10x GENOMICS®

TECHNICAL NOTE

Guidelines for Accurate Target Cell Counts Using 10x Genomics® Single Cell Solutions

INTRODUCTION

10x Genomics® Single Cell Protocols require suspensions of viable, single cells as input (*Single Cell Protocols – Cell Preparation Guide* - CG00053). Accurate counting and qualification of cells during the workflow of the Single Cell 3' v2 Protocol (*Chromium™ Single Cell 3' Reagent Kits v2 User Guide* – CG00052) and the Single Cell V(D)J Protocol *(Chromium™ Single Cell V(D)J Reagent Kits User Guide* – CG000086) is essential to determine the number of cells to load on to the microfluidic chip for analysis on the system. While manual (e.g. hemocytometer) and automated (e.g. Countess® II Automated Cell Counter) counting methods can be used to report cell concentrations, additional factors should be considered that can affect the final count. This Technical Note presents an overview of best practices for cell handling and counting that will increase the accuracy of cell counts and minimize the variability in the number of loaded cells.

METHODS TO QUANTIFY AND QUALIFY CELL SAMPLES

The Countess® II Automated Cell Counter is recommended for the determination of cell concentration and viability for most applications. Sample types with very small cells, highly variable cell sizes or cell aggregation may require alternative counting methods such as a hemocytometer. When characterizing a sample for the first time, review the *Single Cell Protocols – Cell Preparation Guide* (CG00053) which contains additional information.

I. Factors Influencing Cell Recovery

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 in emulsions as the chip is run in the 10x Genomics Controller. Factors that influence cell recovery are listed in Table 1.

Factor	Effect on Cell Recovery	Best Practice Recommendations
Cell Viability	• The presence of a high fraction of non-viable cells may decrease cell recovery.	• Optimization of tissue dissociation protocols. • Prepare cell suspensions with > 90% viable cells. • Additional wash and straining steps may be necessary to remove excess amount of ambient RNA and debris, respectively. • Accurately determine cell viability (e.g. microscopy and LIVE/DEAD™ Cell Viability Assay from ThermoFisher Scientific as an alternative to trypan blue). • Determine cell viability after cell handling steps (e.g. flow-sort) and just prior to loading if the samples will not be processed immediately.

Cell Counting	• Overestimation of cell concentration. decreases cell recovery. • Underestimation of cell concentration can inflate cell recovery. • Flow-sorted cell suspensions can lead to an overestimated cell count. • Small cells (<10 µm) may be difficult to distinguish from debris or other particles and can result in an overestimated cell count. • Very dilute (e.g. < 200 cells/µl) or highly concentrated (e.g. > 1000 cells/µl) cell suspensions may not accurately represent actual cell numbers and can be difficult to get in focus with an automated cell counter (note, this will depend on the dynamic range of counting technique used). • High fraction of cell aggregates can be challenging for both automated and manual cell recognition.	• Sample the cell suspension at least twice and carry out at least two counts on each sample (e.g. a minimum of four counts in total, based on two independent draws from the cell suspension). • When characterizing a sample type for the first time, perform two different counting assays (manual or automated) to assess accuracy of counting practice. • Re-count cell suspensions just prior to chip loading and continue with sample loading without any major delay (< 30 min). • Cell suspensions should be checked visually for debris or cell aggregates as these can result in inaccurate cell counts; filter cell suspensions if needed. • Concentrate or dilute cell suspensions, if necessary, to obtain optimal targeted cell concentration and re-count. • Always re-count cell suspensions after flow-sorting.
Cell Preparation Time	• Some cells (primary cells including PBMCs) form cell aggregates that cannot be resuspended back into a single cell. suspension when kept in PBS for a prolonged period of time (more than 2 hours). This lowers the effective concentration of suspended cells. • Some cell types are more fragile and cell viability may decrease significantly if not processed and loaded immediately.	• Proceed with cell loading immediately after sample preparation. • Cell suspensions should always be kept on ice and incubation time be kept to a minimum (< 30 min). • Cells can be resuspended in their preferred media and kept on ice if they cannot be processed immediately.
Cell Aggregates	• Depending on cell type and heterogeneity of the cell suspension, cells can exhibit “sticky” behavior or a tendency to aggregate, depending on the expression of cell surface proteins characteristic of the cell type. Cell surface proteins can also cause cells to stick to plastic surfaces (e.g. Eppendorf tube, pipette tips).	• Resuspend cells during sample preparation with a regular-bore pipette tip to disrupt cell aggregates. • Filter cells prior to use. • Prior to transfer into the master mix, resuspend the cell suspension with a regular-bore pipette tip. • Cells combined with the master mix should be gently pipetted 5-10 times with a regular-bore pipette tip and immediately loaded with the same pipette tip into the chip.
Pipetting Technique	• Pipetting low cell suspension volumes (< 2 µl) into the master mix can result in greater variance of cell counts. • Insufficient or no mixing of cell suspensions in large resuspension volume prior to transfer to the master mix can result in greater variance of cell counts.	• Pipette mix cells with a P200 or P1000 pipette depending on the total suspension volume just prior to transfer to the master mix. The total volume set on the pipette should be > 50% of the actual volume in which cells are resuspended (e.g. set pipette to > 350 µl if cells are suspended in 700 µl). • Pipette mixing should be done with the full volume at which pipette is set. • Pipette tips should be placed in the middle of the tube when cells are aspirated. • Cells combined with the master mix should be gently pipetted 5-10x with a regular-bore pipette tip and immediately loaded with the same pipette tip into the chip. • Ensure that pipettes are well calibrated.

Table 1. Factors influencing cell recovery.

II. Challenges in Cell Counting

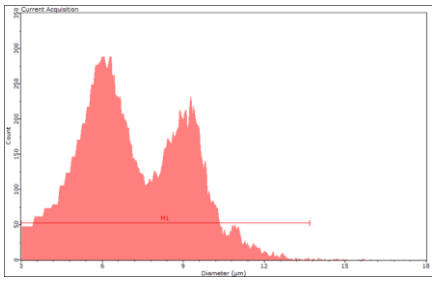
Both manual and automated cell counting methods have inherent error rates that can result in variability in cell counts across multiple counts on the same platform as well as between platforms. The two biggest challenges are:

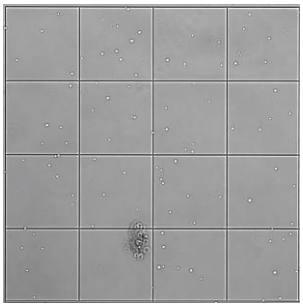
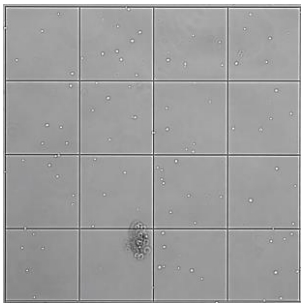
A. Human and automated-algorithm perception and definition of a single cell
B. Counting cells in an optimal cell concentration

A. Human and automated algorithm perception and definition of a single cell

Each algorithm of an automated cell counter will define a cell differently and thus results can vary depending on cell size, cell shape, cell debris and other small particles. Variations in cell counts can also be exacerbated by manual adjustments of brightness and focus. Likewise, manual counting results will primarily depend on specific criteria set by the user that will define a cell. These will differ between users which can lead to substantial variations in cell counts that are significantly higher than the mean of a Poisson distribution (Nielson, L. *et al.*). The examples below (Table 2) highlight the challenges that are associated with:

1. Counting the same cell suspension with two different automated counters
2. Counting the same cell suspension by two different individuals
3. Discriminating cells from debris/particles in a sample cell suspension containing varying degrees of debris

1.	Countess® II Automated Cell Counter	Scepter™ Handheld Automated Cell Counter	Δ**
Automated counts from different automated platforms			
Results	657 cells/µl	773 cells/µl	116 cells/µl

2.	Individual 1	Individual 2	Δ**
Manual counts by different individuals			
Results	96 cells	88 cells	8 cells

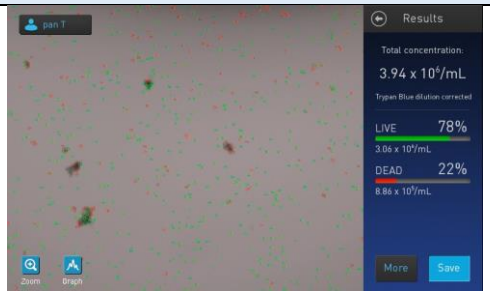
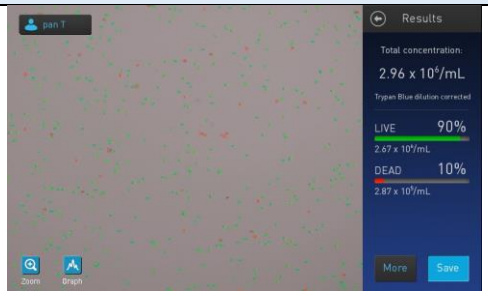
3.	Large Fraction of Debris	Low Fraction of Debris	Δ**
Define cells from debris			
Results	3,940 cells/µl	2,960 cells/µl	980 cells/µl

Table 2. Differences in cell counts for 1) different automated platforms, 2) different users of a hemocytometer and 3) automated counting on the Countess® II Automated Cell Counter for a sample with low and high amounts of debris.

B. Counting cells in an optimal cell concentration

Cell Suspensions that are very dilute or very concentrated may result in unreliable counts since the number of cells counted may not accurately represent the total cell count (dilute) or may be difficult to count (high concentration). For example, if only 100 cells are counted, the variation will be up to 10% even without human-introduced variation. This baseline for variation can be calculated by assuming the variation follows the Poisson distribution where the expected standard deviation is equivalent to the square root of the number of counted events. When using automated cell counters, refer to the product manual to assess the optimal cell concentration range (e.g. Countess® II Automated Cell Counter recommends the range to be between 100 to 1,000 cells/µl). For manual counts using a hemocytometer, we recommend counting a minimum of four squares with cell suspensions that will result in approximately 50-100 cells per square. If the number of cells per square is significantly lower or higher than 50-100, prepare a fresh dilution using a more appropriate dilution factor.

The example below demonstrates the importance of having the appropriate cell suspension concentration to achieve an accurate cell count (see Table 3). An aliquot of a cell suspension with a concentration of ~570 cells/µl was counted using a hemocytometer, then, diluted twice (1:2 and 1:10) and the dilutions. Each concentration was counted using both 1 and 4 squares of the hemocytometer. The cell suspension that was diluted 1:10 resulted in cell counts that were substantially overestimated compared to the actual concentration when both one and four squares of the hemocytometer were counted (1,400 cells/µl and 825 cells/µl, respectively).

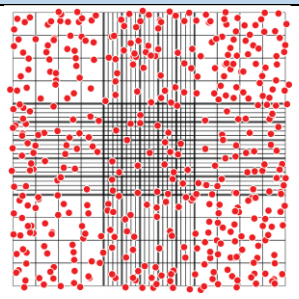
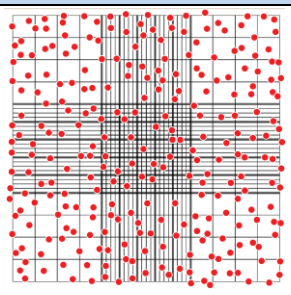
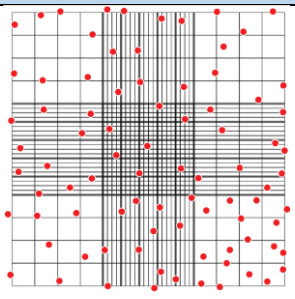
	Original Cell Suspension (~570 cells/µl)		1:2 Dilution (~285 cells/µl)			1:10 Dilution (~57 cells/µl)		
View on Hemocytometer								
	Cells Counted	**Concentration**	**Cells Counted**	**Concentration**	**Δ****	**Cells Counted**	**Concentration**	**Δ****
Four Squares	228 cells	570 cells/µl	116 cells	580 cells/µl	+10 cells/µl	33 cells	825 cells/µl	+255 cells/µl
One Square*	64 cells	640 cells/µl	28 cells	560 cells/µl		14 cells	1,400 cells/µl	
Δ***		+70 cells/µl		-20 cells/µl			+575 cells/µl	

Table 3. Three different cell concentrations are assessed using a hemocytometer. The 1:2 and 1:10 dilution are made from the original cell suspension. *The bottom right square is counted for the One Square measurement for each cell suspension. **Delta is in reference to original versus dilute cell suspensions. ***Delta is in reference to "Four Squares" vs "One Square" counted.

CONCLUSION

Targeting the right cell number is challenging and is dependent on cell type, equipment used, and experience in cell handling and counting. Note, the dynamic range of counting techniques (manual or automated) and/or instrument platforms differ and, thus, it is crucial to pre-determine the optimal range in order to achieve accurate cell counts. Additional factors that can influence cell counts and that are reported in this Technical Note serve as guidelines to re-evaluate and/or troubleshoot existing cell count practices. Adhering to best practices described here will improve overall accuracy of targeted cell counts.

REFERENCES

- Chromium™ Single Cell 3' Reagent Kits v2 User Guide (CG00052)
- Chromium™ Single Cell V(D)J Reagent Kits User Guide (CG000086)
- Demonstrated Protocol – Single Cell Protocols – Cell Preparation Guide (CG00053)
- Nielson, L. *et al.* Hemocytometer Cell Count Distributions: Implications of Non-Poisson Behavior. Biotechnology Progress, 1991.

Notices

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