Using a Counting Chamber (Hemocytometer)

(Adapted from http://www.ruf.rice.edu/~biolabs/methods/microscopy/cellcounting.html; last reviewed on September 7, 2009)

**Note 1:** The counting chamber is used in conjunction with a specially made cover slip. It is important to use the cover slip made for the counting chamber and not a regular microscope slide cover slip. Be sure not to discard the cover slip when you have finished using the counting chamber.

**Note 2:** Both the counting chamber and its cover slip may be stored in a box or plastic container near the microscope. However, if they are not stored in either of these, they are mostly likely setting in a Petri jar containing 70% ethanol. The latter is used for disinfecting the chamber after its use. The ethanol should be periodically replaced with a freshly made solution.

1. Prepare the mirror-like polished surface of the counting chamber by carefully cleaning it with lens paper or a KimWipe. If it is wet from with ethanol from being in the Petri jar, wipe it off with the KimWipe, then allow the chamber to dry before use. The surface can then be further cleaned with lens paper. Similarly, clean the cover slip with lens tissue or a KimWipe being sure it is dry before use.

2. Place the cover slip over the counting surface. Using a micropipette, carefully introduce the cell suspension to be counted into both of the V-shaped wells on the long sides of the counting chamber (Figure 1). The chamber is now considered ‘charged’.

   **Note:** The area under the cover slip fills by capillary action. Therefore, just place the cell suspension in the well and not force the liquid under the cover slip by applying pressure from the pipette. Also, use just enough liquid (about 10-20 µl) should be introduced so that the mirrored surface is just covered following the drawing of the fluid by capillary action.

   ![Figure 1. Diagram of counting chamber.](image)

3. The charged counting chamber is then placed on the microscope stage and the counting grid is brought into focus at **low** power.

   **Note 1:** Allow the counting chamber to rest on the stage or on the bench for a few minutes prior to attempting to count the cells. This permits those cells in suspension to settle out onto the grid pattern.
Note 2: It is essential to be extremely careful with higher power objectives, since the counting chamber is much thicker than a conventional slide. The chamber or an objective lens may be damaged if the user is not careful.

Note 3: One entire grid on standard hemocytometers with Neubauer rulings can be seen at low power (4x or 10x objective). The main divisions separate the grid into 9 large squares (like a tic-tac-toe grid) (Figure 2). Each square has a surface area of one square mm, and the depth of the chamber is 0.1 mm. Thus, the entire counting grid lies under a volume of 0.9 mm-cubed.

Figure 2. Neubauer rulings on counting grid of a hemocytometer. Each of the four corner squares (A-D) is divided into 16 smaller squares. The distance from the left end line of square A to the right end line of square B is 0.3 mm. Similarly, the distance from the top end line of square A to the bottom end line of square D is 0.3 mm. Hence, each of the 9 large squares in the grid has a width of 0.1 mm and a height of 0.1 mm. The depth of the entire grid, when the cover slip is placed over it, is 0.1 mm (see Figure 1). Therefore, the entire volume of the counting grid is 0.9 mm$^3$, or 0.1 mm$^3$ for each of the large squares.

4. To perform the count on yeast cells or conidia, carefully move the “high dry” (40x) objective of the microscope into position. You many need to adjust the light intensity using the rheostat or moving the condenser or diaphragm. Focus on the cells laying on the grid. Systematically count the cells in selected squares, usually A through D. (Method 1). However, if the number of cells is quite high (>300), you may count the four corner squares in each of the large squares (Method 2)

Note 1: Typically, you want to count at least 100 cells per large square (e.g., A in Figure 2). However, you don’t want cell suspensions so dense (e.g., > 500 cells per large square) that they overlap one another. Furthermore, you don’t want the suspension so dilute that there are not at least 100 cells per large square. Until you become well versed in using a counting chamber and making dilutions, it will take trial and error to find the correct cell suspension to use.

Note 2: Always decide on a specific counting patter prior to using the counting chamber to avoid bias. The following is a good rule of thumb for cells that overlap a ruling line: count a cell as “in” if it overlaps the top or right ruling line, and “out” if it overlaps the bottom or left ruling line.
5. Repeat Step 4 for the second counting grid on the counting chamber using the same counting method.

6. To determine a final cell count (concentration in cells per ml) using a standard hemocytometer, use the following:

   **If you used Method 1 –**

   For the one grid on the counting chamber, add the cell counts for squares A through D, and then divide this sum by 4 to get an average cell count. Multiply this last number by $1 \times 10^4$ (this number represents the inverse of the volume of liquid above the large square, effectively a ‘dilution factor’). Finally, multiply the resulting sum by the inverse of the cell suspension dilution used to charge the counting chamber. That is, if you had diluted the cells to $1 \times 10^{-3}$, then multiply the sum by $1 \times 10^3$. The final number derived from this calculation is the concentration (in cells per ml) of the original source of cells, e.g., culture broth. Remember, though, that there are two (2) counting grids on the chamber. Calculated concentrations derived for each should averaged together to generate the actual concentration of the source of cells (in cells per ml).

   As an example of this calculation, let’s suggest that the broth from a yeast culture was diluted 100 fold, or $1 \times 10^{-2}$. After loading the counting chamber and counting one grid, square A was observed to contain 130 cells, squares B and D each contained 120 cells, and square C contained 110 cells. Doing the mathematics,

   $$130 + 120 + 120 + 110 = 480,$$
   $$
   \text{which divided by 4 = 120}
   $$

   This is the average number of cells per large square. This number is then multiplied by the grid dilution factor ($1 \times 10^4$) followed by the broth dilution factor ($1 \times 10^2$), or

   $$120 \times 10^4 \times 10^2 = 120 \times 10^6 \text{ or } 1.2 \times 10^8 \text{ cells per ml}$$

   In short, the yeast broth culture was calculated to contain $1.2 \times 10^8$ yeast cells in every milliliter. However, remember there is a second grid to consider. Let’s assume that similar calculations result in the concentration being $8 \times 10^7$ cells per ml. To achieve the final estimated concentration of the broth culture, you must average the two calculations, or

   $$1.2 \times 10^8 + 8 \times 10^7 = 1.2 \times 10^8 + 0.8 \times 10^8 = 2.0 \times 10^8,$$
   $$
   \text{which divided by 2 equals}
   $$
   $$1.0 \times 10^8 \text{ cells per ml}$$

   The above number is the calculated concentration that one would use if this were an actual experiment in which a cell concentration was needed.

   **If you used Method 2 –**

   For square A, add the counts of the four corner squares, then multiply by 4 to obtain the entire cell count for square A. Repeat this calculation for squares B through D. Using the four numbers derived in this way, determine the cell concentration of the suspension being measured using the calculations described above for Method 1.