

University of Basrah

College of Science

Department of Pathological Analysis

Course title: Tissue Culture Techniques

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By

Dr.Haider Rasheed Daham

College of Veterinary Medicine University of Basrah

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Cell Culture Protocol : Cell Counting Using a Hemocytometer

Harvest cells (as per protocols 3, 4 and 5)

Re-suspend cells in fresh medium

Remove 100-200 μ l of cell suspension

Add Trypan Blue (dilution factor $\times 2$)

Prepare haemocytometer with coverslip

Fill chamber with cell suspension

Count cells

Calculate concentration (refer to procedure)

Aim

For the majority of manipulations using [cell lines](#), such as transfections, cell fusion techniques, cryopreservation and subculture routines it is necessary to quantify the number of cells prior to use. Using a consistent number of cells will maintain optimum growth and also help to standardize procedures using cell cultures. This in turn gives results with better reproducibility.

Materials

- **Cell Culture Media**: pre-warmed to the appropriate temperature (refer to the ECACC cell line data sheet for the correct medium and temperature.)
- 70% (v/v) alcohol in sterile water.
- Trypan Blue Solution .
- 0.05% trypsin/EDTA in HBSS, without Ca^{2+} / Mg^{2+} .

Equipment

- Personal protective equipment (sterile gloves, laboratory coat, safety visor)
- Waterbath set to appropriate temperature
- Microbiological safety cabinet at appropriate containment level
- Centrifuge
- CO₂ incubator
- Hemocytometer
- Inverted phase contrast microscope
- Pre-labelled flasks

Procedure

1. Bring adherent and semi-adherent cells into suspension using trypsin/EDTA as described previously and resuspend in a volume of fresh medium at least equivalent to the volume of trypsin. For cells that grow in clumps centrifuge and resuspend in a small volume and gently pipette to break up clumps.
2. Under sterile conditions remove 100-200 μ l of cell suspension.
3. Add an equal volume of Trypan Blue (dilution factor =2) and mix by gentle pipetting.
4. Clean the hemocytometer.
5. Moisten the coverslip with water or exhaled breath. Slide the coverslip over the chamber back and forth using slight pressure until Newton's refraction rings appear (Newton's refraction rings are seen as rainbow-like rings under the coverslip).
6. Fill both sides of the chamber with cell suspension (approximately 5-10 μ l) and view under an inverted phase contrast microscope using x20 magnification.

7.Count the number of viable (seen as bright cells) and non-viable cells (stained blue). Ideally >100 cells should be counted in order to increase the accuracy of the cell count (see notes below). Note the number of squares counted to obtain your count of >100.

8.Calculate the concentration of viable and non-viable cells and the percentage of viable cells using the equation

$$\text{Viable Cell Count (live cells per millilitre)} = \frac{\text{Number Live Cells Counted}}{\text{Number of large corner Squares counted}} \times \text{Dilution factor (in this example 2) } \times 10,000$$

$$\text{Non-viable Cell Count (dead cells per millilitre)} = \frac{\text{Number Dead Cells Counted}}{\text{Number of large corner Squares counted}} \times \text{Dilution factor (in this example 2) } \times 10,000$$

$$\text{Percentage Viability} = \frac{\text{No of Viable Cells}}{\text{Total No. of Cells}} \times 100$$

Key Points

1. Trypan Blue is toxic and is a potential carcinogen. Protective clothing, gloves and face/eye protection should be worn. Do not breathe the vapor.
2. The central area of the counting chamber is 1mm^2 . This area is subdivided into 25 smaller squares ($1/25\text{mm}^2$). Each of these is surrounded by triple lines and is then further divided into 16 ($1/400\text{mm}^2$). The depth of the chamber is 0.1mm .
3. The correction factor of 10^4 converts 0.1mm^3 to 1ml ($0.1\text{mm}^3 = 1\text{mm}^2 \times 0.1\text{mm}$)
4. There are several sources of inaccuracy:
 - The presence of air bubbles and debris in the chamber
 - Overfilling the chamber such that sample runs into the channels or the other chamber
 - Incomplete filling of the chamber Cells not evenly distributed throughout the chamber
 - Too few cells to count. This can be overcome by centrifuging the cells, re-suspending in a smaller volume and recounting
 - Too many cells to count. This can be overcome by using a higher dilution factor in trypan blue e.g. 1:10
5. The use of a hemocytometer can be time consuming,

