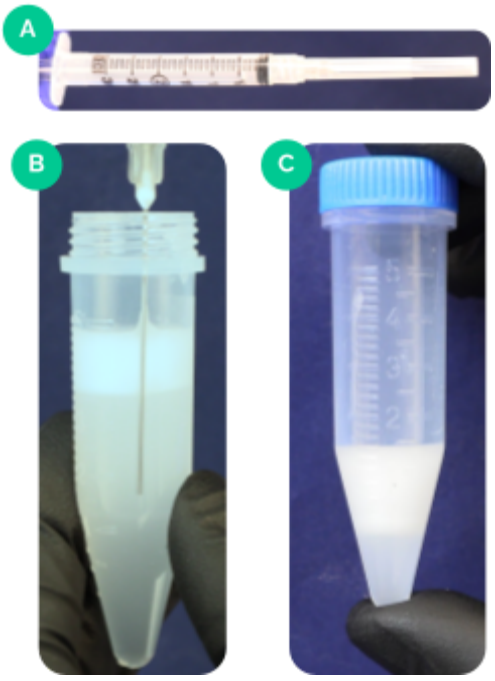


Figure 4. Use a 3mL syringe with a G22 blunt bottom needle shown in **(A)** to remove excess Partitioning Reagent from the bottom phase as shown in **(B)**. Remove excess until the PIPs layer sits at or below the 1.5mL mark **(C)**.

⚠ Tips for aspirating through an emulsion: Be cognizant of 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 2500 µL of Partitioning Reagent to one of the provided CLB2 tubes. Vortex for 10 seconds to generate the emulsion and **immediately** pour the whole volume of the Chemical Lysis Emulsion (~3300 µL) on top of the PIP emulsion. Use one tube of Chemical Lysis Emulsion per sample.

⚠ **WARNING: The volumes of partitioning reagent and chemical lysis buffer are carefully tailored to ensure optimal lysis conditions. Do not deviate from the stated volumes. It is recommended that users add the Partitioning Reagent via multiple dispenses from a P1000 pipette to ensure accuracy and avoid use of serological pipettes, which can drip.**

⚠ 12. Mix by inversion 10 times.

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

13. Verify the PIPseq Dry bath (Figure 5) is preheated to the appropriate temperature according to the sample type in use (see table below). Ensure the heated lid is off by pressing "LidOff" and leaving the lid open. When the heated lid is off there will **not** be a green dot next to the lid temperature. After at least 10 minutes of preheating is complete, insert samples and then skip to the next step to begin the lysis incubation as indicated in the table below.

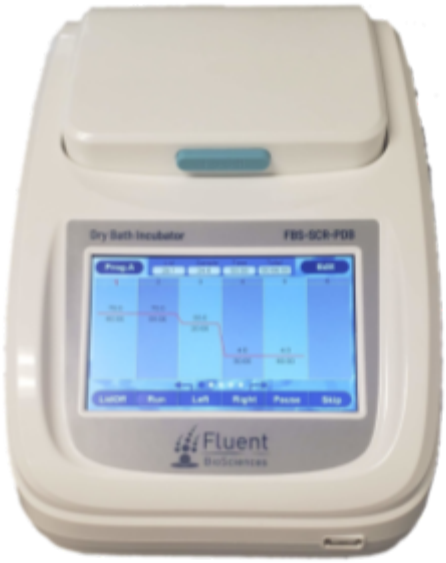


Figure 5. The PIPseq™ Dry bath to be used for the lysis incubation step.

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

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

14. **WORKFLOW TIP**: During incubation, complete steps 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 you can hold the samples at 4˚C overnight or 0˚C for up to 72 hours before proceeding to mRNA isolation. **Do not freeze**! Alternatively, you can move forward to mRNA isolation after the 4˚C incubation. Note: Some condensation may appear on the tubes, which is not a concern.

mRNA Isolation

Breaking Emulsions

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

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

2. Remove and discard any remaining partitioning reagent using a 3 mL syringe attached with a G22 blunt bottom syringe needle. Place the tip through the emulsion slowly to the bottom of the tube, wait for five seconds, and then aspirate out the bottom phase until the top interface aligns with the 2 mL volume marker on the tube.. Be careful not to aspirate out any emulsion.

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

3. Add 2500 µL Breaking Buffer to each sample. Next, add 800 µL De-partitioning reagent to each sample, along the side wall of the tube (Figure 6A).
4. Let the emulsion stand for 1 minute and then invert the tube 5 times to complete breaking.

⚠ Critical: Do NOT vortex the tube during breaking.

5. Centrifuge for 30 seconds on a swinging bucket centrifuge at 1000 x g.

⚠ 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.

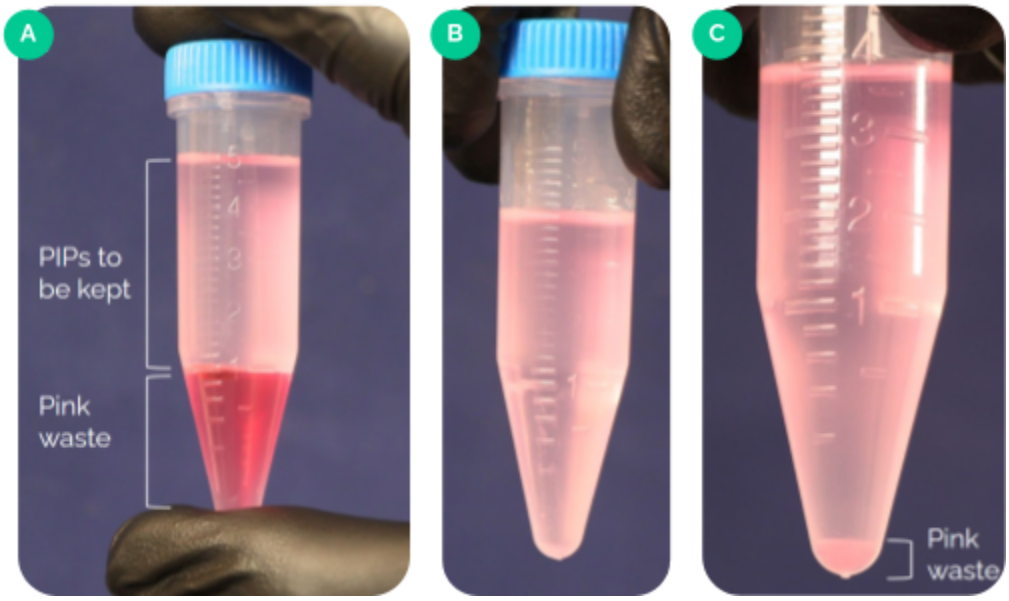


Figure 6. PIPs will break into the 2 layers shown in **(A)** 1 minute after addition of De-Partitioning Reagent. After removal of the pink waste phase with the syringe and needle, your PIPs phase should look like **(B)** before spinning down again for Step 7. After your second spin down inspect your tube for a precipitate pellet, shown in **(C)** that should be removed if present.

6. Remove the pink-colored bottom phase (800 - 1000 µL) from each tube of PIPs using a 3 mL syringe attached with a G22 blunt bottom syringe needle.
7. Centrifuge for 30 seconds on a swinging bucket centrifuge at 1000 x g.
8. Visually inspect the tube and remove any red precipitate using the 3 mL syringe.
9. Keep the tubes on ice or on a compatible cold block.

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

Washing PIPs with 1X Washing Buffer

1. Make one aliquot per sample of 10 mL of Washing Buffer in a 15 mL centrifuge tube. 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 5 mL tube on a swinging bucket centrifuge after the first transfer to collect all the PIPs at the bottom of the tube. Aspirate remaining PIPs from the 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 5 mL tube.

4. Gently mix each tube by tapping the bottom to break up the pellet and invert 10 times. Centrifuge the tubes for 2 minutes at 1000 x g (use ~70-80% of the maximum braking speed) on a centrifuge with a swinging bucket rotor to pack the PIPs (Figure 7).

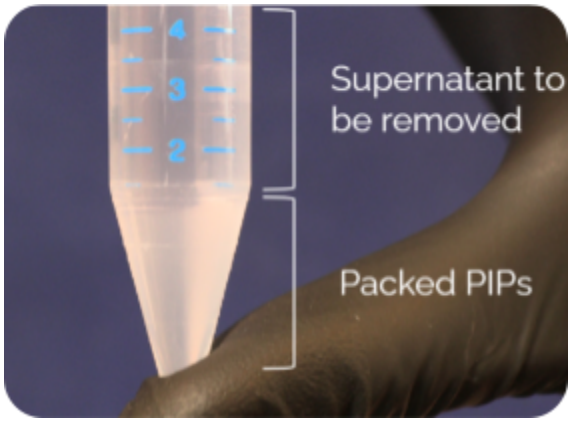


Figure 7. Packed PIPs after centrifuging.

5. Remove supernatant until ~ 1.5- 2 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 1 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. Note: The final combined volume should be 12-14 mL.
7. Repeat steps 4-5 to complete a second wash of the PIPs.
8. Add 12 mL of Washing Buffer from the stock bottle directly into the tube containing packed PIPs.
9. Repeat steps 4-5 to complete a third wash of the PIPs.
10. Add 12 mL of Washing Buffer from the stock bottle directly into the tube containing packed PIPs.
11. Repeat steps 4-5 to complete a fourth and final wash of the PIPs.
12. Centrifuge the tubes for 2 minutes at 1000 x g.
13. Using a 1 mL syringe, remove any remaining supernatant to the 1 mL volume marker on the tube.

    WORKFLOW TIP: If the 15 mL conical used does not have a 1 mL volume marker, use a clean tube and fill with 1 mL of water via pipette to serve as a volume marker.
14. Transfer the 1 mL PIP mixture into a fresh 5 mL tube using a P1000 low retention tip.
15. 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 2 mL reaction. The TSO must be thawed on ice after removal from -80˚C storage. Note that fill volumes assume users will add reagents directly to each reaction tube.
2. Following the table below, add the specified volume of reverse transcription reagents to each sample, in the indicated order.

Reagent	Volume Per Reaction (µL)
RT Additive Mix	860
TSO	80
RT Enzyme Mix	60
Total	**1000**

3. Pipette mix the cDNA synthesis reaction 20 times using a P1000 with a low retention tip. Briefly centrifuge tube to remove droplets from side of tube.
4. Perform cDNA synthesis with the following protocol in the PIPseq Dry bath.

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 thermocycler or the fridge before proceeding to the next step.

Washing PIPs with 0.5X Washing Buffer

1. Centrifuge the 5 mL tubes for 2 minutes at 1000 x g (use ~70-80% of the maximum braking speed) on a centrifuge with a swinging bucket rotor to pellet the PIPs.
2. Aspirate and discard 1000-1200 µL supernatant without disturbing the PIPs pellet.
3. Add 4 mL of 0.5X Washing Buffer to each tube and vortex for 5 seconds to mix.
4. Repeat centrifugation in step 1.
5. Aspirate and discard 4 mL supernatant **WITHOUT** disturbing PIPs pellet.
6. Add 4 mL 0.5X Washing Buffer to each PIP tube.
7. Repeat steps 4-5 to complete a second wash of the PIPs.
8. Add 4 mL 0.5X Washing Buffer to each PIP tube.
9. Repeat steps 4-5 to complete a third wash of the PIPs.
10. Centrifuge to pack the PIPs (repeat step 1).

11. Using a 1 mL syringe, remove any remaining supernatant to the 1 mL volume marker on the tube.
12. Proceed promptly to cDNA amplification.

cDNA Amplification

1. Following the table below, add the specified volume of whole transcriptome amplification (WTA) reagents to each sample, in the indicated order.

Reagent	Volume Per Reaction (µL)
WTA buffer mix	1000
WTA primer	10
Total	**1010**

2. Pipette mix the WTA reaction 20 times using a P1000 with a low retention tip. Briefly centrifuge the tube on a benchtop centrifuge.
3. Distribute the WTA reaction mixture into 8 x 250 µL aliquots (one PCR strip).

⚠ Note: For the last aliquot, briefly centrifuge the 5 mL tubes and transfer all residual volume into the PCR tube.

4. Using a P200 Multichannel pipette, transfer 50 µL from each tube into a new strip. Do not eject the tips. Repeat three more times to generate 4 additional 8 tube PCR strips for a total of five, 8 tube PCR strips.

   WORKFLOW TIP: Users may substitute PCR plates for PCR strips in the above step. The cycle numbers below are the recommended starting points 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 heated lid set to 105˚C.

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

Cells Loaded	Targeted cell capture	Recommended Cycles - high RNA samples (e.g. cell lines, cancer cells)	Recommended Cycles - low RNA samples (e.g. primary cells, nuclei)
<20,000	<10,000	12	16
20,000-80,000	10,000-40,000	11	15
80,000-160,000	40,000-80,000	10	14
160,000-240,000	80,000-120,000	9	13

6. The samples can be stored overnight 4˚C before proceeding to the next step.

Isolate cDNA from PIPs

1. Add 25 uL IDTE to each WTA reaction.
2. Pool 10 diluted WTA products from the same sample onto one centrifuge filter per sample. For every sample, a user will need 4 columns.
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. Combine the flow-through for each sample into a new 15 mL tube.
5. Measure the volume of each sample using a P1000.
6. Add enough IDTE to each sample to achieve a total volume of 2800 µL per sample.

SPRI Purification

1. Make fresh 85% ethanol.
2. Thoroughly vortex bottle of SPRI beads to mix.

WORKFLOW TIP: Users may choose to do the SPRI purification in 5 mL or 15 mL tubes as both are compatible with the recommended magnetic stands.

3. For 2800 µL reaction volumes, add 1680 µL SPRI . This is a 0.6x ratio of SPRI beads.
4. Vortex for 10 seconds and briefly centrifuge (~2 seconds).
5. Incubate for 10 minutes at ambient temperature.
6. Place the 15 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 3 mL 85% ethanol for 30 seconds each. Do not disturb the SPRI beads.
9. Remove final traces of ethanol with P20 or P200 pipette, careful not to disturb the SPRI beads.
10. Air dry for 10 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 280 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 the tube (~ 2 seconds).

13. Incubate for 10 minutes at room temperature.
14. Place tubes into the magnetic rack and bind to the magnet for 5 minutes.
15. Remove and **SAVE** 280 µL of supernatant in a new 1.5 mL tube. Do not disturb the SPRI beads.

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

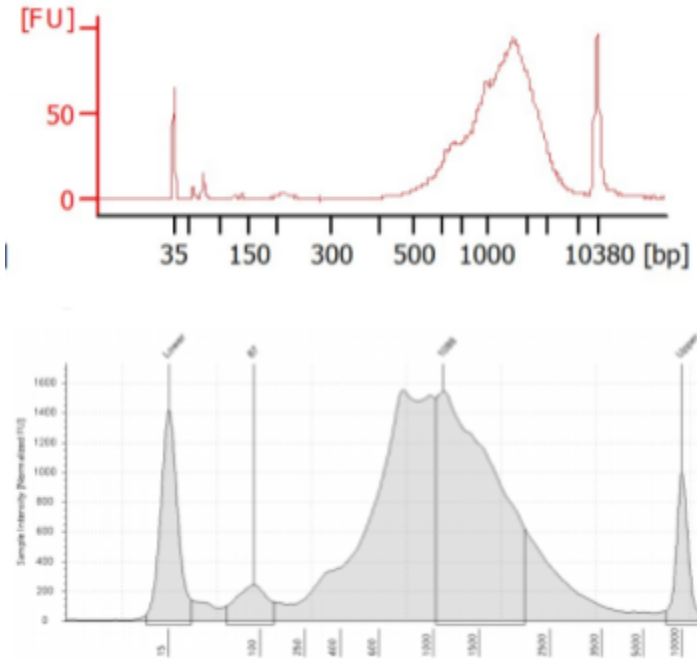
cDNA QC and Quantification

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



Representative cDNA traces for a 1000 cell input human/mouse cell mixture (HEK 293T/NIH 3T3)

using an Agilent High Sensitivity DNA (top) and a high sensitivity D5000 ScreenTape (bottom).

Library Preparation

Fragmentation, End Repair & A-Tailing

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 nuclease-free water in a PCR tube and place it 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 15 µM in 5 µL for 11-500 ng DNA input according to the total number of samples for adapter ligation. If DNA input 1-10 ng is desired the Library Adapter Mix must be diluted to a final concentration of 3 µM in 5 µL. The below table illustrates volumes per reaction for DNA input 11-500 ng.

Library Adapter Mix Per Sample (µL)	Nuclease-free water Per Sample (µL)	Total Volume Per Sample (µL)
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 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 70µL 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 20 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 4 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
501+	6
101-500	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. Proceed immediately 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 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 µL IDTE. Mix the IDTE and SPRI beads by pipetting up and down 10 times at the 13 µL stroke.
18. Incubate for 4 minutes at room temperature.
19. If necessary, do a quick spin down of the SPRI beads
20. Place tubes onto the magnetic rack and bind to the magnet for 5 minutes.
21. STOP Remove and **SAVE** 20 µL of supernatant in a new PCR tube strip. This sample can be stored at -20˚C long-term.

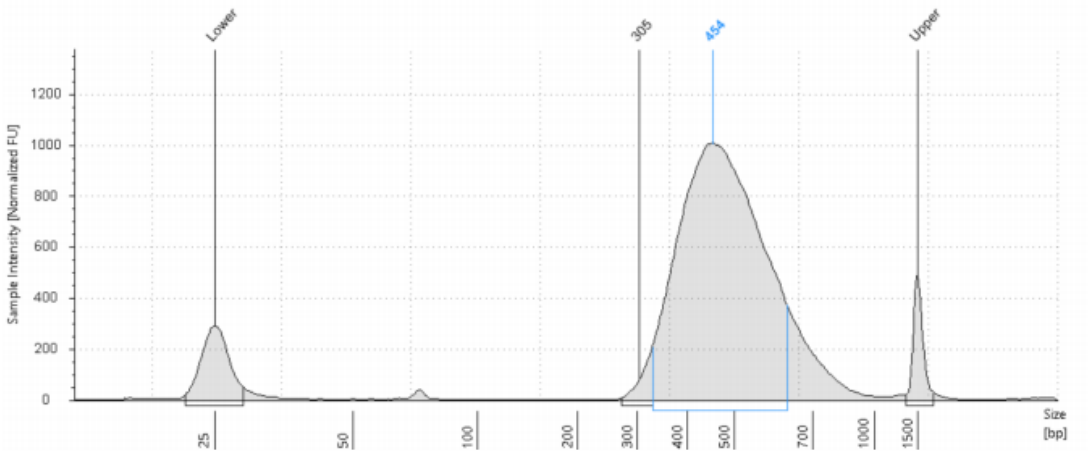
Post Library Preparation QC

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).

Illumina Sequencing

PIPseq T100 3' Single Cell gene expression libraries are composed of standard Illumina paired-end constructs that begin with P5 and end with P7. These libraries are dual-indexed with 8-base i5 and i7 indexes. Read 1 length must be ≥ 51 bases and the recommended read 2 length ≥ 70 bases. Sequencing depth will vary based on your application needs but it is recommended to start with a depth of 20,000 reads per cell.

The PIPseq T100 3' Single Cell gene expression libraries may be pooled for sequencing, taking into account the differences in cell number and read depth requirements. Samples must have distinct index combinations to avoid failures in sample demultiplexing. Refer to Illumina documentation for discussion of appropriate color balance combinations for the selected sequencing platform.

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

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

A simplified pooling scheme for the Illumina NextSeq2000 platform is described below.

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

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

PIPseq 3' Index Sequences

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

Oligonucleotide Sequences

Part Number	Name	Sequence (5' - 3')
FB0001626-1627, FB0001629-1633, FB0002092	Library P7 Index	CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGT GGGCTCGGAGATGTGTATAAGAGACAG
FB0001915-1918, FB0001666-1669	Library P5 Index	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXACA CTCTTTCCCTACACGACGC
FB0003084	WTA Primer	Forward: CTCTTTCCCTACACGACGCTC Reverse: AAGCAGTGGTATCAACGCAGAGT
FB0003078	TSO	AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG
FB0001605	Library Adapter Mix	Ligation primer: /5Phos/CTGTCTCTTATACACATCTCCGAGCC Ligation adapter: TATAAGAGACAGT

Abbreviations

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

Document Revision Summary

Doc ID: FB0003657
Revision: 1.3
Revision date: December 2022

Specific Changes:

- Changes the cycling recommendations in Library Preparation

Legal Notices

Support

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