

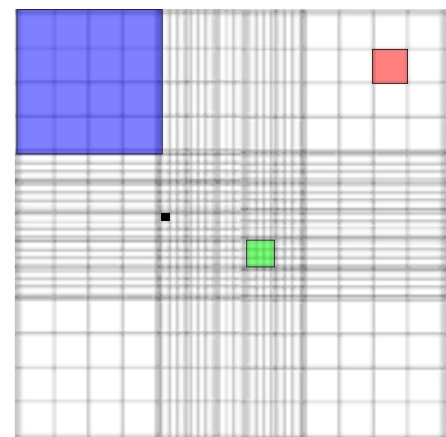
## Cell count and viability assay

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Trypan blue (diamine blue, Niagara blue or vital blue) is a toluidine derived stain with affinity for tissues and dead cells (Ehrlich, 1904). Its name stems from its ability to kill trypanosomes the unicellular protozoa parasites that cause Chagas disease in America and sleeping sickness in Africa. This stain is the most commonly used vital-stain in the world. Vital stains are used to differentiate dead from live cells. In the case of trypan blue, it is normally excluded from live cells with intact cell membranes. Dead or apoptotic cell membranes however, allow the stain to permeate the cells and stain them blue. Trypan blue is not necessary for cell counts but is essential for viability counts. In this procedure a peripheral blood mononuclear cell (PBMC) suspension corresponding to a concentration normally achieved with ficoll preps from blood (see ficoll prep protocol) is mixed with 0.4% trypan blue solution. The stained cells are immediately counted under microscopic observation using a Neubauer haemocytometer. Although the original protocol suggests using equal volumes of cell suspension and 0.4% Trypan blue solution, this ratio is best optimized in each lab as variability in reagents and room temperature may indicate so. In any case, make a mental note of the dilution factor involved. Neubauer haemocytometers are a specialized type of microscope slide that has had a grid of precise dimensions laser etched unto it. The haemocytometer allows an exact volume of cells to be loaded into one or two of its chambers. The grid is composed of nine 1 mm<sup>2</sup> squares (shown in blue on the image below). Each of the four 1 mm<sup>2</sup> squares on each of the corners is in turn divided into sixteen 0.0625 mm<sup>2</sup> squares (shown in red). However, the central 1 mm<sup>2</sup> square is divided into a much finer grid of 25 0.04 mm<sup>2</sup> squares (shown in green) which in turn are composed of 9 squares of 0.0025 mm<sup>2</sup> (shown in black). As the chamber is only 0.1 mm high, each one of these squares has a fixed volume of the cell suspension (see table 1), which allows us to estimate cell concentration based on direct cell counts.

Figure 1

Grid	Colour	Area	Volume
3x3	Blue	1 mm <sup>2</sup>	100 µl
4x4	Red	0.0625 mm <sup>2</sup>	6.25 µl
4x4	Green	0.04 mm <sup>2</sup>	4 µl
1x1	Black	0.0025 mm <sup>2</sup>	0.25 µl



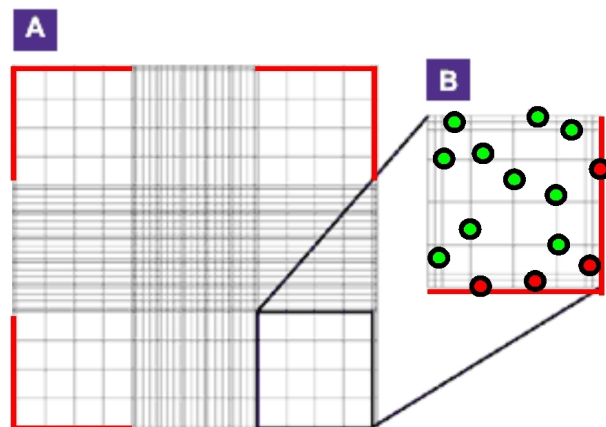
## Procedure

1. Place the 25x microscope objective in the optical path and turn illumination source on.
2. Estimate and record the total volume of the cell suspension from which you will take an aliquot to count the cells.
3. Add 90  $\mu\text{L}$  of 0.4% Trypan blue to a well of a 96 multiwell plate or to a 0.2 mL PCR tube.
4. Mix your cell suspension carefully to homogenize its contents and free cell clusters without killing the cells (finger tapping or flicking of the tube is the norm).
5. Add 10  $\mu\text{L}$  of your cell suspension to the well previously loaded with the trypan blue.
6. Mix the cell suspension and trypan blue by repeat pipetting (5 to 10 times).
7. Take 10  $\mu\text{L}$  of this trypan blue stained cell suspension and loaded unto both chambers of your haemocytometer (see figure 2).
8. Place the haemocytometer on the microscope stage and let the cells stain for 1 minute before you start counting.

Figure 2



Figure 3





9. For PBMC obtained from whole blood samples through ficoll preps we normally count blue (dead) and white (live) cells from the four large corners (i.e., four blue squares such as that one shown in figure 1).
10. Keep a separate count for live and for dead cells.
11. For PBMC obtained from whole blood samples we typically find between 20 and 50 cells (both dead and alive) per each one of these four BLUE squares, which gives us a grand total of between 80 and 200 cells for the four blue squares on the haemocytometer.
12. Cells that are found to lie halfway across the outermost boundary of each square (shown in red on figure 3 A and B) should not be considered for the cell counts.
13. When the cell suspension has more cells than those previously mentioned (more than 50 per blue quadrant), the central quadrant should be used to better estimate their true concentration.
14. Calculate your results.

## Calculations

To calculate cell concentration present in the original cell suspension (the one from which you took the aliquot that was actually used for counting) when the four blue 4x4 corner squares of the haemocytometer are used for counting:

$$\text{Concentration} = \text{Total live count} \div 4 \times \text{dilution factor} \times 10,000.$$

To calculate cell concentration present in the original cell suspension when the central 5x5 square (same size as 4x4 blue square actually) of the haemocytometer is used for counting: (NOTE: This is normally used when cell count given by previous calculation is higher than 200 cells total):

$$\text{Concentration} = \text{Total live count} \div 100 \times \text{dilution factor} \times 10,000.$$

To calculate cell concentration present in the original cell suspension when only 5 green squares belonging to the central 5x5 square is used for counting: (NOTE: This is normally used as an alternative to the previous calculation to speed things up risking accuracy of counts):

$$\text{Concentration} = \text{Total live count} \div 20 \times \text{dilution factor} \times 10,000.$$





To calculate total cells present in the original cell suspension from which your aliquot was taken:

$$\text{Total cells} = \text{cell concentration} \times \text{total volume present in original sample} \\ (\text{the one we recorded at the very beginning}).$$

To calculate cell viability (expressed as a percent):

$$\text{Total number of live cells (white)} \div \text{Total number of cells (both dead and alive)} \times 100$$

## Example

The cell count, concentration and viability is to be assessed on a 3 mL primary lymphocyte culture. Cells are resuspended and cell clusters gently broken up to homogenize suspension. Using a P10 micropipette 10  $\mu\text{L}$  are retrieved from the cell suspension and placed in a PCR tube previously loaded with 90  $\mu\text{L}$  of 0.4% trypan blue (thus generating a 1:10 dilution of our cells). Cells are stained for one minute and then 10  $\mu\text{L}$  are loaded onto the Neubauer slide. The cells present in the four outer 4x4 (blue) squares are counted and classified as dead or alive. After counting we find that we have 455 live cells with 35 dead cells when the cells present in each of the 4 outer blue squares are summed up. Thus:

$$\begin{aligned} \text{Cell Concentration} &= (455/4) \times 10 \times 10 \times 10^4 = 11'375,000 \text{ cells/mL} \\ \text{Total cells present in my primary culture} &= 11'375,000 \times 3 = 34'125,000 \\ \text{Cell viability} &= 455 \div 490 \times 100 = 92.8 \% \end{aligned}$$

## Notes

1. Trypan blue has higher affinity for serum proteins than for intracellular proteins. Should the background of your Neubauer slide appear dark or if dark black clusters show up, wash your cells with PBS or Hanks or any other cell- friendly protein-free medium before staining and counting.
2. Even live cells will ultimately intoxicate with trypan blue and die (and stain blue). Cell viability should not be assessed on cells stained for more than 2 minutes.
3. If cells appear clustered under microscopic observation and after staining, clean the haemocytometer and resuspend or mix cell-trypan blue mix again with your micropipette to break clusters apart.





4. Haemocytometers should be cleaned immediately after use with distilled water and by blotting them with lint-free paper. Do not wipe them clean or dry them out with a wipe as this will scratch the laser-etched grid on them. We normally dry them out with a 70% ethanol spray.

## References

1. Cell Culture Manual 2008-2009, 3rd Edition. Sigma Life Science, pp 230-231.
2. SOP #290 Cell Counting and Viability Determination , Johns Hopkins University . The Genetic Resources Core Facility: The Cell Center . MD, USA.
3. Wikipedia contributors. Hemocytometer. Wikipedia, The Free Encyclopedia. May 22, 2008, at 07:00 UTC. Available at: <http://en.wikipedia.org/wiki/Hemocytometer>.
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## Revision history

- 1.0 Original document.
- 2.0 Changes to document format only.

