DUKE MOLECULAR PHYSIOLOGY INSTITUTE

MGC Molecular Genomics Core

Duke Molecular Genomics Core

10x Genomics® Single Cell/Nuclei Project Workflow

Consult and/or Cost Estimate

- Email karen.abramson@duke.edu to request a cost estimate and/or consult meeting.
- Information required for estimate: assay type, sample species, how many cells/nuclei you want to target (capture with the assay), input material (fresh cells, cryopreserved cells, or frozen tissue for nuclei), how many samples, how many batches, and do you want advanced data analysis.

Scheduling & Ordering

- **A test run is highly recommended!** Performing a test run provides an opportunity for sample prep optimization. Prep your sample as you would for the real run and bring it to us for a free count/viability assessment. **We use AOPI staining which is more accurate than trypan blue.**
- **Submit a service request in CoreResearch@Duke.** We cannot order kits or run samples until this is received.
- **Please note, any kits that we order for your project are non-refundable.**
- Email karen.abramson@duke.edu to schedule your test run and your full 10x run.

Preparing for you run

- For your planned 10x run, fill out and email us the "**New Project Summary and Sample Submission**" forms prior to your actual run. This is not necessary for the test run.
- **Carefully review sample preparation guidelines! This is critical for the success of your project.**
- Our team will reach out to arrange for sample drop off.

Run day

- Let us know approximately what time you will arrive. We prefer to receive samples by 2pm, but exceptions can be made if you discuss it with us in advance.
- Text or call the lab analyst assigned to your project when you arrive.
- **Remain available by phone for at least one hour after drop off. If the cell viability or nuclei intactness is <80% and/or we don't have enough cells to meet your target, we will not proceed without your approval.**

Post-library generation

- We will contact you to verify the sequencing plan.
- Libraries will be either transferred to the Duke Sequencing and Genomics Technologies (SGT) Core or back to you.

Post-sequencing at Duke SGT

- Upon completion of sequencing our bioinformatics team will download the data and begin conversion to fastq files, alignment to transcriptome, and basic analysis via the 10x Genomics® CellRanger software. Basic analysis is required as a part of our service as a quality control step. If you require a **custom reference** (e.g. you have a transgene), you must inform us before we start.
- The data analysis team will contact you for more details regarding your basic analysis and any desired advanced analysis plan.
- **It is important that you download the raw data** within the timeframe listed on the email from the SGT, the link to the data will expire and neither the SGT or MGC will store raw data longterm. **We only store raw data for 3 months.**

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10x Genomics® Single Cell and Single Nuclei Sample Preparation Guidelines

Failure to follow the sample preparation guidelines listed below could result in reduced data quality and/or potential issues on the 10x Genomics® Chromium instrument.

It is essential that you follow the sample preparation protocol provided by 10x Genomics® and the MGC.

The 10x Genomics® Chromium instrument has an expected cell capture rate of approximately 50-60%, meaning if we want to capture ~10,000 cells we will have to load ~16,000. Capture rate is dependent on an accurate cell count and concentration, high viability, and a sample with minimal debris. Due to all variables **we cannot guarantee the expected capture efficiency**, despite adhering to best practices and 10x Genomics® recommendations. In addition, there is a **+/- 20% expected difference between cells targeted and cells captured, meaning that if 10,000 cells are targeted, a successful run would yield between 8,000 and 12,000 cells.**

The best way to ensure good quality data is to strictly follow the provided sample preparation guidelines.

- **Cell counting is critical**. If you do not submit enough cells for us to count them twice, or if the concentration of the cells is below 500 cells/ul ($5x10^5$ cells/ml) the accuracy of the cell count can be compromised. An inaccurate cell count could adversely affect the number of cells loaded, and therefore the number of cells targeted and captured. Cell counts are less accurate at low dilutions and/or viability <80%.
- **Despite all best practices and precautions taken, there is always a risk of clogging or a wetting failure on the 10x Genomics® Chromium instrument.** If we have enough cells and reagents to re-run a sample that has experienced a clog or wetting failure, we will do so free of charge. Low viabilities, cell clumping, and/or debris all increase the risk of clogging or wetting failure on the instrument.
- **Use Eppendorf DNA LoBind Tubes (1.5 ml cat# 022431021). *No substitutions are allowed. Items have been validated by 10x Genomics® and are required for Single Cell workflow, training and system operations.** Use of alternative plastic products increases the risk of clogs and wetting failures.

The full 10x Genomics® Cell Preparation Guide can be found here, including information on cryopreservation & cell thawing.

Key points have been outlined below:

How many cells do we need?

- The minimum is 3-4x the number of cells you want to capture with the assay. We load ~2x the amount you want to capture, and we also need some for the count and viability assessment.

Sample Handling

- After resuspension, samples must be maintained on ice to avoid temperature shock. Some sample types, such as granulocytes and neutrophils should be maintained at room temperature.
- Cell suspensions should be pipetted slowly and gently to minimize damage to the cells. Rough pipetting can negatively impact sample quality metrics, such as the fraction reads in cells and median genes/cell.
- A swinging bucket centrifuge will help to minimize cell loss, and is recommended if available.

Washing & Resuspension

- The recommended cell washing and resuspension buffer is 1X PBS (calcium and magnesium free) containing 0.04% weight/volume BSA (400 µg/ml).
    - BSA is added to minimize cell losses and aggregation.
        - **Do NOT exceed 2% BSA**
- Primary cells, stem cells, and other sensitive cell types may require washing and suspension in alternative buffers to maximize viability.
    - The following alternative buffers have been verified to not influence performance of 10x Genomics® Single Cell Protocols when profiling mixtures of human 293T/17 and mouse NIH/3T3 cell lines:
        - Dulbecco's Phosphate-Buffered Saline (DPBS) with serum
        - Hank's Balanced Salt Solution (HBSS) with serum
        - 1X PBS + up to 10% FBS
        - 1X PBS + up to 2% BSA
- If cell viability cannot be maintained in one of the above buffers, it is also possible to wash and resuspend in most common cell culture media, with or without serum.
  Increasing the BSA/FBS concentration in wash & resuspension buffers may help to maintain cell viability. 0.04% - 1% BSA, or up to 10% FBS have been validated for the 10x single cell assays.
- **Do NOT exceed 0.1nM EDTA**
- **Do NOT exceed 3mM magnesium**
- **Do NOT include any surfactants, such as Tween-20**
- When washing and resuspending cells, always use sufficient volume to maintain concentrations at less than 5000 cells/µl (e.g. less than 5 million cells per tube when using 1 ml washing solution in 1.5ml LoBind tubes).
    - Maintaining cells at higher concentrations can cause aggregation and clumping that will interfere with generation of ideal single cell suspensions.
    - **Final concentration for submission should be 1000-1500 cells/ul (1.0-1.5 x 10^6 cells/ml). Lower concentrations affect the accuracy of the cell counting.**
- When preparing a nuclei sample for single cell gene expression, be sure to add RNase inhibitor to the resuspension buffer to preserve RNA quality.

RBC Lysis

- Red blood cells (RBCs) will be captured by the 10x Genomics® Chromium instrument along with cells of interest and will count towards your total captured cell number.
    - If your samples contain RBCs and they are not a population of interest you should perform a red blood cell lysis.
    - We do not offer this as a service. If RBC lysis is necessary, it must be performed prior to sample drop-off.

Straining Cell Suspensions

- The first steps of the protocol, which take place on the 10x Genomics® Chromium instrument occur in microfluidic channels that are <**50µm**. Debris, fibers, or clumped cells can affect optimal system performance and result in clogging or wetting failures.
    - Filtering cell suspensions with an appropriate cell strainer is helpful for removing large clumps and debris. The strainer should have a pore size that is larger than the maximum cell diameter in the sample, but small enough to catch larger clumps.
        - Filtering should be performed at the last wash step, in a large volume, to minimize cell loss.

Flowmi™ Tip Strainer (VWR catalog# 10032-802) is recommended to minimize volume losses. However, the cell concentration can decrease by 30% or more. It is therefore important to measure the cell concentration after straining for an accurate cell count.Sorting cells

  - Note – Flowmi Tip Strainers are not to be used on cells for Flex (fixed RNA profiling) libraries.

- Sorted cells are often low in concentration, requiring us to centrifuge to concentrate them. Between cells lost during sorting and this extra spin, we often end up seeing ~50% of the sorter counts. Keep this in mind when you're determining how many cells to sort.
- **Ensure that the sheath fluid is compatible with 10x Genomics® workflow (no EDTA or excessive magnesium).**
- Live/dead cell markers should be added to ~5–10 x 10^6 cells/mL 1-5 minutes prior to sorting.
- Collection tube must be coated and filled with collection buffer before sorting.
- Samples should be maintained at 4°C throughout sorting process.
- Using a larger nozzle can minimize stress on your cells.
- Use the optimal collection buffer/media to help avoid stress on cells and maintain high viability. If BSA/FBS/EDTA/Mg^{2+} concentrations are too high, the MGC will centrifuge the sorted samples to perform a buffer exchange. Please note that centrifugation could cause cell stress and/or lower cell. A higher concentration of BSA or serum should be included, since the buffer's concentration will be diluted during sorting.
- After sorting, samples must be maintained on ice and immediately brought to the MGC for processing. Minimize any additional stress on the cells through gentle sample handling.
- For further information on sorting cells, visit https://kb.10xgenomics.com/hc/en-us/articles/360048826911-What-are-the-best-practices-for-flow-sorting-cells-for-10x-Genomics-assays- .

Tissue Handling & Storage for Single Nuclei RNAseq

- Work quickly and minimize handling during all tissue processing steps.
- Wash tissues in a clean **glass** petri dish with cold PBS upon harvest and absorb any excess blood using a laboratory wipe.
- Cut tissues into small pieces (e.g. the size of a rice grain) in a **glass** petri dish for ease of freezing and place in a cryovial. If you use a plastic dish, microscopic pieces of plastic can get into the sample and affect the downstream assay.
- To flash freeze, either submerge the cryovial in liquid nitrogen or a liquid-nitrogen cooled bath (e.g. isopentane) or place the tube deep in a bucket of dry ice. Wait at least 2–3 minutes for the tissue to freeze all the way through, and transfer the tube containing the tissue to vapor phase liquid nitrogen for long-term storage.
  - Tissues should be flash frozen 'dry' (do not include any buffers or media)
- Tissues should be stored long-term in a cryovial in liquid nitrogen for best results. Tissues can be stored short-term (1–2 days) at -80°C if needed.
- Once removed from liquid nitrogen, tissues should be maintained at -80°C or on dry ice until use.
- Nuclei isolation may require optimization.

Difficult Cell Types

Certain cell types are notoriously difficult in single cell/single nuclei assays, such as neutrophils and granulocytes. These cell types are a challenge due to low RNA content, sensitivity to degradation, and elevated levels of inhibitory compounds, such as RNases.

For some specific assays, there will be greater challenges with certain cell types.

The yield/quality of nuclei isolated using the 10x Genomics® Nuclei Isolation Kit will also be affected by the type of input tissue. If your cell/tissue type has not been tested by 10x Genomics®, please look into similar cell/tissue types that have been tested. Reach out to support@10xgenomics.com if you have further questions about cell/tissue type compatibility. All lysis times and occasionally lysis buffer concentration need to be optimized for each tissue type/condition.

For a full list of compatible tissue types for each assay, visit 10xGenomics.com.

Chromium Nuclei Isolation Kit Tested Tissues

Single Cell Flex Tested Tissues

Single Cell Flex from FFPE Tested Tissues

Please don't hesitate to ask questions! We're here to help! Please email DMPI-MGC@dm.duke.edu or karen.abramson@duke.edu.

Single Cell Gene Expression Flex

The Flex assay provides flexibility in sample preparation and experimental design, enabling batching across time and collection sites, cell surface protein labelling, sample multiplexing with unique sample barcodes, infectious agent neutralization, and the potential for cost savings. Flex allows for targeting of 500 - 128k cells with multiplexing and pooling. Fresh frozen tissue, FFPE tissue, nuclei suspensions, or single cell suspensions can be used as input for this assay. For more information, please reference the 10x Genomics® Flex Protocol Planner CG000528.

Please note that at this time, using cells derived from FFPE tissue sections with Feature Barcode technology for cell surface protein detection is not supported by 10x Genomics®.

When working with FFPE samples, we highly recommend checking RNA quality first and only using samples with a DV200>30% (higher DV200 scores may help to rule out samples with poor RNA quality but will not guarantee high quality data)**.** Data quality in FFPE samples is more variable than in other sample types; **poor quality blocks will likely yield data that cannot be interpreted accurately or salvaged**. The quality of the tissue and block is the greatest predictor of data quality; however, DV200 it is not a definitive indicator of results. The capture efficiency of the Flex from FFPE assay also anecdotally experiences greater variation in the number of cells captured, as compared to standard single cell/nuclei assays.

Factors such as storage conditions and sample preparation will affect the RNA quality of the sample. Please note that 10x Genomics® has only tested FFPE samples stored for 1-10 years. For more information on isolation of cells from FFPE sections, please see 10x Genomics® Protocol CG000632.

Recommendations for storage*:

- Use tissue samples less than 5 mm thick to allow complete penetration by formalin
- Fixate tissue samples in 4–10% neutral-buffered formalin (NBF) as quickly as possible after surgical removal
- Use a maximum fixation time of 24 hours (longer fixation times lead to over-fixation and more severe nucleic acid fragmentation, resulting in poor performance in downstream assays)
- Thoroughly dehydrate samples prior to embedding
- Use low-melting paraffin for embedding

Note: From limited testing, 10x found that samples fixed with 10% NBF for longer periods of time (~24 hours) at **4C** are more likely to generate higher-quality data as compared to samples fixed at RT for the same amount of time

If your FFPE samples were not stored and prepared according to these recommendations, we cannot guarantee the quality or yield of data generated by the Flex assay.

Fixation of Cells & Nuclei for Flex

Tissue Fixation & Dissociation for Flex

Cut scrolls can be stored for up to 1 week prior to isolation, so be sure to coordinate timing with the MGC. Once isolated, cells can be stored at -80C until the assay is run. **When providing the MGC with scrolls, be sure to clearly label tubes and communicate the size and number of scrolls.** We request 2x20um scrolls for extraction & QC of RNA. After passing QC we'll request 2 tubes (1.5mL Eppendorf LoBind tubes) per sample of 2x25um scrolls for human tissues or 2x50um scrolls for mouse (totaling 4 scrolls/sample for cells isolation with backup scrolls, if needed). Please note that it is important for the scrolls used for cell isolation to be placed into 1.5mL tubes as the isolation protocol is pestle based and the pestles used are made to fit into 1.5mL tubes. If these scrolls contain insufficient cells, the MGC may request additional scrolls to achieve the minimum input for the Flex assay.