illumina®

TRAINING PACKET

Illumina Single Cell 3’ RNA Prep

CONTENTS

This training packet provides an overview of the Illumina Single Cell 3' RNA Prep kits and is not to be considered a replacement for using the designated documentation.

1. Planning your experiment

Step 1

Refer to the compatible Illumina Single Cell 3' RNA Prep (formerly known as PIPseq V) product documentation applicable to your purchase.

Illumina Single Cell 3' RNA Prep Product Documentation

Product Documentation	Document #	Illumina Catalog #
Illumina Single Cell 3' RNA Prep, T2 Product Documentation	200063178	20135689
Illumina Single Cell 3' RNA Prep, T10 Product Documentation	200063182	20135691
Illumina Single Cell 3' RNA Prep, T20 Product Documentation	200063183	20135692
Illumina Single Cell 3' RNA Prep, T100 Product Documentation	200063184	20135693

Step 2

If you purchased an Illumina Single Cell Supplemental Enrichment and Amplification (SEA) Kit, refer to the Illumina Single Cell Supplemental Enrichment and Amplification (SEA) Kit section in this Training Packet for important information and links needed to plan your experiment.

Step 3

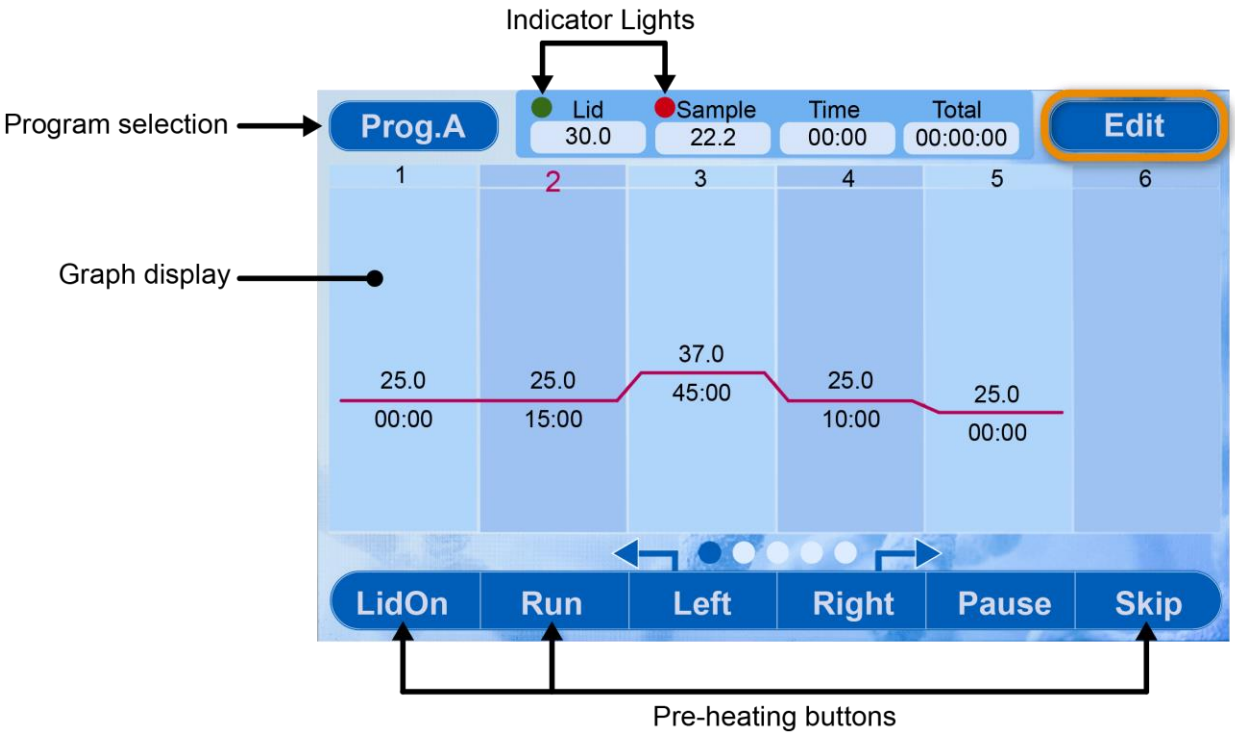
Make sure you have purchased and received the Illumina Single Cell Prep Starter Equipment Kit. The equipment included in the kit are required for successful results. See the Appendix Starter Equipment Kit section for equipment and accessories included in the kit.

Review the 'Equipment Preparation' section of the product documentation. The PIPseq Vortexer comes preinstalled with the vortex adapter head for T2 and T10 reactions (8 tube holes). If you are using a T20 kit (4 tube holes) or T100 kit (2 large tube holes), you will need to change out the vortex adapter head using the thumbscrew before starting your experiment.

Review the 'Dry Bath Operation' instructions in the product documentation to learn how to change between the different Dry Bath Protocols and how to manually set the lid mode for each program. The lid mode is not set

automatically when you select a program. You must choose the correct program, then select "**Edit**" and use the "**LidMode**" button of the editing screen to toggle to the correct lid mode setting.

Figure 1: Dry Bath Interface



Be sure that the correct tube block is installed in the Dry Bath for the kit being used (0.5 ml tube block for T2/T10, 1.5 ml tube block for T20, and 5 ml tube block for T100).

It is important to start Capture and Lysis immediately after preparing your final cell or nuclei suspension (there is no safe stopping point). The mRNA in the samples is not fully protected until samples are lysed at the end of the Capture and Lysis step. Equipment must be prepared before starting any sample preparation processes for your experiment.

Step 4

Make sure that you have received the materials shipped to you as outlined in the 'Product Contents' section of the product documentation and that they are stored at the proper temperatures. Complete kit should include both capture and barcoding kit and library preparation kit.

Step 5

Make sure that you have all the necessary third-party equipment and materials as outlined in the 'User-Supplied Consumables & Equipment', 'Consumables', and 'Equipment' sections of the product documentation.

Starting March 2025, the emulsion safe plasticware will no longer be provided in the consumable kits. Customers must purchase the specific consumables listed in the product documentation. Substituting these materials can adversely affect performance.

Step 6

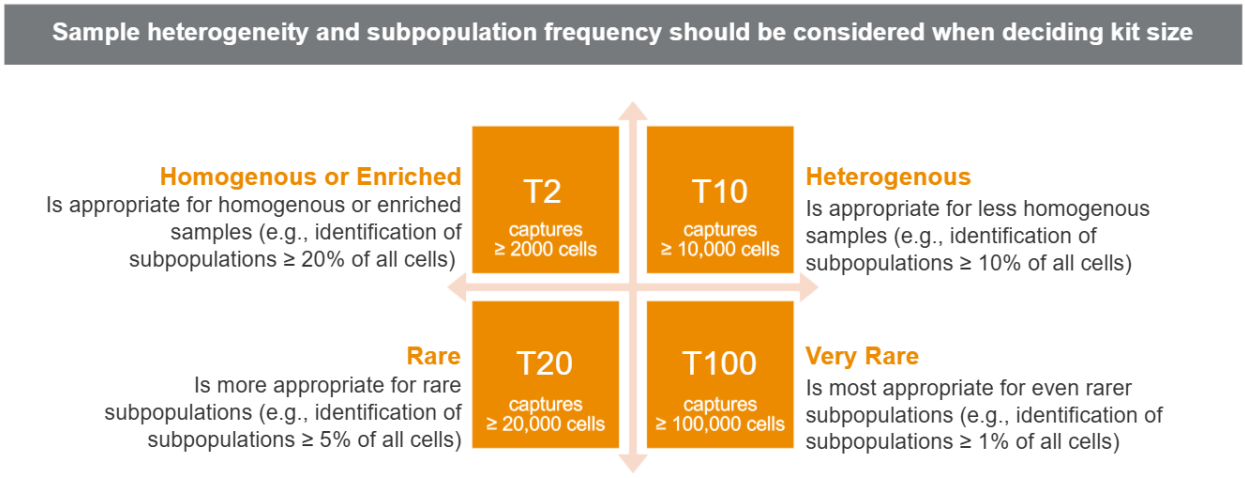
If your Illumina Single Cell 3' RNA libraries will be sequenced on a NovaSeq X Series or NextSeq 2000 sequencing instrument using the latest XLEAP-SBS chemistry, it is important to review the recommendations in the Appendix when pooling ≤ 4 libraries together. You will need to determine the UDI Library Index Mixes (1–8) to use for proper color balancing in a single lane or sequencing run. Improper color balancing can result in difficulties in demultiplexing the data.

1.1 Experimental design

If you are new to single cell sequencing, review the literature for further information on sample preparation methods compatible with single cell sequencing for your sample type, single cell experimental designs that have been used for similar projects, and best practices for completing single cell sequencing studies.

Both sample heterogeneity and subpopulation frequency should be taken into consideration to define the adequate cell or nuclei numbers required for an experiment. If unsure about sample type heterogeneity, it might be necessary to run a pilot experiment with T20 reactions to identify the rarity of the subpopulations you are trying to identify. Refer to Figure 2 for kit specific criteria.

Figure 2



1.2 Importance of pilot studies

PIPseq technology is optimized for efficient and sensitive transcript capture from live cells. Quick isolation and mild dissociation of cell types is essential and should be confirmed in a pilot experiment before committing to the actual

experiment. Completion of a pilot study before scale up is especially important when working with any new sample types. It is also valuable for customers who are new to single cell sequencing.

Purchasing a T2 is an economical way to complete an inexpensive pilot study before committing to large-scale data production. The T2 kits have the same chemistry as the higher cell capture kits, so you can assess each step of the scRNAseq process and complete any optimization needed, before scaling up with larger reactions that may require a significantly higher read depth and more expensive sequencing runs.

Processing a small number of samples before scale up is key to an informed experimental design for generating the type of sequencing results that you are looking for. There are many processes to evaluate during a single cell sequencing project. For some projects, the sample preparation process might be complex. Steps such as dissociation, enrichment, labeling cells, accurate quantitation of cells, determining cell viability, proper pipetting force and centrifuge speeds, and evaluation of third-party methods to remove debris and dead cells should all be evaluated before scale up. These steps can vary for each sample type.

When researchers are comparing wild type vs. treated or mutant conditions, the cDNA in these treated groups may have lower quality mRNA that is an inherent part of their mutant/treatment system. Discovering this during a pilot study allows for designing a more informed experiment. It might be necessary to prepare two or more PIPseq reactions from the same single cell suspension to reach the desired cell number for the experiment or to reach an equivalent cell capture rate as the wild-type sample. After the technical replicates are sequenced (ideally in the same sequencing run to limit batch effects), the replicates can be merged using the PIPseeker software and analyzed as a single sample.

An assessment of the mRNA quality from the new sample type being processed is necessary to determine if the associated upstream processes are sufficiently optimized. This can be evaluated in a quick pilot study without even taking the T2 reactions through to sequencing. Running through the Illumina Single Cell workflow and verifying that the cDNA QC and the final library pass both the QC steps is essential for producing quality sequencing results. Alternatively, after completing all sample preparation processes upstream of the Illumina Single Cell workflow, the total RNA can be isolated and run with an Agilent TapeStation RNA Assay to evaluate the quality and to verify the total RNA is not degraded (RIN value ≥ 7 is recommended).

If you take your pilot experiment through sequencing, this will provide even more information to help guide your experimental design. To assess sample quality, it is informative to review the rank plot and clustering results. You can also review key sequencing metrics to verify the data quality is good before deeper sequencing with larger cell numbers. These metrics include the percentage of mapped reads, captured cell count, percentage of reads in cells, percentage of reads in mitochondria, duplication rate, genes/cell, and transcripts/cell.

2. Sample preparation

Sample quality is critical for optimal results. Ideal cell suspensions are 90% viable and contain minimal debris or aggregates. The best option is to start with fresh cells or tissue whenever possible. Nuclei sequencing is required when starting from frozen tissue. It is important to work quickly and minimize unnecessary handling steps. If cells

are handled too roughly, cells will lyse which can increase background mRNA. Incorporating additional wash steps is recommended to remove debris. Refer to the Enrichment strategies (FACS, FANS, MACS) section of this Training Packet for third-party options to help improve cell viability if it is too low.

To minimize damage during sample preparation, pipetting and centrifugation should be kept to a minimum. A tightly packed cell pellet requires extra pipetting, which can damage cells from shearing effects. Pipetting steps should be slow and gentle. The use of wide-bore pipette tips is recommended to minimize the shear force on the cell suspension during certain points in the cell preparation protocol, even when working with small cell types. It might be necessary to use narrow-bore pipette tips in some cell types to reduce clumps. When optimizing the workflow, pay close attention to using the minimum speed and force necessary for a given cell type, as this will vary.

It is important to note that Illumina Single Cell 3' RNA kits are not compatible with Fetal Bovine Serum (FBS), which is commonly used in cell thawing media and in pre- and post- flow sorting buffers. FBS inhibition can cause low cDNA yield and/or fragment sizes. Users should closely follow the product documentation instructions to pellet their cells or nuclei and to complete a wash step before resuspending in the appropriate Illumina sample suspension buffer. There should be no more than 1% BSA or 0.1% FBS in the final cell or nuclei suspensions before starting the workflow. The wash step is also important for removing components that could disrupt emulsion stability or RNA hybridization, such as high calcium ion concentrations, high-volume fractions of carryover organic solvents (eg DMSO, methanol), or high molecular weight polymeric additives (eg PVSA, DEPC) that might be used in earlier sample treatment steps.

Note that Accutase is preferable to Papain and DNase I for dissociations during sample preparation. DNase 1 can result in breaking down the cell barcode structures in the particle templated instant partitions (PIPs). If no other options are available, make sure the DNase 1 is thoroughly washed out of the cell suspension (at least three 2 ml wash steps in PBS, followed by a 1 ml wash step in Cell or Nuclei Suspension Buffer that is included in the kit).

2.1 General recommendations for cells

The Illumina Single Cell 3' RNA kits require completing Capture and Lysis to get to a safe stopping point. It is very important for customers to minimize the amount of time that it takes for completing Sample Preparation through Capture and Lysis.

It is recommended for customers to prepare their equipment in advance and to thaw one PIP tube for each sample being processed before, or during sample preparation to prevent unnecessary delays between steps. **The PIP tubes can take up to 30 minutes to thaw on ice and are stable on ice for up to 5 hours.** The Capture and Lysis step only takes 10 minutes of hands-on-time after sample preparation is complete. After cells are lysed in the emulsions, they are stable at 20°C on the Dry Bath.

Customer provided RNase inhibitor (0.4–1 U/µl final concentration) may need to be added to any buffers during sample preparation being used for time-consuming steps (eg antibody staining buffers, buffers used in sorting collection tubes, and to the cell suspension during cell counting).

Including RNase inhibitor in upstream processes is especially important for challenging sample types that may have high endogenous RNase and/or low RNA content (eg neutrophils, eosinophils, adipose, pancreas, spleen, macrophages, plants, and so on). It is usually not necessary to add RNase inhibitor for cell wash steps unless working with granulocytes.

The Illumina Single Cell 3' RNA prep kits provide enough RNase inhibitor to add with the samples into the PIP tube during Capture and Lysis. **If there will be a delay between completing sample preparation and starting Capture and Lysis, RNase inhibitor should be added directly into the diluted cells instead of separately into the PIP tube.** It is not recommended to use DEPC in place of RNase inhibitor, as it required deactivating and is inhibitory if not fully washed from cells before starting the Illumina Single Cell workflow.

2.2 Illumina Single Cell Nuclei Isolation Kit

The Illumina Single Cell Nuclei Isolation Kit (formerly known as the Fluent V Nuclei Isolation kit) has been validated for frozen mammalian tissue.

Illumina Single Cell Nuclei Isolation Product Documentation

Product Documentation	Document #	Illumina Catalog #
Illumina Single Cell Nuclei Isolation Kit Product Documentation	200063146	20132795

Certain reagents and steps might be too harsh for use with other sample types. It is not recommended for use with non-mammalian sample types. The lysis incubation time, the number of steps using a Dounce homogenizer, and the centrifuge speed/time used to pellet nuclei might need to be adjusted for fresh mammalian tissue and we cannot guarantee performance. Contact support for further recommendations if you are interested in isolating nuclei from cell lines or cells in culture.

2.3 General recommendations for nuclei

Refer to the recommendations in the Illumina Single Cell Nuclei Isolation Kit Product Documentation for assessing nuclei quality before starting the Illumina Single Cell workflow. The quicker you can move from cells to encapsulated nuclei, the better. We recommend minimal centrifugation and making sure the preps remain ice cold the entire time. (Keep all buffers, reagents, equipment, consumables, and plasticware on ice throughout the isolation.)

When isolating nuclei for many samples, it is best to prep samples in small batches through Capture and Lysis (10–15 minutes of hands-on time), then use the stopping point to prep another batch of samples. The samples can then be processed together through the remaining Illumina Single Cell workflow steps to eliminate batch effects. Try to minimize the amount of time between completing nuclei isolation and starting Capture and Lysis. After isolation, nuclei will start to degrade and clump.

Be sure not to freeze and store isolated nuclei (it disrupts the nuclear membrane) unless you are fixing nuclei first according to our demonstrated DSP-Methanol Fixation Protocol for Nuclei. Refer to the Fixation section of this Training Packet for further information.

A high-quality nuclei suspension will have minimal debris and aggregates with intact membranes that are round and smooth. Nuclei with compromised membranes will appear disjointed, an indication of blebbing. For tissues with high amounts of debris, a smaller tissue input is recommended to reduce levels of debris. We recommend using live/dead fluorescent staining combined with size gating to determine the count and to use high magnification (60X) to check for blebbing.

For nuclei counting, we recommend AO/PI with size gating using the Luna FL Dual-Fluorescence counter by Logos. Refer to the Size gating recommendations section below.

Always conduct replicate counts to ensure accuracy. It is not recommended to use trypan blue, as debris can easily be counted as nuclei, which will overestimate the count (leading to a lower-than-expected capture rate), especially when size gating is not implemented.

2.4 Size gating recommendations (cells and nuclei)

Size gating recommendations using an automated fluorescent cell counter with various mouse tissue types:

	Minimum size	Maximum size
Nuclei	4 µm	11 µm (16 µm maximum size for brain)
Cells	11 µm	60 µm (would need to adjust for larger cell types)
PBMCs	6 µm	60 µm

2.5 Use of alternative nuclei isolations kits or protocols

Alternative nuclei isolation protocols:

- When using alternative nuclei isolation protocols, it is necessary to include final concentrations of 0.8 U/µl RNase inhibitor and 1X protease inhibitor into the nuclei extraction buffer/nuclei lysis buffer during tissue lysis. Failure to do so can result in degraded cDNA.
- After nuclei extraction is complete, centrifuge the nuclei suspension (preferably, 500 × g for 5 minutes at 4°C) to pellet and aspirate the supernatant. Use a P200 to carefully remove as much supernatant as possible without disturbing the pellet.

  ***Warning**: using a fixed angle centrifuge for pelleting nuclei for wash steps can result in excessive sample loss. Tubes compatible with swinging bucket centrifuges should be used to ensure a flat pellet.*

- Add 1 ml of Nuclei Suspension Buffer (NSB) working solution, mix well and spin at 500 × g for 5 minutes at 4°C.
- Next, aspirate supernatant without disturbing the pellet and resuspend nuclei using NSB working solution.

Note: centrifuge speeds/times listed above might need to be adjusted for non-mammalian sample types.

Customers can use other kits or alternative protocols for nuclei isolation, but the extra time-consuming steps to minimize large debris to prevent clogging of microfluidic devices (eg sucrose or other density gradients, magnetic bead purifications) can be harsh on the nuclei. This can increase nuclei leakage and prevent transcripts from paranuclear membranes from being captured.

Using filter steps is a gentle method for debris removal and is optimal for PIPseq technology, which has no instrument clogging issues from cellular or nuclear debris. After Dounce homogenizer steps, it is recommended to use two filter steps to remove debris. It is recommended to start with a 40 µm gravity filter. If the first filter step takes more than ~3 minutes, customers can optionally use an Uberstrainer with negative pressure for the first filter step (but this will let through more debris), then immediately follow with a 40 µm gravity filter step to remove any remaining debris, which should then go much quicker.

For the second filter step, it is recommended to use a smaller sized filter, such as a 10 µm Uberstrainer while applying negative pressure. For nuclei from mouse brain or other larger nuclei sample types, a 20 µm Uberstrainer may be used. The faster this step can be completed, the better quality the isolated nuclei will be. Using negative pressure is preferable to gravity filtration.

For customers using alternative nuclei isolation kits or protocols, the RNase inhibitor is customer supplied when adding it to the nuclei extraction buffer or when formulating the NSB working solution. The Illumina Single Cell 3' RNA kits only provide enough RNase inhibitor for use during Capture and Lysis.

We recommend using one of the compatible alternative RNase inhibitors listed in the 'User Supplied Consumables and Equipment' section of the product documentation. Protector RNase Inhibitor (40 U/µl) and RiboGrip RNase Inhibitor (220 U/µl) are optimal for nuclei, with RiboGrip being the best choice for challenging sample types, such as those with lower RNA content and/or higher endogenous RNase (eg plants).

We provide enough BSA in the kits for the final nuclei suspension, but if you would like to include BSA in the NSB used for wash steps (this is optional, and not used internally), you will need to purchase your own BSA (molecular-biology grade).

DSP-Methanol fixation is an alternative option that can be used to help protect the mRNA in isolated nuclei before the Illumina Single Cell workflow and is especially recommended if it is not possible to proceed immediately to Capture and Lysis following nuclei isolation.

2.6 Formulating Nuclei Suspension Buffer Working Solution

Refer to the 'Prepare NSB Working Solution' section of the Illumina Single Cell 3' RNA Product Documentation for instructions on how to formulate the NSB working solution using the NSB provided in the kits. This should be

formulated based on whether you plan to move forward with freshly isolated nuclei or if you plan to fix nuclei first using the DSP-Methanol Fixation for Nuclei Demonstrated Protocol. If proceeding with fixation, it is a prerequisite to use NSB working solution without BSA or RNase inhibitor for the wash steps and final resuspension before fixing the isolated nuclei according to the fixation protocol.

2.7 Fixation

We have two demonstrated DSP-Methanol Fixation Protocols. One is for fixing cells and the other is for fixing nuclei. Note: Illumina Single Cell 3' RNA Prep kits are not compatible with FFPE or formalin fixed samples.

DSP-Methanol Fixation Demonstrated Protocols

DSP-Methanol Fixation for Cells Protocol Instructions (Document # 200067419)

DSP-Methanol Fixation for Nuclei Protocol Instructions (Document # 200067424)

Cells and nuclei should be washed according to the Illumina Single Cell workflow to remove any residual proteins and resuspended in Fluent Cell Suspension Buffer or Nuclei Suspension Buffer (with no RNase inhibitor or BSA added). If there are any proteins remaining in the cell or nuclei suspension before fixation, it can form a precipitate with the methanol and cause fixation to fail.

Fixed cells may be stored for up to 7 days at -20°C and fixed nuclei may be stored for up to 2 months at -20°C. Fixation is helpful if you need to ship samples in dry ice to another location. When counting cells or nuclei after fixation, both cells and nuclei will stain red with AO/PI because the cell membrane is permeabilized. Refer to the recommendations for the final post-fixation count listed at the end of the fixation protocols. If there are some crystal structures remaining in the samples post-fixation, this is residual DSP and should not cause issues with the Illumina Single Cell workflow.

2.8 Shipping samples

It is recommended to fix cells or nuclei using the DSP-Methanol Fixation Demonstrated Protocol for cells or nuclei before shipping samples to another location for processing the Illumina Single Cell workflow. They should be rehydrated according to the protocol on arrival and just before starting Capture and Lysis.

2.9 Enrichment strategies (FACS, FANS, MACS)

Fluorescence-activated cell or nuclei sorting (FACS or FANS) and magnetic-activated cell sorting (MACS) can be beneficial for the enrichment of target cell populations and can facilitate the exclusion of dead or damaged cells. Enrichment steps also add a lot of time and can stress the cells causing an overall decrease in cell health. It might be necessary to do a second live/dead cell sort following cell sorting to ensure sufficient cell viability.

It is not recommended to move forward with cells that are less than 75% viable. Adding additional cell wash steps is best if only minor viability improvement is needed. If significant improvement is required, it is recommended to

either optimize sample preparation processes and reduce sample preparation time, or to incorporate a Dead Cell Removal Kit (eg Akadeum Life Sciences, Miltenyi Biotec). These kits require starting with a high number of cells (500k minimum cells for Akadeum and 1 M cells for Miltenyi).

It is common for FBS to be used in pre-sorting buffers and in collection buffers. As an alternative to FBS (which is inhibitory to PIPseq), BSA can be used in buffers and for tube blocking. It is critical to complete the cell or nuclei wash step according to the product documentation after sorting if the sorted samples contain any potentially inhibitory reagents such as FBS, as described in the Sample preparation section.

It is recommended for cells to be sorted in a collection tube containing a buffer with 0.4–1 U/µl of RNAse inhibitor. It is recommended for nuclei to be sorted into a collection tube with up to 2 U/µl of RNAse inhibitor and 1% BSA. This will help prevent clumping and degradation.

We do not recommend relying on cell counts or nuclei counts from sorting, as these are often overestimated and will not result in the expected capture rate from PIPseq. It is best practice to complete the wash step and to then count cells or nuclei after resuspending samples in the appropriate Fluent suspension buffer. **Note that each wash step can reduce the number of cells or nuclei by up to 50%** (note: excessive sample loss will occur if using a fixed angle centrifuge). Always use tubes compatible with swinging bucket centrifuges to ensure a flat pellet to minimize sample loss during wash steps and to prevent cell shearing).

If you are working with low cell or nuclei numbers and there will be no inhibitory reagents in the final suspension (refer to the Sample preparation section of this document for further information), a low volume of Cell Suspension Buffer or Nuclei Suspension Buffer may be used in the collection tube (200–400 µl). Be sure to include the recommended final concentrations of RNase inhibitor and BSA for the sample type you are working with as described in this section. The 1–2 ml wash step may be eliminated if there are no potential inhibitors, **but this increases the risk of additional background noise**. Do a post-enrichment count and determine the count for the entire tube. The cells or nuclei may then be concentrated by centrifugation to pellet the samples, then removing a safe volume of the supernatant so as not to disturb the cell or nuclei pellet. Make sure you have a sufficient volume of supernatant remaining in the tube to load into the PIP tube for the kit size you are using. Resuspend the samples and determine the new count by adjusting the tube count by the new final volume.

3. Illumina Single Cell Workflow

The workflow is an open system (does not rely on an enclosed microfluidic device), so the samples are more exposed to exogenous RNases that are in the environment or from people. Be sure not to touch any surfaces, reagents or consumables with bare hands, as most exogenous RNases come from the skin. All lab personnel should wear full PPE. Carefully review the 'Best Practices' section of the product documentation for important recommendations for working with RNA and with PCR products before starting your first experiment.

Review the workflow in the product documentation for workflow timing and safe stopping points. If no safe stopping point is listed, be sure to proceed immediately to the next step.

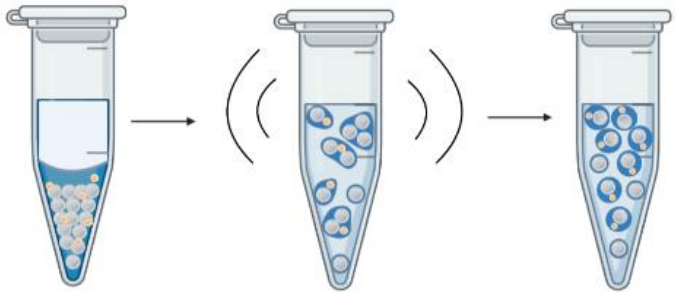
3.1 Video tutorials for key steps

It is highly recommended to view the short video tutorials for key steps in the workflow before starting your experiment.

Videos are available on the Illumina Single Cell Prep training page

All Kits: Capture and Lysis

At the beginning of the Illumina Single Cell workflow, cells or nuclei are loaded into a tube containing PIPs. An overlay of Partitioning Reagent is added and vortexing is used to subdivide this aqueous solution into smaller and smaller partitions, until each has captured a template particle that may or may not include a captured a single cell.



The PIPs in the tube during this step are viscous and can be sticky (similar to the consistency of honey). It is very important to use low retention pipette tips and proper pipetting technique when working with PIPs.

When mixing samples with the PIPs it is important to pipette mix slowly to the first stop only to avoid creating foam or excessive bubbles. After mixing, bring the pipette tip out of the liquid and go to the second stop to dispense any remaining liquid on the side wall of the tube. The samples will be further mixed during the subsequent vortexing steps, so light mixing is all that is needed for this step.

All Kits: Pelleting PIPs during wash steps

Depending on the kit size, certain wash steps use a benchtop mini microcentrifuge or "minifuge" to pellet the PIPs (eg USA Scientific, #2641-0016). The minifuge should be able to spin at speeds of ~2000 × g. If you observe PIPs that are floating or the PIPs pellet is not well packed, it is recommended to increase the amount of spin time. **Do not use speeds over 2000 × g.**

It is best practice to use the power button and to turn the power "off" to achieve a gradual brake after spinning to ensure a well-packed PIP pellet, before removing the supernatant. Using standard braking such as using the brake button or opening the lid can potentially disrupt the PIP pellet depending on the type of minifuge being used.

When removing the supernatant during wash steps, **keep the pipette tip towards the top of the liquid and follow the volume down to avoid PIP loss from aspirating too close to the PIP pellet**. The PIP pellet does not look like a cell pellet but is more translucent. It is recommended to check the PIP volume in the tube before and after each wash step and to make a note of any PIP loss. For T2 reactions, it is recommended to remove the 8-tube strip from the red guide rack to check the volume of the PIP pellet after the 1X washing steps and after the 0.5X washing steps.

3.2 Quality checks

cDNA QC

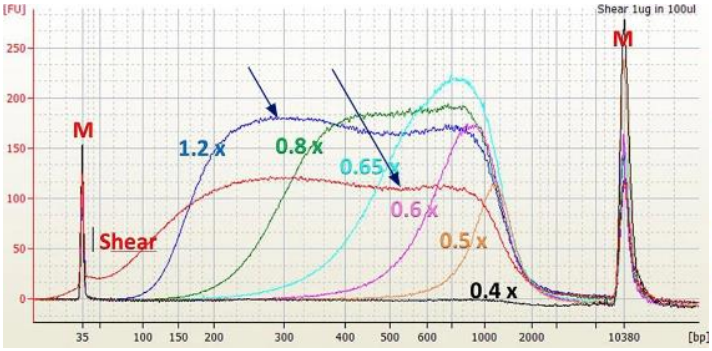
It is recommended to set the region table to 200–5k bp when determining average fragment size for the cDNA. If the average fragment size is below 500 bp, be sure to carefully review the next section on cDNA degradation. Reach out to Illumina Technical Support if further troubleshooting is needed.

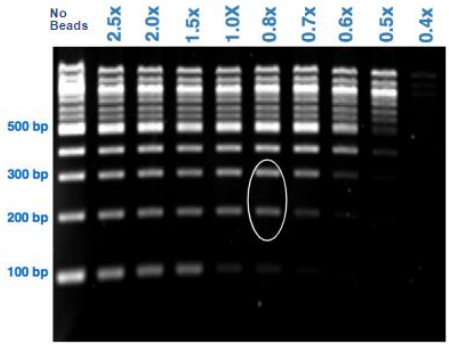
The Magnetic Bead Cleanup ratio used for size selection after generating cDNA during Whole Transcriptome Amplification is tuned to balance two different factors:

- Removal of short (low bp) contaminants (eg primer dimers, off-target primer interactions)
- Retaining captured mRNA, even from samples with lower RIN values

Note: Other single cell platforms use a 0.6x ratio, while the Illumina Single Cell workflow uses a 0.8x ratio. This results in fragment sizes between 200–400 bp that one would not see in other platform's cDNA QC traces to be retained.

This allows for more gene diversity to be retained through library prep, resulting in increased sensitivity metrics but with a slight reduction in sequencing efficiency. When comparing the average fragment size of the cDNA generated with the Illumina Single Cell workflow to the cDNA generated from other single cell platforms, the Illumina Single Cell workflow average fragment size is expected to be lower due to this difference.





Library QC

The final library average fragment size is expected to range between 370–550 bp when the region table is set from 200 to 800 bp.

If there are large adapter peaks remaining in the final library, these can significantly reduce sequencing efficiency. If these are present, it might be necessary to bring the sample volume up to 100 µl and to do an additional 0.8x SPRI-Select Size Selection (add 80 µl of SPRI-Select, not provided) to remove more of the smaller fragments from the library. This extra size selection is not recommended if the library prep concentrations are low. Contact support for additional information.

3.3 cDNA degradation

To prevent lower cDNA fragment sizes and yield, be sure to complete the following steps correctly during the Illumina Single Cell workflow:

1) Be sure to complete the 1–2 ml wash step during sample preparation to remove any potentially inhibitor reagents (eg FBS, ≥ 1% BSA, high calcium concentrations).

2) Be sure the PIPs are fully broken during the Breaking emulsions step and ensure **ALL the pink waste is removed.**

If these steps are completed correctly and cDNA degradation is still experienced (average fragment size < 500 bp), then it is likely that processes upstream of the Illumina Single Cell workflow require further optimization.

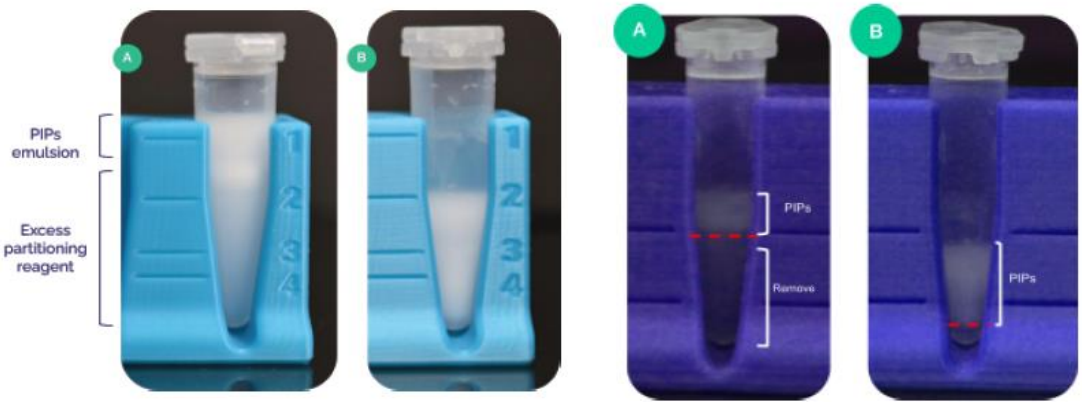
If cell viability was low, it might be necessary to increase wash steps to remove dead cells and debris. If wash steps are not sufficient to improve cell viability, try using live/dead cell sorting, or incorporate the use of a Dead Cell Removal Kit.

If cell viability was not an issue, then it is recommended to include RNase inhibitor during any time-consuming sample preparation steps by following the recommendations provided in the Sample preparation section of this Training Packet.

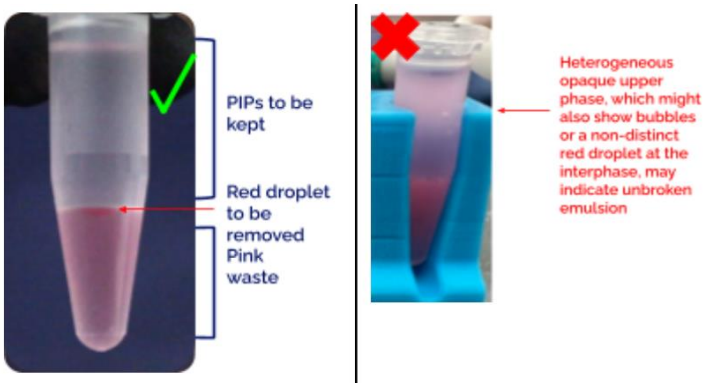
If cDNA degradation is experienced with nuclei that were not isolated using the Illumina Single Cell Nuclei Isolation Kit, it is recommended to review the recommendations listed in the Use of alternative nuclei isolation kits or protocols section of this Training Packet and to adjust the protocol you are using accordingly. Please contact support if further troubleshooting is needed.

3.4 Breaking emulsions

When removing excess partitioning reagent during step 2 of 'Break Emulsions' in the product documentation, be sure to remove **as much of the bottom phase as possible** without disturbing the upper PIPs + Cells phase. If PIP loss is experienced during the workflow, it might be necessary to go below the marker line on the tube stands. To prevent PIP loss, please refer to the Video tutorials for key steps. No more than a few µl of excess partitioning reagent should be remaining in the tube before breaking (refer to example images below).



Following the inversion and centrifuge steps there should be two distinct phases. There should not be a gradient between the two phases or a dispersed red droplet. An upper phase that is heterogenous and showing small bubbles is another sign of unbroken emulsions.



If any samples are showing signs of unbroken PIPs, it could indicate there was too much partitioning reagent remaining in the tube before breaking or that there was more than 1% BSA in the final cell or nuclei suspension. Customers should add more de-partitioning reagent (pink) as instructed in the Illumina Single Cell 3' RNA Prep

Product Documentation and repeat the inversion and centrifuge steps to ensure that the emulsions are completely broken.

Important: When the emulsions are fully broken, it is critical to remove all pink waste, including the red droplet. The pink waste contains harmful organics that are inhibitory to downstream enzymatic steps such as reverse transcription, which can result in lower cDNA yield and fragment sizes. Be sure to complete the second centrifuge step and use good lighting to make sure the pink waste has been removed, even if you need to aspirate a few µl of the clear aqueous phase in the process. This will not reduce sensitivity, as the sample in the upper phase is diluted during the breaking emulsions step.

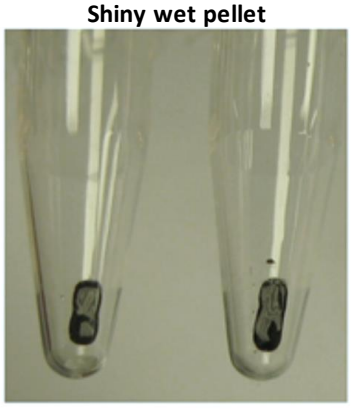
3.5 Magnetic cleanup bead purifications

Insufficient mixing of sample and Magnetic Cleanup Beads will lead to inconsistent size selection results. Make sure to mix well.

During the air-dry step to remove residual ethanol, immediately resuspend the magnetic bead pellet as soon as it only has a slight gloss remaining. When the magnetic bead pellet no longer looks shiny, it can quickly become cracked (overdried). An overdried bead pellet can trap cDNA in bead clumps that are hard to break up, which can significantly reduce the elution efficiency.

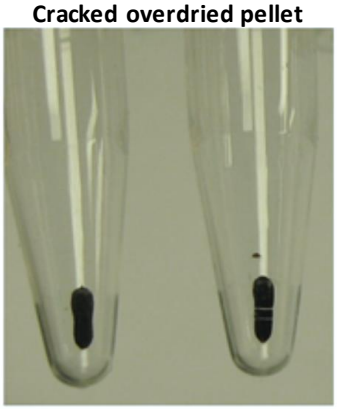
Workflow tip: If beads are accidentally overdried and showing cracks, after resuspending the beads in IDTE buffer, use a multi-channel pipette to mix up and down periodically (every 30 seconds or so) during the 5 minute incubation off the magnetic rack. This helps to break up any clumps that might be trapping cDNA.

Shiny wet pellet



Matte dry pellet

Cracked overdried pellet



4. Illumina Single Cell Supplemental Enrichment and Amplification (SEA) Kit

4.1 PIPseq Replay

The Illumina Single Cell Supplemental Enrichment and Amplification (SEA) Kit (formerly known as the PIPseq Supplemental Enrichment and Amplification Kit) was designed to facilitate custom single-cell RNA-seq applications with Illumina Single Cell 3' RNA Prep kits including the unique opportunity for sample recovery after three months of storage through re-amplification from retained hydrogel bead-bound cDNA. During the Illumina Single Cell workflow, if the cDNA passes the QC step, but something goes wrong during library preparation and one or more of the libraries failed the library QC, it is possible to re-amplify cDNA from the PIPs pellet stored at -80°C using the Illumina Single Cell SEA Kit. The re-amplified cDNA may then be used with the Illumina Single Cell 3' RNA library kit to generate new libraries. Contact Illumina Support for further information.

4.2 Cell labeling applications with the SEA kit

Begin by labeling the cells or cellular proteins of interest with **polyadenylated** synthetic nucleotide tags (SNTs) using a preferred protocol (not provided). These labeled cells are then used as input into one of the standard Illumina Single Cell 3' RNA Prep kits (T2, T10, T20, T100). Refer to the recommendations listed in the Experimental design section for determining the kit size that is best for your application needs. It is important to note that staining protocols for labeling cells with SNTs can increase background noise by up to 30%.

Follow the standard Illumina Single Cell workflow to generate 3' gene expression libraries according to the instructions in the Illumina Single Cell 3' RNA Prep Product Documentation linked in the Planning your experiment section of this Training Packet. During the "Isolate cDNA from PIPs" step of the standard Illumina Single Cell workflow, a PIPs pellet is generated for each sample being processed, which is saved at -80°C. This PIPs pellet is then used as input into the Supplementary Amplification and Enrichment of SNTs workflow, according to the instructions in the supplementary user guides linked below. This supplementary protocol is used for targeted amplification of the SNT-derived cDNA, which is then used for SNT library preparation. Customers will then pool and sequence their gene expression and SNT libraries together. There should be no overlapping index combinations for any of the libraries being pooled together.

What is needed:

Purchase at least one Illumina Single Cell 3' RNA Prep Kit (T2, T10, T20, T100) and at least one Illumina Single Cell Supplemental Enrichment and Amplification (SEA) Kit. More kits might be needed depending on the kit size and number of samples being processed. You will also need to supply the polyadenylated SNTs being used to label the cells or cellular proteins of interest and to order the oligos used to amplify their SNTs from the PIPs pellet. Finally, order the Index Adapters used for the Index SNT Samples step for the SNT library. Review the "Oligonucleotides" section of the SNT User Guides linked below for further information.

Single Cell RNA Synthetic Nucleotide Tag Enrichment Product Documentation

Illumina Single Cell RNAT2 Synthetic Nucleotide Tag Enrichment Product Documentation (Doc # 200064127)

Illumina Single Cell RNAT10 Synthetic Nucleotide Tag Enrichment Product Documentation (Doc # 200064128)

Illumina Single Cell RNAT20 Synthetic Nucleotide Tag Enrichment Product Documentation (Doc # 200064129)

Illumina Single Cell RNAT100 Synthetic Nucleotide Tag Enrichment Product Documentation (Doc # 200064130)

4.3 sgRNA Applications with the SEA Kit

CROPseq is a powerful method for identifying the roles of thousands of genes simultaneously. Users will generate perturbed cells using **polyadenylated** single guide RNAs (sgRNA) using a preferred protocol (not provided). These modified cells are then used as input into one of the standard Illumina Single Cell 3' RNA Kits (T2, T10, T20, T100). Please refer to the recommendations listed in the Experimental design section for determining the kit size that is best for your application needs.

Follow the standard Illumina Single Cell workflow to generate 3' gene expression libraries according to the instructions in the Illumina Single Cell 3' RNA Prep Product Documentation linked in the Planning your experiment section of this Training Packet. During section 5.5.1 of the standard PIPseq workflow, a PIPs pellet is generated for each sample being processed, which is saved at -80°C. This PIPs pellet is then used as input into the Supplementary Enrichment of sgRNA workflow, according to the instructions in the supplementary user guides for the SEA kit linked below. This supplementary protocol is used to amplify sgRNA-derived cDNA. The sgRNA-derived cDNA will then be used as input for library preparation. Then, pool and sequence the gene expression and sgRNA libraries together. There should be no overlapping index combinations for any of the libraries being pooled together.

What is needed:

Purchase at least one Illumina Single Cell 3' RNA Kit (T2, T10, T20, T100) and at least one Illumina Single Cell Supplemental Enrichment and Amplification (SEA) Kit. More kits might be needed depending on the kit size/number of samples for your project. Order the oligos used to amplify the cDNA generated from the captured sgRNAs from the PIPs pellet (refer to the "Oligonucleotides" section in the sgRNA user guides for further information). The Illumina Single Cell UD Index (96 index, 96 samples, sold separately) can be used for sgRNA Library Preparation except when modifications are made to the PE2 Nextera – U6 site reverse primer sequence, in which case custom unique dual indices must be ordered.

Illumina Single Cell RNA Guide RNA Enrichment Product Documentation

Illumina Single Cell RNAT2 Guide RNA Enrichment Product Documentation (Doc # 200064131)

Illumina Single Cell RNAT10 Guide RNA Enrichment Product Documentation (Doc # 200064132)

Illumina Single Cell RNAT20 Guide RNA Enrichment Product Documentation (Doc # 200064133)

Illumina Single Cell RNAT100 Guide RNA Enrichment Product Documentation (Doc # 200064134)

5. Sequencing

If working with a sequencing core facility, it can be helpful to share the following information and to provide them with a copy of the product documentation, if needed.

5.1 Sample indexing for sequencing

Remember to document which samples are used with each index combination. There should be a unique index combination used for every library that will be pooled together in a sequencing lane or run. Refer to the Illumina Single Cell RNA Indexes section of the Illumina Adapter Sequences documentation to prepare sample sheets for sequencing. Sequencing core facilities and sequencing centers typically request a sample sheet to be populated with the sequences from the "i7 sequence for sample sheet" and "i5 adapter sequence" columns. If preparing a sample sheet to use with BCL convert, the rev comp of the i5 adapter column might be required. Please refer to Illumina guidance on sample sheets.

The "i7 adapter sequence" column is provided because those are the sequences used to fill in the Xs for the Library P7 Index Adapter. (Refer to "Illumina Single Cell RNA Indexes" section of the Illumina Adapter Sequences documentation)

Shipping date	Component name	i7 sequence (for sample sheet)	i7 adapter sequence
Before 2025	UDI Library Index Mix 2	TAGAATTGGA	TCCAATTCTA
From January 6, 2025	UDI Library Index Mix 2	AGAGGCAACC	GGTTGCCTCT

Note: The UDI Library Index Mix 2 sequences were updated on January 6, 2025.

The Illumina Single Cell Unique Dual Indexes set offers 96 unique index combinations that can be used for the Sample Index PCR step. This kit is required for customers that want to pool more than 8 libraries together in a single sequencing lane or run. The index sequences provided in the Illumina Single Cell UD Kit match the index sequences in the Illumina UD Index Plate A. (Note that the PCR handles are different, so these kits are not interchangeable.)

It is important to note that the 8 index combinations provided in the T2, T10, T20, and T100 kits are identical to column 3 of the Illumina Single Cell UD Kit. Be sure not to mix and match these index combinations for samples sequencing in the same lane or sequencing run.

5.2 Sequencing read depth

T2 Kit	T10 Kit	T20 Kit	T100 Kit
20,000 reads per input cell	20,000 reads per input cell	20,000 reads per input cell	20,000 reads per input cell
• 5000 cells input	• 17,000 cells input	• 40,000 cells input	• 200,000 cells input
• 100M reads per T2	• 340M reads per T10	• 800M reads per T20	• 4 billion reads per T100

After the first run with a particular sample type and kit, you can evaluate the capture rate and sequencing saturation metrics to determine if the sequencing depth can be adjusted in future experiments based on your sample type and specific experiment needs.

5.3 Sequencing read length

Read 1 must be at least 45 cycles and Read 2 must be at least 72 cycles. It is recommended to maximize the cycle number for Read 2 (cDNA read) if you are using a 150-cycle paired-end kit.

If you are sequencing libraries using 100 cycle kits (containing reagent overage for 138 cycles max) use the recipe shown below:

45 + 10 + 10 + 72 = 137 cycles total

It is important to tell the core lab to not use any trimming during or after sequencing (**no QC or adapter trimming when converting BCL to fastq.gz files)**, as this will result in barcode errors and the data will require re-analysis. The single-cell analysis software does not require trimmed fastq.gz files.

5.4 Sequencing recommendations

It is recommended for the sequencing core lab to pool libraries together from all experimental conditions before single-cell sequencing with an Illumina sequencing system, as this will minimize batch effects and can help prevent color balancing issues with the indexes being used.

It is required to add a minimum of 1% PhiX in the final library loading pool. This should be increased to a minimum of 2% PhiX if a NovaSeq X Series instrument will be used. Increased PhiX concentrations are needed when pooling PIPseq libraries together with other library types due to the limited base diversity of Read 1 starting at cycle 46 (poly A region).

The final library loading concentrations listed below are used by our internal team for sequencing Illumina Single Cell 3' RNA Prep libraries. These concentrations may need to be adjusted when combined with other library types:

6. Data analysis

Illumina provides various options for secondary and tertiary analysis of Single Cell Library Prep data.

DRAGEN Secondary Analysis

The DRAGEN Single Cell RNA App is available in the cloud computing environments Illumina Connected Analytics (ICA) and BaseSpace™ Sequence Hub (BSSH). It is not available for DRAGEN onboard NovaSeq™ X Series or NextSeq™ 1000/2000 instruments currently.

Learn more: Illumina DRAGEN Secondary Analysis Online Help

Partek Flow software

For the tertiary analysis of Illumina Single Cell RNA Prep data, we recommend customers use Partek Flow. Partek Flow can utilize the sparse matrix output from the DRAGEN Single Cell RNA App v4.4 and PIPseeker. Partek Flow offers multiple methods for differential expression, cell clustering, dimension reduction and cell typing. The solution is available as Illumina-hosted or customer-deployed configurations.

Learn more: Partek Flow software

PIPseeker

Customers are recommended to transition to the DRAGEN Single Cell RNA App v4.4 which has faster analysis time and supports secondary analysis for all Illumina Single Cell RNA Preps (formerly Fluent PIPseq™ V products).

There will be no further development, improvements or bug fixes to the existing PIPseeker software, which will be hosted on the Fluent BioSciences website until the last available download date.

Below is a link where you can explore example PIPseq data sets, download the PIPseeker tutorial, and the PIPseeker User Guide. Note that at least 20 GB of available RAM are needed to complete the tutorial. Much higher

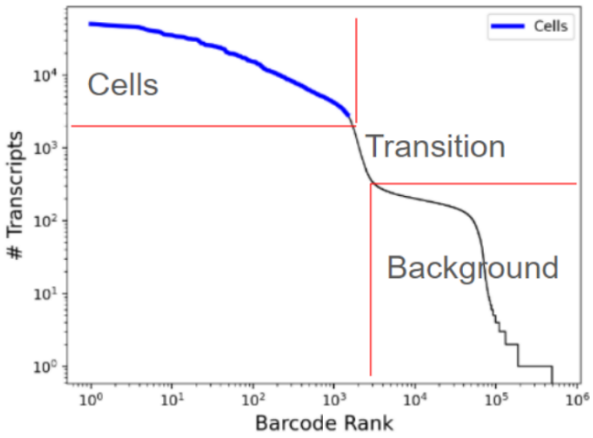
computing resources are required for analyzing the full data set. (Refer to the PIPseeker User Guide to calculate the computing resources required.) This usually requires working through a supercomputing cluster.

https://www.fluentbio.com/products/pipseeker-software-for-data-analysis/

Choosing sensitivity levels in PIPseeker:

Sensitivity levels are a feature that's unique to PIPseeker. The different sensitivity levels give you the ability to include or exclude more of the cell barcodes in the transition zone between cells and background. You should have gene expression matrices corresponding to all 5 sensitivity levels, so feel free to explore the different data sets. A sensitivity level should be chosen for each sample separately depending on the shape of the barcode rank plot and where the transition zone falls.

- If you work with homogenous cell populations (eg cell lines), calling to the "knee" of the rank plot is generally advised (blue line extends to the top of the transition zone as shown below).
- For heterogeneous populations, a general heuristic is to identify the "knee" of the rank plot, and then call ~30% more barcodes than the number identified at the knee point (this will usually lead to calling ⅓ to ½ of the way down the "cliff" of the rank plot, in the region labeled "Transition" zone below.



The filtered matrix files from the sensitivity level chosen for each sample may be used as input for tertiary analysis using other programs such as Seurat, Partek, or BioTuring. Note that PIPseeker data is not compatible with other single cell platform analysis tools such as Cell Ranger.

Resources

General Support

Illumina Technical Support
For information about products and services, answers to questions about technology, or to log any issues you may be experiencing.

Illumina Knowledge
For FAQs, troubleshooting articles, and reference material for products and workflows.

Illumina Single Cell Prep Support

Illumina Single Cell Prep Support
For product documentation and training on the Illumina Single Cell Prep workflow.

Note: For information on how to use Illumina Single Cell Prep accessories, such as starter equipment, refer to the reference guides available on the Fluent Biosciences website.

Index adapters

Index Adapters Pooling Guide
For UDI strip pooling strategies and UDI 96 plate pooling strategies.

Illumina Adapter Sequences
For the Index Adapter Sequences Guide.

Data analysis

Illumina DRAGEN Secondary Analysis Online Help

DRAGEN Single Cell RNA App

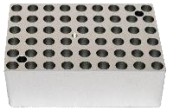
Partek Flow software

PIPseeker

Appendix

Starter Equipment Kit

Component name	Used for kit size	Image
PIPseq Vortex Mixer	All	
PIPseq rotating vortex assembly for 5 ml tubes	T100	
PIPseq rotating vortex assembly for 1.5 ml tubes	T20	
PIPseq rotating vortex assembly for 0.5 ml tubes	T2 & T10	
PIPseq Dry Bath with heated lid, which includes the following materials: • 2.5 mm Allen key wrench, silver • PIPseq Dry Bath Stylus • Replacement tips for PIPseq Dry Bath Stylus • 5x20 mm 5A 250V Fast blow fuse for PIPseq Dry Bath • Dry block removal tool, white	All	
PIPseq Dry Bath 5 mL block	T100	

PIPseq Dry Bath 1.5 mL block	T20	
PIPseq Dry Bath 0.5 mL block	T2 & T10	
PIPseq Combination 1.5 ml and 0.5 ml tube stand, blue	T2, T10, T20	
PIPseq 8-tube stand, for 0.2 ml tubes, black	T2	
PIPseq guide rack, red	T2	

Starter Equipment reference guides

PIPseq Vortexer Adapter Installation User Guide (Doc ID: 200064267)

PIPseq Vortex Mixer Operating Instructions (Doc ID: 200064269)

Dry Bath Incubator with Heated Lid Operation Manual (Doc ID: 200064268)

Recommended indexes for pooling ≤ four-plex using UDI Mix Strip

It is recommended to pool at least three Illumina Single Cell 3' RNA Prep libraries together when using the NovaSeq X series or the NextSeq 2000 sequencing instruments with the latest XLEAP chemistry. Refer to the table below for index combinations recommended to ensure proper color balancing according to Illumina guidelines based on the instrument that will be used for sequencing. Not all combinations have been experimentally validated.

Sample Name	Index Combination to Use	Instrument(s)
Sample 1	UDI Library Index mix 5	Only three-plex option for NovaSeq X Series
Sample 2	UDI Library Index mix 6	
Sample 3	UDI Library Index mix 7	
Sample 1	UDI Library Index mix 1	Three-plex option for NextSeq 2000
Sample 2	UDI Library Index mix 2	

Sample 3	UDI Library Index mix 3	
Sample 1	UDI Library Index mix 4	Three-plex option for NextSeq 2000
Sample 2	UDI Library Index mix 5	
Sample 3	UDI Library Index mix 6	
Sample 1	UDI Library Index mix 6	Three-plex option for NextSeq 2000
Sample 2	UDI Library Index mix 7	
Sample 3	UDI Library Index mix 8	
Sample 1	UDI Library Index mix 1	Four-plex option for NovaSeq X Series or NextSeq 2000
Sample 2	UDI Library Index mix 2	
Sample 3	UDI Library Index mix 3	
Sample 4	UDI Library Index mix 4	
Sample 1	UDI Library Index mix 5	Four-plex option for NovaSeq X Series or NextSeq 2000
Sample 2	UDI Library Index mix 6	
Sample 3	UDI Library Index mix 7	
Sample 4	UDI Library Index mix 8	

Recommended indexes for pooling two-plex through nine-plex using UDI Plate

It is recommended purchase the UDI Index Plate (20132788) for pooling > 8 libraries together. Refer to the Illumina Single Cell Unique Dual Indexes (96 Indexes, 96 Samples) of the Illumina Adapters Pooling Guide for all recommendations.

Revision history

Version	Date	Summary of Changes
00	January 2025	Initial release.

01	April 2025	• Added Starter Equipment Kit details to Appendix.
		• Aligned content with updated product documentation:
		  ○ Illumina Single Cell 3’ RNA Prep, T2 Product Documentation (Document # 200063178)
		  ○ Illumina Single Cell 3’ RNA Prep, T10 Product Documentation (Document # 200063182)
		  ○ Illumina Single Cell 3’ RNA Prep, T20 Product Documentation (Document # 200063183)
		  ○ Illumina Single Cell 3’ RNA Prep, T100 Product Documentation (Document #200063184)
		  ○ Illumina Single Cell Nuclei Isolation Product Documentation (Document # 200063146)
		  ○ DSP-Methanol Fixation for Cells Protocol Instructions (Document # 200067419)
		  ○ DSP-Methanol Fixation for Nuclei Protocol Instructions (Document # 200067424)
		  ○ Illumina Single Cell RNAT2 Guide RNA Enrichment Product Documentation (Document # 200064131)
		  ○ Illumina Single Cell RNAT10 Guide RNA Enrichment Product Documentation (Document # 200064132)
		  ○ Illumina Single Cell RNAT20 Guide RNA Enrichment Product Documentation (Document # 200064133)
		  ○ Illumina Single Cell RNAT100 Guide RNA Enrichment Product Documentation (Document # 200064134)
		  ○ Illumina Single Cell RNAT2 Synthetic Nucleotide Tag Enrichment Product Documentation (Document # 200064127)
		  ○ Illumina Single Cell RNAT10 Synthetic Nucleotide Tag Enrichment Product Documentation (Document # 200064128)
		  ○ Illumina Single Cell RNAT20 Synthetic Nucleotide Tag Enrichment Product Documentation (Document # 200064129)
		  ○ Illumina Single Cell RNAT100 Synthetic Nucleotide Tag Enrichment Product Documentation (Document # 200064130)
		• Updated UDI Library Index Mix 2 to the correct i7 adapter sequence.
		• Added Resources section with support links.