Performing Cell Counts with a Hemacytometer

Cell counting is an integral part of assessing the results of cell isolation procedures. For best results, both a total nucleated cell count (using 3% acetic acid with methylene blue) and a viability count (using Trypan Blue) should be performed.

Total Nucleated Cell Counts Using 3% Acetic Acid With Methylene Blue

This counting method determines the total nucleated cell count in a sample. The acetic acid lyses the cellular membranes, and the methylene blue stains the exposed nuclei. Because mature red blood cells lack nuclei, they are excluded from this count.

1. Prepare an appropriate dilution of the well-mixed single cell suspension using PBS or serum-free medium. For an accurate representation of concentration, use at least 20 µL of cell suspension to make the dilution.
   Example - Preparing a 10-fold dilution
   • Add 180 µL of 3% acetic acid with methylene blue to a microcentrifuge tube or well of a 96-well plate
   • Mix the cell suspension and add 20 µL to the tube or well containing 3% acetic acid with methylene blue
   Note: The appropriate dilution will result in a cell concentration that gives 50-100 cells per square in the hemacytometer. We recommend starting with a 10-fold dilution, but the appropriate dilution factor will depend on the approximate number of cells present in the starting sample.
2. Cover a clean hemacytometer with a clean cover slip.
3. Using a capillary tube or pipettor, apply a small drop (about 10 µL) of the stained, diluted cell suspension to the surface of the hemacytometer at the edge of the coverslip, being careful not to move the coverslip.
4. Place the hemacytometer on the stage of a binocular light microscope, adjust the microscope to 10x power and focus on the cells. Count the cells (stained nuclei) in each of the four outside squares of the hemacytometer (Figure 1), including cells that lie on the bottom and left-hand perimeters, but not those that lie on the top and right-hand perimeters (Figure 2). A minimum of 100 cells should be counted.

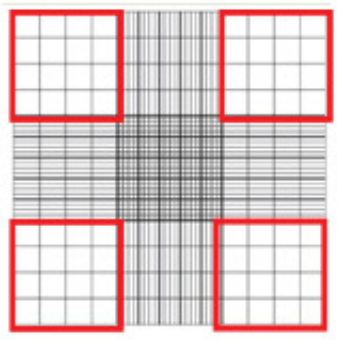

Figure 1: Hemacytometer grid

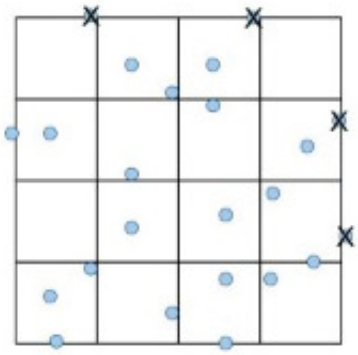

Figure 2: Cell counting guidelines

Note: If there are more or fewer than approximately 100 cells per square on the hemacytometer, prepare a new diluted sample using a greater or smaller dilution factor.

5. Each of the nine major squares of the hemacytometer represents a total volume of 0.1 mm^3. Since 1 cm^3 is equivalent to 1 mL, the cell concentration can be determined using the following equation:
   Total number of nucleated cells/mL = average cell count per square x dilution factor x10^4
   Note: An online hemacytometer calculation tool may be used to easily convert the cell counts into cell concentrations.

Viability Counts Using 0.4% Trypan Blue

Trypan Blue is used to distinguish between live and dead cells. Live cells possess intact cell membranes that exclude dyes such as trypan blue, whereas dead cells absorb/take on these dyes. Therefore the cytoplasm of dead cells stains blue and that of viable cells appears clear. To obtain an accurate representation of cell viability, all reagents, including the cell sample, must be warmed to room temperature before preparing a diluted sample of the cell suspension for counting.

1. Prepare an appropriate dilution of the well-mixed single cell suspension using PBS or serum-free medium. For an accurate representation of concentration, use at least 20 µL of cell suspension to make the dilution.
   Example - Preparing a 10-fold dilution
   • Add 80 µL of PBS or serum-free medium to a microcentrifuge tube or well of a 96-well plate
   • Add 20 µL of well-mixed cell suspension to the tube or well containing medium
   • To a fresh tube or well add 20 µL of 0.4% Trypan Blue
   • Add 20 µL of well-mixed diluted cell suspension to tube or well containing 20 µL 0.4% Trypan Blue
   Note: The appropriate dilution will result in a cell concentration that gives 50-100 cells per square in the hemacytometer. We recommend starting with a 10-fold dilution, but the appropriate dilution factor will depend on the approximate number of cells present in the starting sample.
   Note: To obtain an accurate representation of cell viability, cells should be counted within 15 minutes of staining as cell viability will decrease over time.
2. Cover a clean hemacytometer with a clean cover slip.
3. Using a capillary tube or pipettor, apply a small drop (about 10 µL) of the stained, diluted cell suspension to the surface of the hemacytometer at the edge of the coverslip, being careful not to move the coverslip.
4. Place the hemacytometer on the stage of a binocular light microscope, adjust the microscope to 10x power and focus on the cells. Count the unstained (viable) and stained (non-viable) cells separately. Count the cells in each of the four outside squares of the hemacytometer (Figure 1 above), including those that lie on the bottom and left-hand perimeters, but not those that lie on the top and right-hand perimeters (Figure 2). A minimum of 100 cells should be counted.
   Note: If there are more or fewer than approximately 100 cells per square on the hemacytometer, prepare a new diluted sample using a greater or smaller dilution factor.
5. To obtain the total number of viable cells/mL in the diluted sample, use the following equation:
   Total number of viable cells/mL = (average viable cell count per square) x dilution factor x 10^4
6. To obtain the total number of cells/mL in the diluted sample, use the same equation as above, but include both the viable and non-viable cells in the total cell count. Correct for dilution factor as above to obtain the total number of cells/mL in the undiluted aliquot.
7. The percentage of viable cells can be determined using the following equation:

$$\text{Viable cells (\%)} = \frac{\text{number of viable cells} \times 100}{\text{number of viable cells + number of non-viable cells}}$$

Note: An online hemacytometer calculation tool may be used to easily convert the cell counts into cell concentrations and determine % viability.