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1.0 Purpose

This procedure instructs how to count mammalian cells to determine density, viability, cell generation time and doubling time using a Hemacytometer.

2.0 Scope

This SOP applies to Biopharmaceutical Development Program (BDP) personnel counting mammalian cells with a Hemacytometer.

3.0 Authority and Responsibility

1.1 The Program and Technical Director, Biopharmaceutical Development Program (BDP) has the authority to define this procedure.

1.2 Laboratory personnel are responsible for the implementation of this procedure.

1.3 Biopharmaceutical Quality Assurance (BQA) is responsible for quality oversight of this procedure.

4.0 Equipment and Supplies

4.1 Bright Line Phase Hemacytometer (Counting Chamber), BDP PN 20739.
4.2 12 x 75 mm tubes or equivalent, BDP PN 20751.
4.3 Sterile disposable pipets, BDP PN's 20101, 20103, 20104.

4.4 0.4% Trypan Blue Dye, BDP PN 10095.
4.5 Septihol, BDP PN 30129.
4.6 Inverted Microscope with 10X objective.
4.7 Pipetting device.
4.8 Counting Device.

5.0 Description and Procedure for using a Hemacytometer

5.1 Description of a Hemacytometer (See Attachments 1 and 2)

A Hemacytometer is a modified glass slide that consists of 2 raised chambers. Each chamber is divided into nine 1 mm squares. On the outer edge of each chamber is a beveled groove that guides the cell suspension into the chamber. A cover slip is suspended over the chambers at a distance of 0.1 mm. The volume of cell suspension that will occupy one of the 9 squares will equal 1 X 10^{-4} mL. In practice, the primary 5 squares in each chamber are counted to equal 10 squares. This will give the number of cells within 1 mm^3 or 1X10^{-3} mL (1 mm^3 = 1 µL and 1000 µL = 1 mL). One cm^2 is approximately equivalent to 1 mL. If 10 squares are counted to obtain cells/mL, the total number in 10 squares is multiplied by 1000. If the average per square is calculated from less than 10 squares counted, then the number will need to be multiplied by 10,000 to convert the cell count to cells/mL.

5.2 Procedure for Using a Hemacytometer

5.2.1 Refer to ***SOP 13209, Mammalian Cell Culture – Initiation and Maintenance of Serial Cell Cultures*****,** to prepare a sample of the cells for counting.

5.2.2 In practice, a concentration of 100 to 300 cells per 5 squares (1 chamber, refer to Attachment 3) of the Hemacytometer is desirable. Using a pipet, transfer a sample of the cell suspension to a tube and add the desired volume of Trypan Blue to the cells. Trypan Blue is a stain that will penetrate the membrane of non-viable cells and stain them blue. This stain is required to determine the number of non-viable cells (viability of the culture)

Example: If it is necessary to dilute the cells1:4 to fall within the range stated in 3.2.2, then 1 part of cells are added to 3 parts Trypan Blue. A typical dilution would then be 100 µL cells to 300 µL of Trypan Blue.

5.2.3 Mix gently by pipetting several times with the Eppendorf tip to avoid excessive bubbling.

5.2.4 Position the cover slip that comes with the Hemacytometer over the chambers so that it is even (this cover slip is made especially for a Hemacytometer and a microscope slide cover slip cannot be used). Load both chambers of the Hemacytometer through the groove with the stained cell suspension. Allow the chamber to fill quickly and smoothly by capillary action from the transfer pipet. If visible unevenness is noted in filling, the count will not be accurate. Do not allow the liquid to overfill or underfill the chamber.

5.2.5 Follow the below guidelines when counting cells.

5.2.5.1 All cells in contact with the visual bottom and/or right-hand boundary of each of the 5 squares are not counted.

5.2.5.2 All cells in contact with the visual top and/or left-hand boundary lines are counted. Any cells lying outside of the triple boundary lines are not included in the count.

5.2.5.3 All cells showing dye uptake, and/or darkened small appearance, even though faint, are counted as nonviable cells

6.0 Calculating Viable Cells, Non-Viable Cells and Viability

6.1 Viable and Non-viable Cell Densities

Viable Cells/mL = Number of viable cells counted in 10 squares x dilution factor x 1,000 (1000 µL = 1 mL)

Example:400 cells counted in 10 squares X 2(dilution made in Trypan Blue)

X 1,000 = 800,000/mL

Nonviable cells/mL = Number of non viable cells counted in 10 squares x

dilution factor x 1,000 (1000 µL = 1 mL).

Example:

50 cells X 2 (dilution made in Trypan Blue) X 1,000 = 100,000/mL

NOTE: If an average is calculated per square out of the total squares counted less then 10, multiply the average by 10^4 (Example: average cells counted per square = 40 then the calculation would be 40 X 2 X 10,000 = 800,000/mL.

6.2 Percent Cell Viability

$$\frac{\text{Viable cells/mL}}{\text{Total cells/mL}} \text{X100} = \text{Percent viabiltiy}$$

Example: $\frac{800{,}000}{900{,}000} = 0.888 \text{ X } 100 = 89\%$

7.0 Calculating Generation Number

When growing cells and determining the parameters for growth, it may be necessary, in some situations, to determine how many times the cells will replicate over a given period of time. Below are the equations and examples of how to determine these factors.

NOTE: Since these calculations are performed during the log phase of the growth cycle, the $\log_{10}$ of cell counts in the formula will need to be determined.

7.1 Cell Generation Number over time

Number of generations = 3.32 ($\log_{10}$ final cell count - $\log_{10}$ initial seed)

(The number 3.32 is the reciprocal of the $\log_{10}$ of 2.)

Example: A cell culture was seeded at 1.5 X 10^5 viable cells/mL and grew to 1.6 X 10^6 at day 3.

The $\log_{10}$ of 1.5 X 10^5 = 5.176

The $\log_{10}$ of 1.6 X 10^6 = 6.204

Therefore, 3.32 X (6.204 – 5.176)

3.32 1.028 = 3.41 generations

8.0 Calculating Population Doubling

To determine the population doubling time, the number of generations above will be used along with the total time to determine a multiplication rate first.

The multiplication rate **r** = $\frac{\text{generation number}}{\text{Total time}}$

In the example below, the cells grew for 72 hours and a sample was taken to determine the population doubling time in 24 hours.

Example: $\frac{3.41 \text{ generations}}{72 \text{ hours}}$ = 0.047 generations per hour or 1.128 generations per 24 hours

The Population Doubling time is then $\frac{24 \text{ hours}}{1.128}$ = 21.3 hours per doubling

9.0 Hemacytometer Maintenance

9.1 Chambers and cover slips

9.1.1 Do not allow the cell solution on the Hemacytometer to dry out. Rinse with Septihol immediately after use.

9.1.2 Dry with lint-free wipes or towel and allow to air dry completely before the next use.

9.1.3 Periodically flush the Hemacytometer and cover slip with RODI water or WFI.

9.1.4 Store chambers and matching cover slips in a protective container.

10.0 Documentation

10.1 Permanent records not requiring GMP documentation will be maintained in BQA issued laboratory notebooks. Data on these processes will be recorded daily as work is performed.

10.2 Permanent records of GMP documented work will be maintained in BQA issued laboratory notebooks, Form C-00-09, Cell Split Record for Suspension Cells, C-00-16, Cell Split Record for Adherent Cells, approved Batch Production Records (BPR), and/or other forms as required.

11.0 References and related documents

SOP 13209 ***Mammalian Cell Culture – Initiation and Maintenance of Serial Cell Cultures***

12.0 Attachments

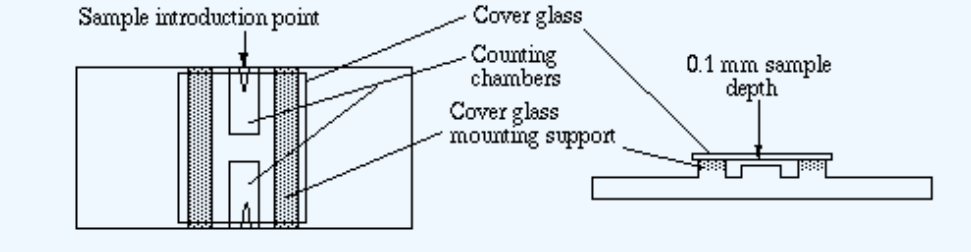
12.1 Top and Side View of a Hemacytometer

12.2 Counting Grids in One Chamber of a Hemacytometer

12.3 Area to Count on One Side of a Hemacytomete

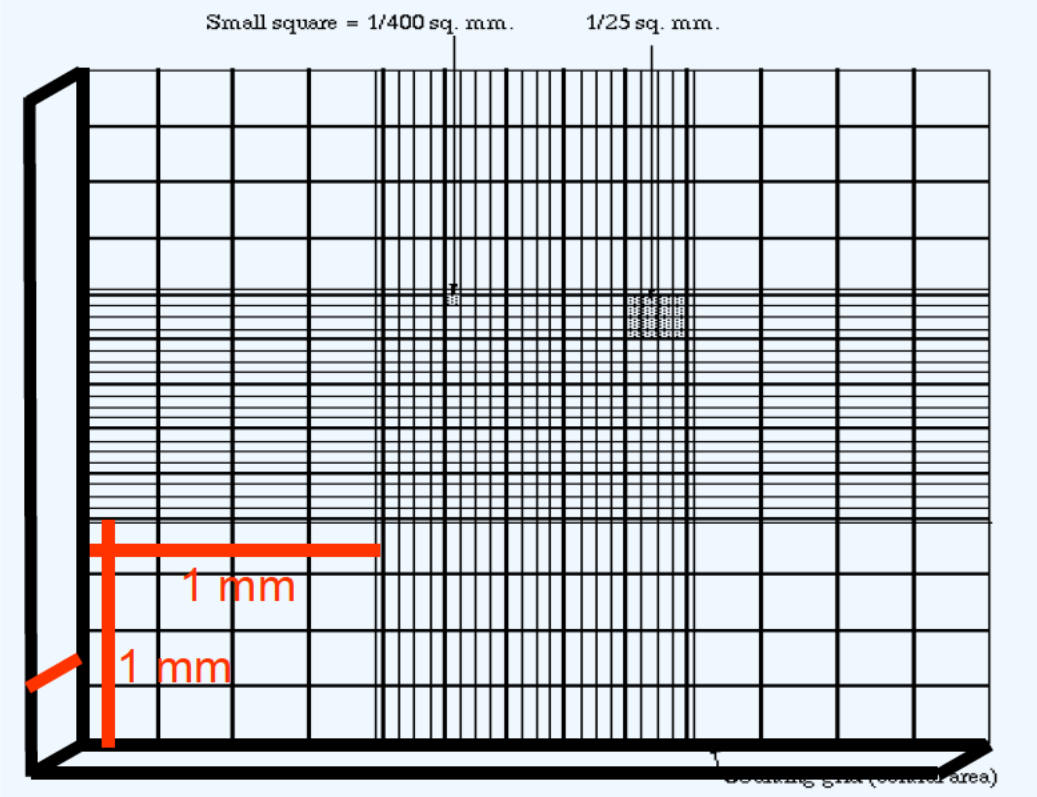
ATTACHMENT 1

Top and Side View of a Hemacytometer



ATTACHMENT 2

Counting Grids in One Chamber of a Hemacytometer



ATTACHMENT 3

Area to Count on One Side of a Hemacytometer