## Materials and Equipment

Materials

- Fresh cells to be counted
- 0.4% Trypan Blue
- PBS
- 2-1000µl Pipets
- 2-1000µl Pipet Tips

Equipment

- Hemocytometer

## Procedure

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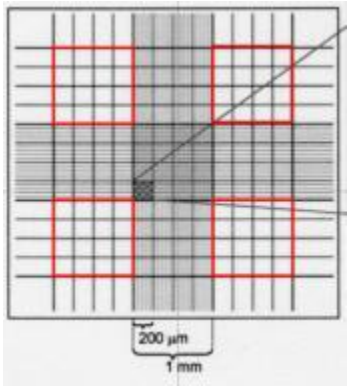
| 1. | Prepare a clean, dry hemocytometer and a cover slip by cleaning both with 70% Ethanol and drying with lens paper to prevent scratching. Lay cover glass on top of hemocytometer to cover counting chamber. |
| 2. | Prepare cell suspension from thawed cells or detached cells. (See How to Passage Cells). Write the volume of the starting cell suspension in milliliters. |
| 3. | Mix the cell suspension thoroughly but carefully trying to avoid bubbles using a sterile serological pipette. Make sure that suspension is homogeneous.<br>NOTE: If there are groups or clusters of cells in the suspension the count will not be accurate. |
| 4. | Transfer 20 µL aliquot of the cell suspension to small tube. |
| 5. | Add equal 20 µl volume of 0.4%(w/v) Trypan Blue to cell suspension and mix well. This makes a 1:2 dilution<br>NOTE: Do not keep cells in TB solution for more than 15 minutes before cell counting (after 10-15min in TB cells start dying and the count will not be accurate). |
| 6. | Mix cells again and transfer 10 µl to hemocytometer chamber, filling the chamber by capillary action.<br>Do not under-fill or over-fill the chamber. Avoid bubbles inside the chamber. |
| 7. | Using the 10X objective, focus on the gridlines of the chamber. Count the viable cells (non-blue) in four corner squares of one chamber. (Reference Figure 1 below) Count the nonviable cells (blue) in the four big squares in each corner of one chamber. Cells that lie on the lines of squares should only be counted if they are touching the top and left-hand lines of each corner square (make this rule to avoid counting the same cell two times).<br>  <br>*Figure 1 Hemocytometer Counting Chamber*<br>NOTE: If the cell number in one small square is vastly more than 100 discard the sample, clean the hemocytometer and prepare new solution by additional dilution of the initial cell suspension. |

<table>
<tr><td>8.</td><td>For cell suspension dilution take a new 20 µl aliquot of cell suspension and add different amount of PBS until there is no greater than 100 cells per each hemocytometer square (Figure 1).<br><br>For example, mix 20 µl of cell suspension with x amount of PBS. Add 20 µl of this solution to 20 µl of TB to achieve desired total dilution (Table below).
<table>
<tr><th>x</th><th>Dilution</th><th>Final Dilution</th></tr>
<tr><td>20 µl</td><td>1:4 (1:2 Cells:PBS dilution x 1:2 Cells in PBS:Trypan dilution)</td><td>4</td></tr>
<tr><td>40 µl</td><td>1:6 (1:3 Cells:PBS dilution x 1:2 Cells in PBS:Trypan dilution)</td><td>6</td></tr>
<tr><td>60 µl</td><td>1:8 (1:4 Cells:PBS dilution x 1:2 Cells in PBS:Trypan dilution)</td><td>8</td></tr>
</table></td></tr>
<tr><td>9.</td><td>If the cell number per square is very low (<5) then the original suspension should be centrifuged down and resuspended in a smaller volume.</td></tr>
<tr><td>10.</td><td>Calculations<br>$$\boldsymbol{Average\ Viable\ Cell\ Number\ per\ square} = \frac{\#\ of\ cells\ per\ square}{4}$$<br>$$\boldsymbol{Total\ \#\ of\ cells} = Avg\ Viable\ Cell\ \#\ per\ square \times dilution\ factor \times 10{,}000 \times Vol.of\ suspension\ (m)$$<br>$$\%\ \boldsymbol{Viability} = \frac{Total\ \#\ of\ viable\ cells}{\text{Total \# of viable} + \text{nonviable cells}} \times 100$$<br>$$\boldsymbol{\#\ of\ cells\ per\ mL\ of\ the\ solution} = Average\ Cell\ Number\ per\ square \times dilution\ factor \times 10{,}000$$</td></tr>
<tr><td>11.</td><td>Clean the hemocytometer by rinsing with $dH_2O$ then 70% Ethanol and drying thoroughly with lens paper.</td></tr>
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