Tips & Tricks

# Sample Preparation Tips for Single Cell Gene Expression

## Introduction

Single cell transcriptomics is a powerful tool for unraveling complex biological systems, giving you the ability to study gene expression dynamics on a cell-by-cell basis. Underlying the ability to dissect cell-type specific differences in complex samples is single cell RNA-sequencing (scRNA-seq) which generates single cell profiles for populations of cells. Single cell gene expression profiling provides a critical understanding of cellular processes during disease development and progression. The technological advancements of scRNA-seq allow researchers to explore the true diversity of gene expression at the single cell level by providing access to critical data, such as rare or novel cell types, that are often masked by bulk RNA-seq methods (Figure 1). To take full advantage of this transformative technology, however, it is essential to properly prepare single cell samples. This article outlines recommendations for the preparation of single cell samples for use with the 10x Genomics Single Cell Gene Expression or Immune Profiling Solutions.

### Sample Preparation Protocol for scRNA-seq

- Cell Dissociation Protocol Tips
- Pipetting Technique and Tip Choice
- Cell Washing & Straining
- Counting and Viability Assessment
- Storage After Preparation

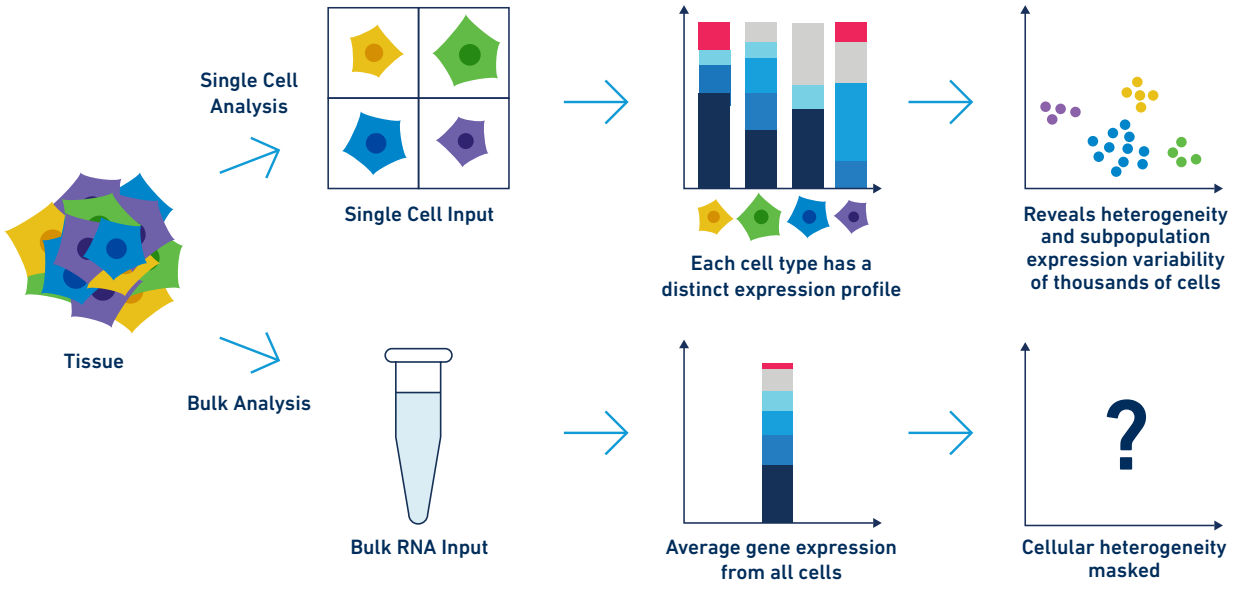


Figure 1. scRNA-seq reveals cellular heterogeneity that is masked by traditional bulk RNA-seq methods.

| Cells Tested | Species | Cell Source | Total RNA (pg/cell) | Cell Size (μm) |
|---|---|---|---|---|
| PBMC | Human | Extracted from blood | ~0.75 | ~5-10 |
| E18 neuron | Mouse | Brain Tissue | ~2-3 | ~9 |
| Jurkat | Human | Suspension | 5.5 | ~12 |
| Raji | Human | Suspension | 7.3 | ~12 |
| HEK293T | Human | Adherent | 14.2 | ~18 |
| NIH3T3 | Mouse | Adherent | 16.1 | ~18 |
| HCC1954 | Human | Adherent | 15.7 | ~18 |
| HCC38 | Human | Adherent | 21.6 | ~30 |

Table 1. Cell types tested by 10x Genomics. RNA Extracted using Maxwell® RSC SimplyRNA cells kit and determined by Qubit™ assay.

## Sample Preparation Protocol for scRNA-Seq

### Cell Dissociation Protocol Tips

Optimizing the cell dissociation protocol for your particular sample type will be critical in order to retrieve highly viable cells and minimize cell death and lysis. Dead cells can lyse, resulting in the release of ambient RNA; this cell-free RNA can contribute to the background noise of the assay and will compromise the quality of single cell data. Proper handling of cells will have a direct effect on the measurements that determine the number of cells loaded onto the system and the number of cells recovered. Following the guidelines below increases the accuracy of cell counts and minimizes variability from sample to sample.

Understanding the limitations and requirements of a given sample type will result in more robust data. A number of available sample preparation protocols address these issues, but may need to be adapted for your particular sample type. For example, suspension cell lines, bead-enriched cells, and flow-sorted cells are already suspended and only need to be washed and counted before use with the Chromium Single Cell Gene Expression or Immune Profiling Solutions. However, when working with tissue, it is important to optimize the dissociation protocol well in advance of your first library preparation to ensure maximum quality. We offer a number of Demonstrated Protocols to help guide you in optimizing sample preparation (see page 4).

Another consideration is that different cell types display a wide range of initial RNA content, and accurate RNA input measurements will impact several key workflow decisions, such as the number of PCR cycles used in library preparation. For example, human peripheral blood mononuclear cells (PBMCs) are RNA poor with about 0.75 pg/cell, while HCC38 cell lines are very RNA rich with 21.6 pg/cell (Table 1).

### Pipetting Technique and Tip Choice

Pipetting technique is critical when dissociating tissue or working with cells already in suspension. For example, as cells sit in a tube, they begin to settle. When pulling from either the top or the bottom of the solution, it is possible to aspirate very different numbers of cells, especially if the cells settle quickly. Instead, cell suspensions should be pipette-mixed immediately prior to transfer and then aspirated from the middle of the tube each time, generally below the halfway mark of the solution.

Additionally, it is critical that cells are handled gently when pipetting. Pipette mixing roughly, even with a wide-bore pipette tip, can negatively impact sample quality. Table 2 compares key single cell data metrics from four different cell-mixing experiments performed on HEK293T cells. Both rough pipette mixing and vortexing can cause premature cell lysis, creating high ambient RNA levels that contribute to background noise in the system and ultimately decreasing the fraction of reads in cells (highlighted in blue). Similarly, the number of median genes per cell also decreases as a result of rough cell handling (Table 2). Instead, the cell suspension should be mixed gently with a wide-bore pipette tip. This will yield cleaner data, increase library complexity (i.e. increase number of median genes per cell) and result in a higher fraction of reads in cells.

| Metric | Control | Wide Bore (Rough) | Narrow Bore (Rough) | Vortex 5s |
|---|---|---|---|---|
| Number of Cells | 1,118 | 846 | 1,012 | 983 |
| Reads per Cell | 50,000 | 50,000 | 50,000 | 50,000 |
| Valid Barcodes | 95.40% | 95.50% | 95.30% | 95.50% |
| Fraction Reads in Cells | 79.40% | 72.80% | 54.00% | 63.10% |
| Reads Mapped Confidently to Transcriptome | 70.50% | 71.40% | 71.80% | 71.00% |
| Valid UMIs | 99.40% | 99.40% | 99.40% | 99.40% |
| Median Genes per Cell | 3,137 | 3,180 | 2,833 | 2,934 |

Table 2. Pipette mixing roughly, regardless of pipette tip size, can negatively affect sample quality.

### Cell Washing & Straining

When working with suspension cell lines, dissociated tissues, or large numbers of cells, it is highly recommended that a thorough washing protocol is followed and will influence cell recovery after being loaded into the Chromium Controller. This can vary based on sample type as well as scRNA-seq method. Our scRNA-seq washing protocol calls for a solution of PBS containing 0.04% BSA when washing and resuspending cells.

Large cell aggregates or debris can increase the risk of clogs on a microfluidic chip and also affect cell recovery by interfering with accurate cell counting, especially when using an automated cell counter. To avoid this, we recommend using a cell strainer, with pore sizes of 30 to 40 microns, before loading the cell suspension onto the microfluidic chip. A number of strainers have been successfully tested on samples loaded into our Single Cell Gene Expression and Immune Profiling Solutions.

It is important to note that each straining will result in cell suspension volume loss as well as changes in cell concentration. If straining is required, the single cell suspension should be counted again after straining is complete.

### Counting and Viability Assessment

To assess sample quality, it is crucial to measure cell viability after washing and straining. There are a number of methods, but we have found that the Trypan blue exclusion method works well to identify the proportion of live cells to dead cells.

The accuracy of the final cell count and, ultimately, the agreement between targeted and calculated cell recovery is dependent on knowing how many cells are in a sample. Once the straining and washing steps have been completed, a cell counting device, such as a hemocytometer or an automated cell counter, can be used for quantification. These devices not only provide accurate counts but are also critical to calculate the volume of cell suspension required to load the desired number of cells onto the microfluidic chip. Another important factor that can impact the accuracy of the final cell count is cell stock concentration. We found cell stock concentrations between 700 and 1,200 cells/µL to be optimal to achieve the targeted number of recovered cells (Figure 2). Cell suspensions that are outside this optimal concentration range may result in unreliable cell counts. If samples are outside this range, we recommend adjusting the cell stock concentrations accordingly.

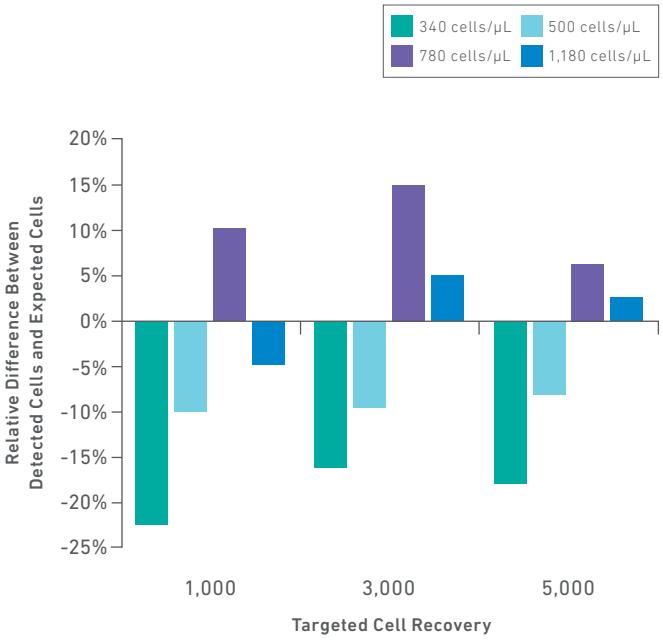


Figure 2. Relative difference of recovered cells from targeted cell counts, calculated by Cell Ranger Analysis Pipelines vs. the targeted cell counts for each library. Cell Suspensions #1 (green, 340 cells/µL) and #2 (light blue, 500 cells/µL) showed the greatest deviation from the targeted cell counts, 16-23% and 8-10% respectively. Target accuracy was improved with cell suspensions that were prepared at higher stock concentrations, Cell Suspensions #3 (purple, 780 cells/µL) and #4 (medium blue, 1,180 cells/µL).

A high percentage of non-viable cells may impact the targeted cell recovery numbers, and in samples with a high fraction of dead cells (>30%), we recommend using our Demonstrated Protocol on Removal of Dead Cells from Single Cell Suspensions. This protocol outlines best practices for reducing the percentage of dead cells in a single cell suspension.

**Storage After Preparation**

To minimize changes to the transcriptome, once the cells are washed and counted, single-cell suspensions should be stored on ice until they are used in partitioning and library construction. Ideally, once samples are prepared, they should be utilized for downstream steps within 30 minutes.

It is important, however, to be aware of a cell type's unique characteristics as these will impact both how long your cells should be left on ice and how quickly they should be used after preparation. For example, some cells, such as PBMCs, will begin to form clumps if they are sitting on ice for extended periods of time. These clumps are difficult to dissociate, increasing the risk of clogs and, with each clump being counted as a single cell, decreasing counting accuracy. Additionally, some cells are stickier than others and clump at a faster rate. For these, it is particularly important to minimize the time between preparation and use.

## Conclusions

Having a plan in place for the best handling and preparation of your samples is key when preparing single cell suspensions as there are many factors that need to be considered in order to get high quality data. For additional guidance on single cell sample preparation, there are a number of Demonstrated Protocols available, which provide cell-type specific protocols. For cell types that are difficult to isolate as whole intact cells, nuclei isolation may be an effective alternative approach. Guidelines are also available for the steps following sample preparation, such as library preparation, sequencing, and data analysis and visualization. Furthermore, our how-to videos, especially Chapters 4 through 7, should help walk you through the sample and library preparation processes.

**Demonstrated Protocols for Single Cell Isolation**

Single Cell Protocols - Cell Preparation Guide
This Cell Preparation Guide describes best practices and general protocols for washing, counting and concentrating cells from both abundant and limited cell suspensions (greater than or less than 100,000 total cells, respectively) in preparation for use in 10x Genomics Single Cell Protocols.

Tumor Dissociation for Single Cell RNA Sequencing
This protocol describes best practices and general protocols for cell lysis, washing, debris removal, counting, and concentrating nuclei from both single cell suspensions and neural tissue in preparation for use in 10x Genomics Single Cell Protocols.

Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing
This protocol outlines how to prepare single cell suspensions from cultured cell lines in preparation for use in 10x Genomics Single Cell Protocols.

Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing
This protocol outlines best practices for reducing the percentage of non-viable or dead cells from a single cell suspension.

Isolation of Nuclei for Single Cell RNA Sequencing
This protocol describes best practices and general protocols for cell lysis, washing, debris removal, counting, and concentrating nuclei from both single cell suspensions and neural tissue in preparation for use in 10x Genomics Single Cell Protocols.

Enrichment of CD3+ T Cells from Dissociated Tissues for Single Cell RNA Sequencing and Immune Repertoire Profiling
This protocol outlines best practices for enriching the per centage of Cluster of Differentiation 3 positive (CD3+) T cells obtained from dissociated tumors in preparation for use in 10x Genomics Single Cell Protocols.

Click here to view additional protocols




LIT000037 *Rev A Sample Preparation Tips for Single Cell Gene Expression*