

4.A2 Cell Enumeration

(True 2000)

Rarely are cells counted during routine propagation of cell lines; however, the use of a hemocytometer is a practical method for determining cell numbers in cell suspensions. The Improved Neubauer Hemocytometer consists of two chambers, each of which is divided into nine 1.0-mm² squares. A matching cover glass that is supplied with the chamber is supported 0.1 mm over the squares so that the total volume over each square is 1.0 mm² x 0.1 mm or 0.1 mm³ or 10⁻⁴ cm³. Since 1 cm³ is approximately equal to 1 mL, the cell concentration/mL is the average count per square x 10⁴. Routinely, cells are counted in a total of ten 1 mm squares (fill both sides of the chamber and count the four corner and the middle squares on each side).

To reduce counting errors, count cells that touch the outer line to the left and top of the square, but do not count cells touching the outer line to the right and bottom of the square. Hemocytometer counts do not distinguish between living and dead cells unless a vital stain is used such as Trypan Blue.

Trypan Blue stain is not absorbed by living cells and can be used to distinguish between viable and nonviable cells in cell counts. Use a 1:1 dilution of cell suspension with 0.1% Trypan Blue stain and count only unstained cells. Do not count debris or dead cells that stain blue.

Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted (10 minutes at 500 rpm) and re-suspended in protein-free medium or Hanks salt solution prior to counting.

A. Materials

- Hemocytometer chamber
- 75 cm² flask of cells
- Trypan Blue (0.1% in PBS)
- Microscope
- Dilution tubes (12 x 75 mm)
- Pasteur pipet
- Hanks balanced salt solution, or MEM-0 (MEM w/o serum)
- Trypsin - EDTA
- Pipets 1-mL, sterile, cotton plugged
- 22 x 22 mm cover-slips

B. Procedure

1. Select a healthy (log phase) 75 cm² flask of cells and remove cells from flask surface as described in Section 2, 4.3.A “Subculture Procedures for Flasks.”
2. Re-suspend cells in tissue culture medium (MEM-0). For ease and accuracy in counting, the hemocytometer should be filled with cell suspensions containing approximately 20 to 50 cells/mm² (1×10^5 to 2×10^5 cells/mL). Dilutions vary depending on age of the cells, cell density, and cell aggregation.
3. Aseptically transfer 0.5 mL of the cell suspension into a dilution tube.
 - a. Add 0.5 mL Trypan Blue stain (0.1%).

Note: If cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye.

4. Gently mix to suspend the cells evenly. With a 22 x 22 mm cover-slip in place on top of the hemocytometer, use a Pasteur pipet to transfer a small drop of Trypan Blue-cell suspension mixture to both chambers. Carefully touch the edge of the cover-slip with the pipet tip and allow each chamber to fill by capillary action. Do not overfill or underfill the chambers.
5. Using a microscope with a 10x ocular and a 10x objective, count 10 squares (five from each chamber) as outlined above.
6. Calculate the number of cells/mL and the total number of cells as follows:
 - a. Cells/mL = x (mean) count per square $\times 10^4$ \times Trypan Blue dilution factor.
 - b. Total cells in flask = cells/mL \times total volume of cell suspension.

Example: Total number cells counted in 10 squares = 300 cells
 x count/square = 300 cells/10 squares = 30 cells

$$\begin{aligned} \text{cells/mL} &= 30 \times 10^4 \times 2 \text{ (dilution factor)} \\ \text{cells/mL} &= 60 \times 10^4 \text{ cells/mL} \\ \text{cells/mL} &= 6.0 \times 10^5 \text{ cells/mL} \end{aligned}$$

$$\begin{aligned} \text{Total cells} &= 6.0 \times 10^5 \text{ cells/mL} \times 8 \text{ mL (original volume cell suspension)} \\ \text{Total cells} &= 48.0 \times 10^5 \text{ cells} \\ \text{Total cells} &= 4.80 \times 10^6 \text{ cells} \end{aligned}$$

If the cells/mL calculated is not within the recommended range of cell density, use the following formula to adjust the dilution in your flask before splitting.

$$\text{a. mL medium needed} = (\text{actual cells/mL}) (\text{vol. of cell suspension}) / \text{desired cells/mL}$$

Example: actual count = 6×10^6 cells/mL
 desired count = 1×10^6 cells/mL
 volume of cell suspension = 8 mL

mL medium needed = x

$$\begin{aligned}x &= \text{mL medium needed} = 6 \times 10^6 \text{ cells/mL} \times 8 \text{ mL} / 1 \times 10^6 \text{ cells/mL} \\ \text{mL medium needed} &= 48 \times 10^6 \text{ mL} / 1 \times 10^6 \\ &= 48 \text{ mL}\end{aligned}$$

Since you have 8 mL already in the flask, you would need to add 40 mL of medium to the flask before splitting to get the recommended seeding cell density for each new culture.

5.1 Parasitology Introduction

The following chapter describes identification procedures for four parasitic infections of fish that are commonly included in a fish health inspection. The target parasite species include three myxozoan parasites of salmonid fishes: *Myxobolus cerebralis*, *Ceratomyxa Shasta*, and *Tetracapsula bryosalmonae*; and the cestode: *Bothriocephalus acheilognathi*, which infects members of the Family Cyprinidae. Section 2, Chapter 2 Sampling describes procedures for proper sampling of fish tissues to ensure detection of any of these pathogens during a fish health inspection.

For *Myxobolus cerebralis*, presumptive identification is based on identification of the myxozoan spore stage from pepsin-trypsin digested (PTD) cartilage. Tissues from up to five fish may be pooled for screening by PTD. Identification of the spores is based on morphology. Confirmatory identification is based on identification of the spores in histological sections or on amplification of *M. cerebralis* DNA by the polymerase chain reaction.

For *Ceratomyxa shasta*, presumptive identification is based on identification of myxozoan spore or trophozoite/presporogonic stages from intestinal tissue. Identification of the myxospore stage is sufficient for confirmation of infection. Identification of the earlier stages must be confirmed by amplification of *C. shasta* DNA by the polymerase chain reaction.

For *Tetracapsula bryosalmonae*, presumptive identification is based on identification of the presporogonic stages of the parasite in Leishman-Giemsa or lectin stained imprints of kidney or spleen tissue. Infection is confirmed by identification of these stages in histological sections of kidney tissue or on amplification of *T. bryosalmonae* DNA by the polymerase chain reaction.

For the cestode, *Bothriocephalus acheilognathi*, visualization of any cestode with a pyramidal scolex results in a presumptive positive classification. Confirmation requires verification of morphological characteristics of the scolex.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service, the United States government, and /or the American Fisheries Society. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.