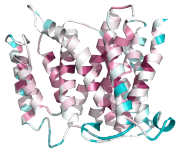
Hemocytometer Cell Counting Protocol



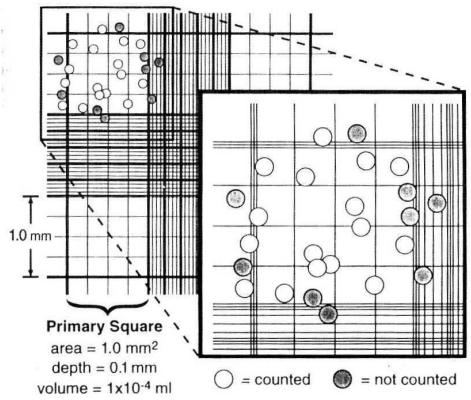
INTRODUCTION

For basic protocol, see At the Bench, page 227 or http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html

We often skip the trypan blue step as most of the cells are viable. You should avoid counting cells that are obvious cell debris vs. rounded cells with "halos". The latter are most likely to be living cells. If using trypan blue, just count the cells that have excluded the dye.

Cell suspensions should be dilute enough so that the cells do not overlap each other on the grid, and should be uniformly distributed. If there are more than 200 cells per well, dilute your suspension with PBS by an appropriate volume. If the cell density per large square is less than 50 then go back to your culture and use more cells and less PBS.

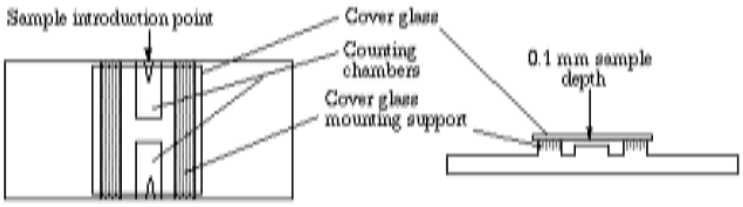
Cell suspensions should be dilute enough so that the cells do not overlap each other on the grid, and should be uniformly distributed as it is assumed that the total volume in the chamber represents a random sample. This will not be a valid assumption unless the suspension consists of individual well-separated cells. Cell clumps will distribute in the same way as single cells and can distort the result. Unless 90% or more of the cells are free from contact with other cells, the count should be repeated with a new sample. Also, the sample will not be representative if the cells are allowed to settle before a sample is taken. Mix the cell suspension thoroughly before taking a sample to count.



Count the cells in selected squares so that the total count is 100 cells or so (number of cells needed for a statistically significant count). For large cells this may mean counting the four large corner squares and the middle one. For a dense suspension of small cells you may wish to count the cells in the four 1/25 sq. mm corners plus the middle square in the central square. Always decide on a specific counting pattern to avoid bias. For cells that overlap a ruling, count a cell as "in" if it overlaps the top or right ruling, and "out" if it overlaps the bottom or left ruling.

PREPARING THE HEMOCYTOMETER

To prepare the hemocytometer, the mirror-like polished surface is carefully cleaned with lens paper and ethanol. The coverslips (which is thicker than those for conventional microscopy) should also be cleaned. The coverslip is placed over the counting surface prior to adding the cell suspension. The cell suspension is introduced into one of the V-shaped wells using a pasteur pipet. Allow the area under the coverslip to fill by capillary action. Enough liquid should be introduced so that the mirrored surface is just covered. The counting chamber is then placed on the microscope stage and the counting grid is brought into focus at low power. One entire grid on standard hemacytometers with Neubauer rulings can be seen at 40x (4x objective).The main divisions separate the grid into 9 large squares. Each square has a surface area of 1mm2, and the depth of the chamber is 0.1mm. Each square of the hemacytometer (with cover slip in place) represents a total volume of 0.1 mm3or 10-4 cm3. Since 1 cm3 =1 ml, the subsequent cell concentration per ml can be determined.



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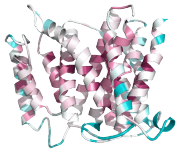

PROTOCOL

When Culturing from:

35 mm or 6 well dishes	T-25 Flask (60-90% confluent)	T-75 Flask (60-90% confluent)
Trypsinize in 0.5 ml of trypsin solution. Suspend culture in 1 ml of complete media.	Trypsinize in 1 ml of trypsin solution. Suspend culture in 4 ml of complete media.	Trypsinize in 3 ml of trypsin solution. Suspend culture in 9 ml of complete media.

1. Transfer 200 µl of the cell suspension into a 1.5 ml microfuge tube.

2. Add 300 µl of PBS and 500 µl of 0.4% trypan blue solution to the cell suspension (creating a dilution factor of 5) in the centrifuge tube.

3. Mix thoroughly and allow to stand 5 to 15 minutes. **Note:** If cells are exposed to trypan blue for extended periods of time, viable cells may begin to take up dye as well as non-viable cells, thus, try to do cell counts within one hour after dye solution is added.

4. With a cover-slip in place, use a pasteur pipette and transfer a small amount of the trypan blue-cell suspension to a chamber on the hemocytometer.

5. This is done by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber to fill by capillary action. Do not overfill or underfill the chambers. 20µl USING CAPILARY ACTION should be enough. Do not directly pipet into the chamber.

6. Count all the cells (non-viable cells stain blue, viable cells will remain opaque) in the 1mm center square **and** the four corner squares.

7. Refer to diagram above. Keep a separate count of viable and non-viable cells. If greater than 25% of cells are non-viable, the culture is not being maintained on the appropriate amount of media; reincubate culture and adjust the volume of media according to the confluency of the cells and the appearance of the media.

8. If there are less than 50% or more than 200 cells per large square, repeat the procedure adjusting to an appropriate dilution factor.

9. Repeat the count using the other chamber of the hemocytometer.

10. Each square of the hemocytometer (with cover slip in place) represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations.

    - Cells per ml = the average count per square x the dilution factor x 10^4 (count 10 squares)
    - Example: If the average count per square is 45 cells x 5 x104 = 2,250,000 or 2.25 x 106 cells/ml.
    - Total cell number = cells per ml x the original volume of fluid from which cell sample was removed.
    - Example: 2.25 x 10^6 (cell per ml) x 10 ml (original volume) = 2.25 x 10^7 total cells.