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

Download and review the compatible Illumina Single Cell 3’ RNA Prep (formerly known as PIPseq V) user guides applicable to your purchase. **Before each experiment, make sure to download the latest revision of the user guide.** The revision number is listed under “Doc ID” on the title page.

Illumina Single Cell 3’ RNA Prep user guides

User guide	Doc ID	Illumina catalog #
Illumina Single Cell 3’ RNA Prep, T2 / PIPseq V T2 3’ Single Cell RNA Kit User Guide	FB0005260	20135689
Illumina Single Cell 3’ RNA Prep, T10 / PIPseq V T10 3’ Single Cell RNA Kit User Guide	FB0004762	20135691
Illumina Single Cell 3’ RNA Prep, T20 / PIPseq V T20 3’ Single Cell RNA Kit User Guide	FB0005261	20135692
Illumina Single Cell 3’ RNA Prep, T100 / PIPseq V T100 3’ Single Cell RNA Kit User Guide	FB0005262	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 that you have received the materials shipped to you as outlined in the ‘PIPseq Platform Overview’ section of the user guide and that they are stored at the proper temperatures. Be sure to remove the Breaking Buffer, Washing Buffer, and Fluent Magnetic Cleanup Beads from the Capture & Barcoding Ambient Kit box and store at 2°C to 8°C.

Step 4

Make sure that you have all the necessary third-party equipment and materials as outlined in the ‘Required Third-Party Reagents’, ‘Consumables’, and ‘Equipment’ sections of the user guide.

Step 5

If your Illumina Single Cell 3’ Prep libraries will be sequenced with 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 of this document before starting your experiment to determine the UDI Library Index Mixes (1–8) to use for proper color balancing when pooling ≤ 4 libraries together in a single lane or sequencing run with these specific instruments. Improper color balancing can result in difficulties in demultiplexing the data.

Step 6

It is important to start Capture and Lysis as soon as possible after preparing your final cell or nuclei suspension (there is no safe stopping point between these two steps). Therefore, it is important to have the equipment prepared before starting any sample preparation processes for your experiment.

Review the ‘PIPseq Equipment Preparation’ section of the user guide. 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 according to the user guide instructions before starting your experiment.

It is also recommended to review the ‘PIPseq Dry Bath Operation’ instructions in the user guide to learn how to change between the different Dry Bath Protocols and how to manually set the lid mode for each program (refer to User Guide section 3.2.3). 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 in the bottom, left corner of the editing screen to toggle to the correct lid mode setting. The “**+5.0**” setting is used for the Cell Lysis (program A), the “**105**” setting is used for both the Nuclei Lysis (program B) and the cDNA Synthesis (program C). Select “**Save/Return**” to return to the operation screen. Also, 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).

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.

- The use of a T2 kit (captures ≥ 2000 cells or nuclei) is appropriate for homogenous or enriched samples (eg identification of subpopulations ≥ 20% of all cells or nuclei).
- The use of a T10 kit (captures ≥ 10k cells or nuclei) is appropriate for less homogenous or enriched samples (eg identification of subpopulations ≥ 10% of all cells or nuclei).
- The use of a T20 kit (captures ≥ 20k cells or nuclei) is more appropriate for rare subpopulations without using prior enrichment steps which can be damaging to cell health (eg identification of subpopulations ≥ 5% of all cells or nuclei).
- The use of a T100 kit (captures ≥ 100k cells or nuclei) is most appropriate for even rarer subpopulations (eg identification of subpopulations ≥ 1% of all cells or nuclei), as the additional cells or nuclei captured would make the rare cell population more likely to cluster separately from the other cell types.

1.2 Importance of pilot studies

PIPseq technology is optimized for efficient and sensitive transcript capture from live cells, and therefore, 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 kit along with the T10, T20, or T100 kits planned for the actual experiment, 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. Users should closely follow the user guide instructions to pellet their cells or nuclei and to complete a wash step before resuspending in the appropriate Fluent 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 Fluent Cell or Nuclei Suspension Buffer).

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. Therefore, 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 or in a sterile environment at room temperature.

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 User Guide

User guide	Doc ID	Illumina catalog #
Illumina Single Cell Nuclei Isolation Kit / Fluent V Nuclei Isolation Kit User Guide	FB0005260	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)
PMBCs	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 1X Nuclei Suspension Buffer (1X NSB), mix well and spin at 500 × g for 5 minutes at 4°C.
- Next, aspirate supernatant without disturbing the pellet and resuspend nuclei using 1X NSB.

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 1X Nuclei Suspension Buffer. The Illumina Single Cell 3' RNA kits provide enough RNase inhibitor to add with the samples into the PIP tube during Capture and Lysis.

We recommend using one of the compatible alternative RNase inhibitors listed in the 'PIPseq-Compatible Alternative RNase Inhibitors' section of the PIPseq V User Guides. 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 1X 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 1X Nuclei Suspension Buffer

Refer to PIPseq V User Guide 'Nuclei Suspension Buffer Preparation' section for instructions on how to formulate 1X Nuclei Suspension Buffer (1X NSB) using the 6X 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 1X NSB with no 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*

Demonstrated Protocol DSP-Methanol Fixation for Cells (Doc ID: FB0004708)

Demonstrated Protocol DSP-Methanol Fixation for Nuclei (Doc ID: FB0004745)

**Note: On the Resources page, you may need to navigate to page 2 or later.*

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 1X 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 user guide 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 1X 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. 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 user guides for important recommendations for working with RNA and with PCR products before starting your first experimen..

The ‘Protocol Timing’ section in the ‘Introduction’ section of the user guide lists all the safe stopping points during the workflow. If no safe stopping point is listed, be sure to proceed immediately to the next step. The ‘Hands-on-Time’ column on the right will tell you how long it will take to get to the next safe stopping point to help you plan out your day.

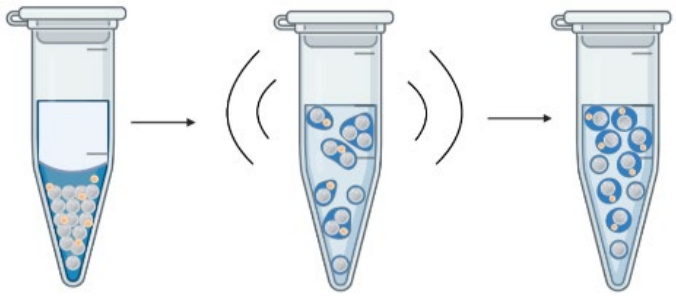
3.1 Video tutorials for key steps

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 using a standard bore, **low-retention** pipette tip, 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

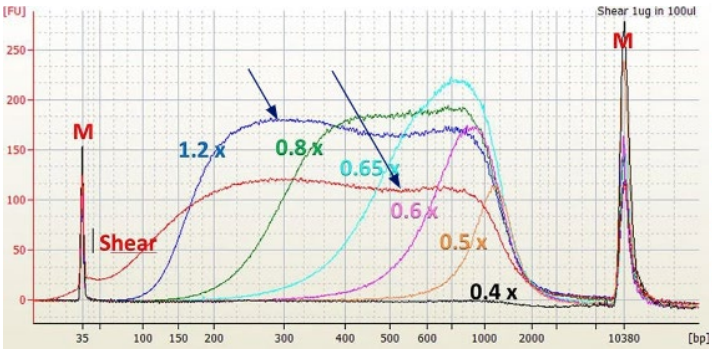
It is recommended to set the region table to 200–5k bp when determining average fragment size for the cDNA. If the average fragment size is below 500 bp, be sure to carefully review the next section on cDNA degradation. Reach out to Illumina Technical Support if further troubleshooting is needed.

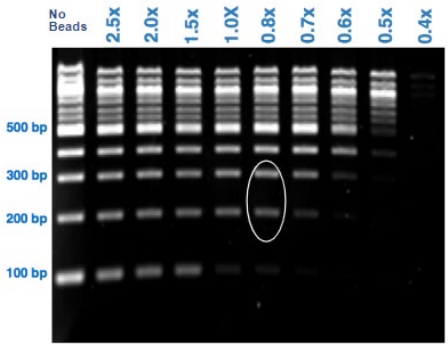
The Magnetic Bead Cleanup ratio used for size selection after generating cDNA during Whole Transcriptome Amplification is tuned to balance two different factors:

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

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 wouldn't 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.

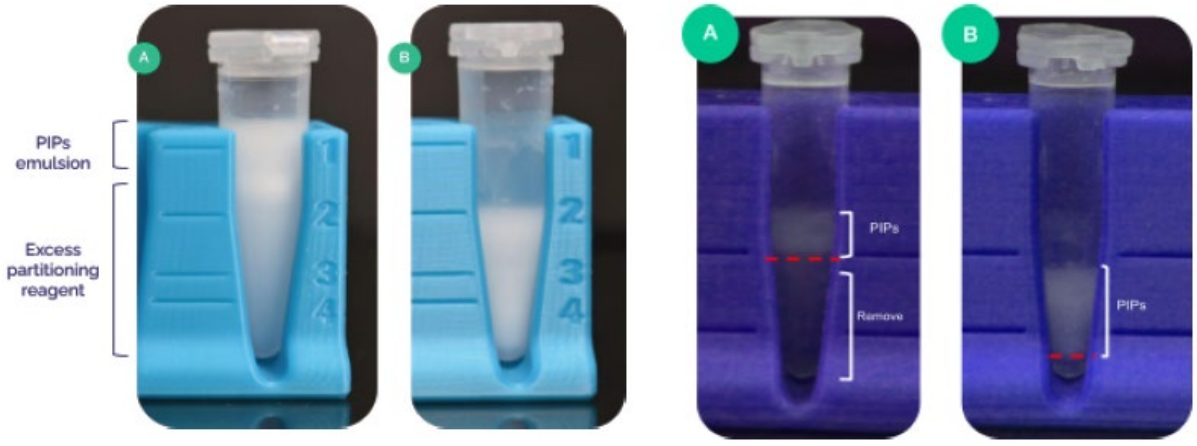
If cell viability was low, it might be necessary to increase wash steps to remove dead cells and debris. If wash steps are not sufficient to improve cell viability, try using live/dead cell sorting, or incorporate the use of a Dead Cell Removal Kit.

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.

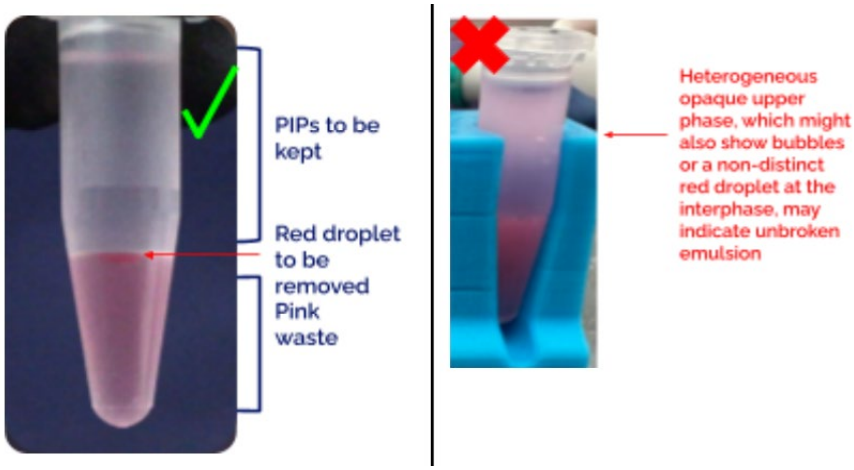
If cDNA degradation is experienced with nuclei that were not isolated using the Illumina Single Cell Nuclei Isolation Kit, it is recommended to review the recommendations listed in the Use of alternative nuclei isolation kits or protocols section of this Training Packet and to adjust the protocol you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

3.4 Breaking emulsions

When removing excess partitioning reagent during step 2 of 'Breaking Emulsions' in the user guide, 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 User Guides 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.

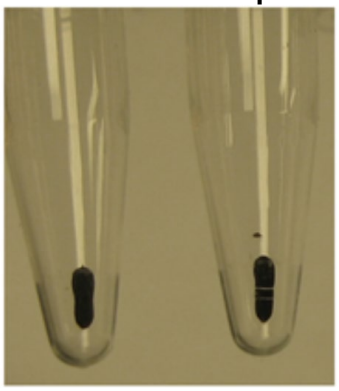
During the air-dry step to remove residual ethanol, immediately resuspend the magnetic bead pellet as soon as it only has a slight gloss remaining. When the magnetic bead pellet no longer looks shiny, it can quickly become cracked (overdried). **The air-dry step typically only takes 2 minutes in strip tubes and 0.5 ml tubes and up to 3 minutes in 1.5 ml tubes.** 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 five 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.

PIPseq Replay User Guide

Illumina / PIPseq Replay User Guide (Doc ID: FB0005446)

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 User Guides linked in the Planning your experiment section of this Training Packet. During section 5.5.1 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 Sample Index PCR step for the SNT library. Review Section 1 of the SNT User Guides linked below for further information.

Supplementary user guides used to prepare SNT libraries:

Illumina / PIPseq V T2 Supplemental Enrichment of Synthetic Nucleotide Tags Kit User Guide (Doc ID: FB00005389)

Illumina / PIPseq V T10 Supplemental Enrichment of Synthetic Nucleotide Tags Kit User Guide (Doc ID: FB0005390)

Illumina / PIPseq V T20 Supplemental Enrichment of Synthetic Nucleotide Tags Kit User Guide (Doc ID: FB0005391)

Illumina / PIPseq V T100 Supplemental Enrichment of Synthetic Nucleotide Tags Kit User Guide (Doc ID: FB0005392)

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 User Guides 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 section 1 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.

Supplementary user guides used to prepare the sgRNA libraries

Illumina / PIPseq V T2 Supplemental Enrichment of sgRNA Kit User Guide (Doc ID: FB00005393)

Illumina / PIPseq V T10 Supplemental Enrichment of sgRNA Kit User Guide (Doc ID: FB00005394)

Illumina / PIPseq V T20 Supplemental Enrichment of sgRNA Kit User Guide (Doc ID: FB00005393)

Illumina / PIPseq V T100 Supplemental Enrichment of sgRNA Kit User Guide (Doc ID: FB00005393)

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 user guide, 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 PIPseq 3’ Index Sequences table in the user guides to prepare sample sheets for sequencing. Sequencing core facilities and sequencing centers (eg NovoGene) 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 Oligonucleotide Sequences table at the end of the Illumina / PIPseq V User Guides.)

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	CTAATGATGG

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.) Refer to the Illumina pooling recommendations for the Illumina UD Indexes Set A when planning your experiment.

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

The recommended sequencing read depth for the T2, T10, and T20 kits is 20,000 reads per input cell.

- For T2, if you load 5K cells, you will need 100 M reads per sample.
- For T10, if you load 17K cells, you will need 340 M reads per sample.
- For T20, if you load 40K cells, you will need 800 M reads per sample.

The recommended sequencing read depth for the T100 kit is 10,000 reads per input cell.

- For T100, if you load 200K cells, you will need 2 B reads per sample.

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:

- NextSeq 500/550 recommended final library loading concentration 1.6 pM including ≥ 1% PhiX
- NextSeq 2000 recommended final library loading concentration: 550 pM including ≥ 1% PhiX.
- NovaSeq 6000 final library loading concentration 210 pM including ≥ 1% PhiX (equivalent to Pooled Loading Concentration of 1.05 nM).
- NovaSeq X Series final library loading concentration 190 - 200 pM including ≥ 2% PhiX.

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 about DRAGEN secondary analysis

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 about 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.

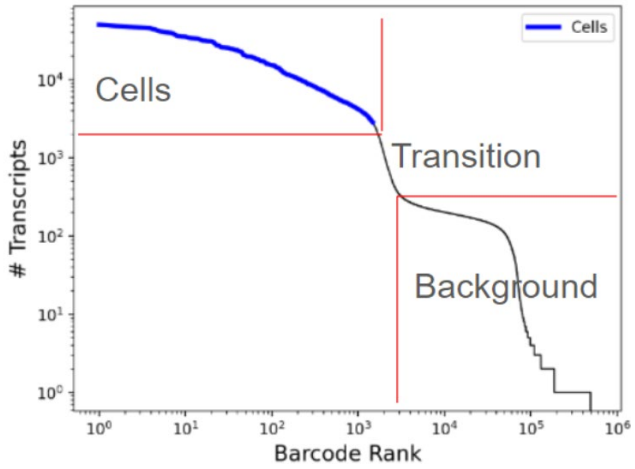
Below is a link where you can explore example PIPseq data sets, download the PIPseeker tutorial, and the PIPseeker User Guide. Note that at least 20 GB of available RAM are needed to complete the tutorial. Much higher computing resources are required for analyzing the full data set. (Refer to the PIPseeker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

Appendix

Recommended indexes for pooling ≤ 4 PIPseq libraries

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 3 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	3 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	3 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	3 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	4 PLEX option for NovaSeq X 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	4 PLEX option for NovaSeq X or NextSeq 2000
Sample 2	UDI Library Index mix 6	
Sample 3	UDI Library Index mix 7	
Sample 4	UDI Library Index mix 8	

Revision history

Version	Date	Summary of Changes
00	January 2025	Initial release.