

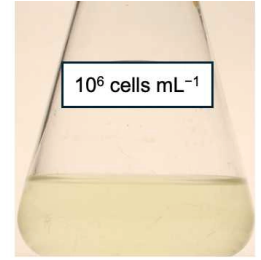


Jul 30, 2024

Determination of *Auxenochlorella protothecoides* Cell Density With a Hemocytometer

DOI

dx.doi.org/10.17504/protocols.io.rm7vzjk12lx1/v1



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DOI: dx.doi.org/10.17504/protocols.io.rm7vzjk12lx1/v1

Protocol Citation: Dimitrios Camacho, Sabeeha Merchant 2024. Determination of *Auxenochlorella protothecoides* Cell Density With a Hemocytometer. [protocols.io https://dx.doi.org/10.17504/protocols.io.rm7vzjk12lx1/v1](https://dx.doi.org/10.17504/protocols.io.rm7vzjk12lx1/v1)

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Protocol status: Working

We use this protocol and it's working

Created: June 12, 2024

Last Modified: July 30, 2024

Protocol Integer ID: 101702

Keywords: Hemocytometer, Cell, Counting, auxenochlorella, cell density



Funders Acknowledgement:

**National Institutes of Health:
Nutritional Copper Signaling
and Homeostasis Grant**

Grant ID: GM 042143

**National Institutes of Health:
Molecular Basis of Cell
Function T32 Training Grant**

Grant ID: 5T32GM007232-44

**US Department of Energy
(DOE), Office of Biological
and Environmental Research
(BER): Systems Engineering
of *Auxenochlorella***

***protothecoides*: from
Photosynthesis to Biofuels
and Bioproducts Grant**

Grant ID: DE-SC0023027

**University of California,
Berkeley, Chancellor's
Fellowship**

Grant ID: N/A

Disclaimer

We would like to acknowledge Usha F. Lingappa and Jeffrey L. Moseley for comments and suggestions.

Abstract

This protocol describes a reliable method for counting the cell densities of *Auxenochlorella protothecoides* cultures using a Neubauer hemocytometer. This method is accurate for cell densities $\geq 10^6$ cells / mL.

Guidelines

The key to obtaining accurate and precise cell counts is to mix the samples well before every step. Cells will settle to the bottom of the tube or flask if left standing, even for a few minutes.



Materials

Consumables:

1. 1.5 mL tubes
2. RAININ P20, P200, P1000 tips
3. Milli-Q H₂O
4. Kimtech Science Kimwipes™
5. 10 mM phosphate buffer, pH 7.
6. 10% bleach
7. 70% ethanol

Equipment:

1. Sterile hood
2. Bunsen burner
3. Igniter
4. Hausser Scientific hemocytometer, cell depth 0.1 mm
5. Hemocytometer cover slide
6. Zeiss Compound microscope (Model AXIO Lab 10) with magnification of 100x, 200x, 400x (Objective x Ocular lens)
7. RIANIN P20, P200, P1000 and respective RAININ tips
8. Tactile mechanical tally counter



Before start

Make the phosphate buffer solution.

1. Make a stock of 1 Molarity (M) NaH₂PO₄ and 1 Molarity (M) Na₂HPO₄
2. Dissolve 5.38 g of NaH₂PO₄ • H₂O (monobasic monohydrate, M.W. = 138 g / mol) in 39 mL of Milli-Q H₂O.
3. Dissolve 8.66 g of Na₂HPO₄ (dibasic anhydrous M.W. = 142 g / mol) in 61 mL of Milli-Q H₂O.
4. Mix solutions together to make 100 mL 1 Molarity (M) sodium-phosphate buffer, pH 7 .
5. Add 10 mL of 1 Molarity (M) sodium-phosphate buffer to 990 mL of MilliQ-H₂O to make a 10 millimolar (mM) phosphate buffer, pH 7 .

Procedure

10s

- 1 Move the culture flask to a clean sterile hood.
- 2 Swirl the culture several times to ensure thorough mixing.
- 3 Using sterile technique, pipette  100 μL of culture into a  1.5 mL tube.
- 4 Dilute the sample until the suspension is almost clear (fig. 1). Record your dilution factors.

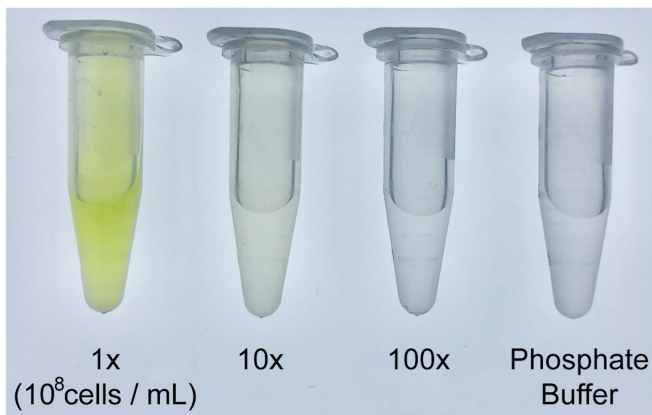





Fig. 1. Dilution of a sample containing 10^8 cells/ mL. 100x was chosen for counting

- 4.1 Dilute samples with  10 millimolar (mM) sodium-phosphate  7 or with the growth medium in which the cells were cultured. Dilutions generally do not exceed 200-fold.
- 5 Remove the hemocytometer from its protective case and wipe both the hemocytometer and cover slide with a KimwipeTM.
- 6 Place the hemocytometer on a KimwipeTM. Place the cover slide over the hemocytometer
- 7 Pipette  10 μL of water into both chambers of the hemocytometer. The cover slides have a coating on one side which alters the dispersion of the Milli-Q H₂O and your sample on the hemocytometer.

- 7.1 ⌚ 00:00:05 If the H₂O is not uniformly dispersed within ⌚ 00:00:05, (fig. 2 A) you may have the wrong side of the cover slide facing down. Wipe the cover slide and hemocytometer with Kimwipe™, then flip the cover slide over. Retest the dispersion of H₂O. 10s

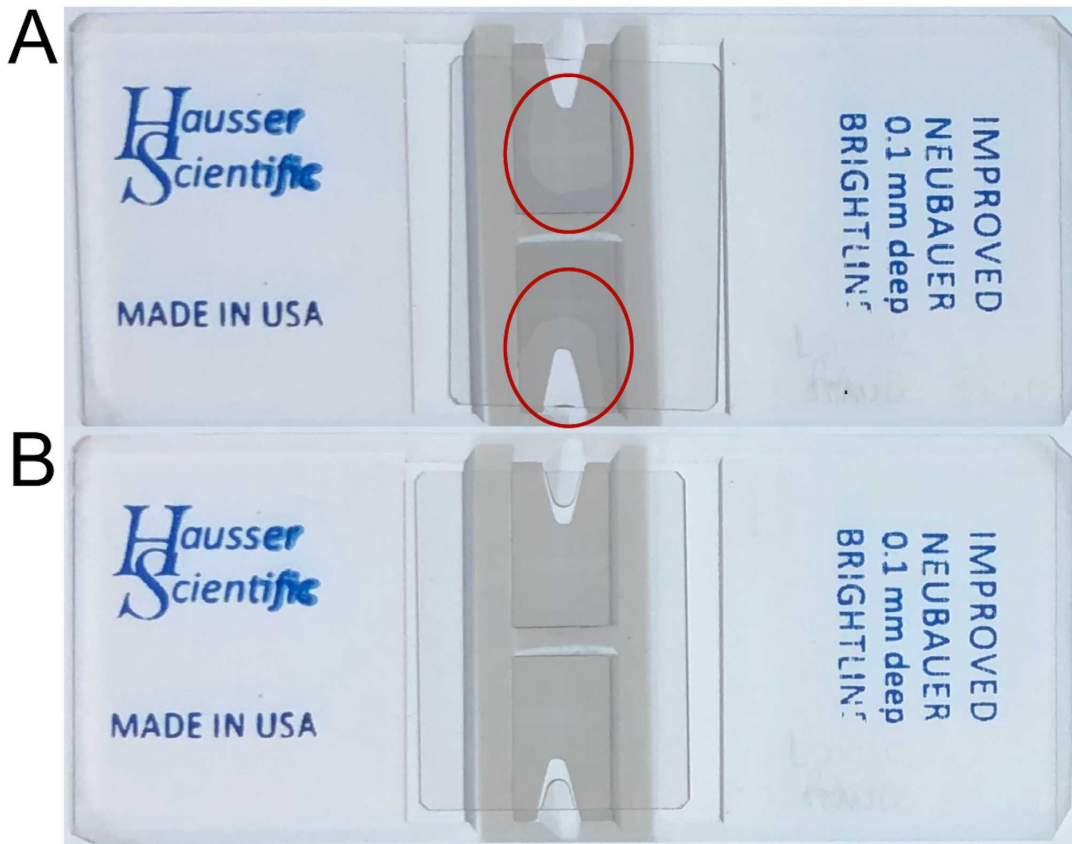


Fig. 2. Picture of Neubauer hemocytometers 5 s after H₂O is dispensed into each well. A) Incorrect cover slide orientation results in incomplete dispersion of H₂O highlighted in red circles. B) Correct orientation of cover slide allows for homogeneous dispersion of H₂O within 5 s.

- 8 Use a Kimwipe™ to wipe the hemocytometers dry.
- 9 Ensure that the sample is well mixed and pipette 🧴 10 µL of sample into each of the two chambers.

- 10 Remove the dust cover from the microscope. Rotate the nosepiece to set the objective lens to 20x. Rotate the turret condenser to the brightfield setting denoted as BF. Lower the microscope stage using the coarse focus knob. Switch the light source on.

Safety information

Warning: For your safety, decrease the light intensity before looking into the eyepiece.

Adjust the inter-pupillary distance by pushing or pulling the eye tubes together or apart.

- 11 Hold the stage clip open, set the hemocytometer on the microscope stage, and release the stage clip gently to secure the hemocytometer.

- 11.1 Carefully raise the stage using the fine focus knob until the gridlines of the hemocytometer come into focus.

Warning: The cover slide will collide with the objective lens if the stage is raised too high. This may result in damage to the cover slide, hemocytometer, and objective lens.

- 11.2 Use the diopter adjustment to compensate for the difference of vision between your two eyes.

Close one eye and adjust the stage using the fine focus knob to focus on the gridlines of the hemocytometer.

Open the opposite eye and close the other eye. Adjust the focus using the diopter adjustment without touching the fine adjustment knob.


- 11.3 Slowly increase the light intensity until you are comfortable with the contrast of cells and the background.

- 12 The contrast for transmitted light brightfield microscopy can be optimized according to the Köhler illumination technique, summarized below.

- 12.1 Raise the condenser using the substage height adjustment knob to max height.

Make sure that the condenser does not collide with the bottom of the hemocytometer. There is an adjustable stop screw that you may set so that the condenser never collides with the hemocytometer.

- 12.2 Close the luminous-field diaphragm until a ring is visible.

- 12.3 Center the luminous field diaphragm using the two centering screws.
- 12.4 Open the luminous field diaphragm until the edge of the diaphragm disappears from the field of vision.
- 12.5 Remove one eyepiece from the tube socket.
- 12.6 Look into socket with your naked eye.
- 12.7 Use the lever on the condenser to adjust the aperture so that the luminous field diaphragm is about $\frac{2}{3}$ – $\frac{4}{5}$ of the diameter of the exit pupil of the objective.
- 12.8 Replace the eyepiece. These are the optimal settings for most applications. Repeat steps 12 to 12.7 after switching between objective lenses.
- 13 After pipetting the sample into the chambers, wait  00:01:00 for the sample to disperse evenly in both chambers. Cells should settle in place and should stop moving. All cells should also come into the same plane of focus as the gridlines as they become evenly suspended in the Z-axis.
- 14 Count between 100 – 200 cells inside the triple grid lines that outline the border of the 1 mm x 1 mm grid (5 × 5 squares) (fig. 3). Cells that are partially through the triple grid line should be counted only if more than half of the cell is inside of the innermost line; if more than half of the cell is outside the innermost line, it should not be counted (panel B, fig. 3).

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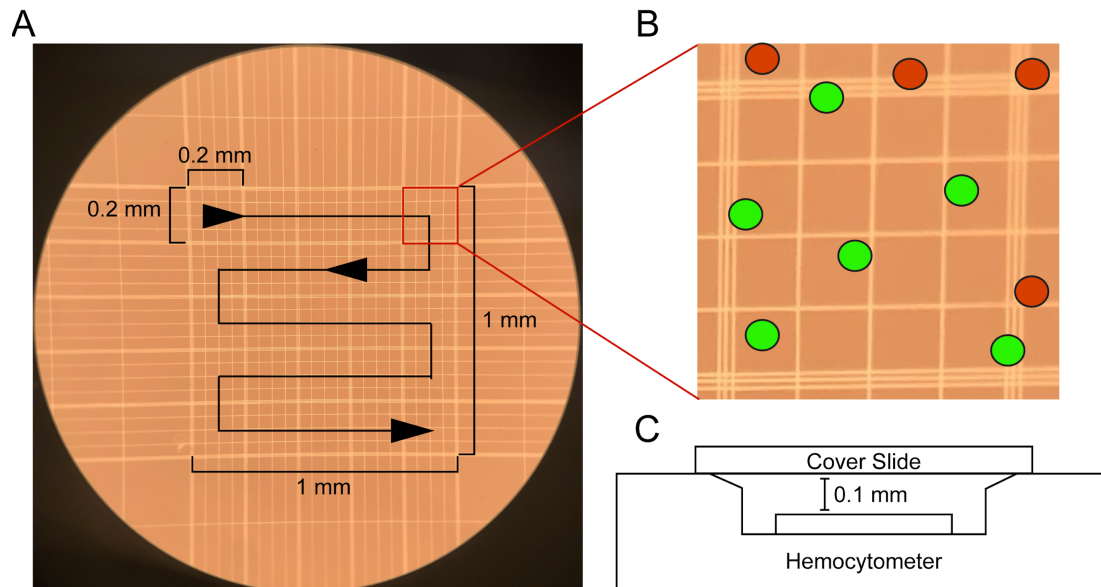


Fig. 3. View of hemocytometer through a brightfield compound microscope. A) 100x magnification of hemocytometer without cells. Distances between gridlines and the recommended counting pattern are indicated. B) Zoomed in image of 0.2 mm × 0.2 mm grid. Green circles represent cells that should be counted whereas red circles represent cells that should not be counted. C) Side view of a hemocytometer indicating the distance between the cover slide and the hemocytometer.

- 14.1 If more than 10 cells are present within the first 0.2 mm × 0.2 mm grid alone, re-dilute the sample and record the new dilution factor.
- 15 Calculate the average number of cells counted across both chambers and multiply this number by your dilution factor and 10^4 cells. This number is the cell density (cells / mL). See calculation below.

$$\text{volume} = l \times w \times h = 1 \text{ mm} \times 1 \text{ mm} \times 0.1 \text{ mm} = 0.1 \text{ mm}^3 = 0.1 \mu\text{L}$$

$$\text{cell density} = (\text{cells counted} / 0.1 \mu\text{L}) \times 10^4 = \text{cells} / \text{mL}$$

- 16 After completing the count, carefully lower the microscope stage and remove the hemocytometer. Turn off the microscope's light source and replace the dust cover.
- 17 Remove the cover glass and wipe the cover slide and hemocytometer clean with Kimwipe™. Pipette 10 μL of Milli-Q H_2O into each chamber. Remove the cover slide and wipe it dry with a Kimwipe™. Clean the hemocytometers with 70% ethanol or 10% bleach, rinse with Milli-



Q H₂O, and dry with a Kimwipe™. Store the hemocytometer and cover slide in their protective case.

- 18 Autoclave or kill all samples and dilutions with [M] 10 % (v/v) Bleach.