1. Overview/Purpose

The purpose of this SOP is to determine proper techniques for counting yeast cells in beer samples.

2. Summary

This document applies to cellermen and brewers needing to quantitate the amount of cells (living and dead) and cell viability in a beer sample.

3. Safety/Environmental Considerations

Hazard	Precaution
Methylene Blue	Wear gloves when handling the reagent or samples containing the reagent. Dispose of samples containing the reagent in the specifically-labeled waste bottle.

4. Incident History

4.1. None

5. Definitions and Abbreviations

5.1. Definitions

5.1.1. Viable = Living cells

5.1.2. Non-viable = Dead or non-living cells

5.1.3. Weak Cells =Living cells that stain slightly blue

5.2. Abbreviations

5.2.1. SOP = Standard Operating Procedure

5.2.2. PPE = Personal Protective Equipment

5.2.3. mL = milliliters

5.2.4. µL = microliters

6. Equipment Illustration Drawings

Illustration #1

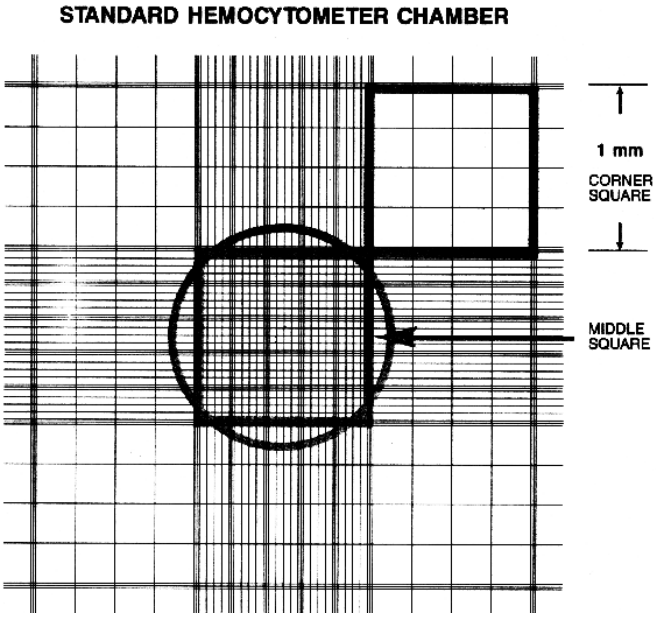

Illustration #2 5x5 grids

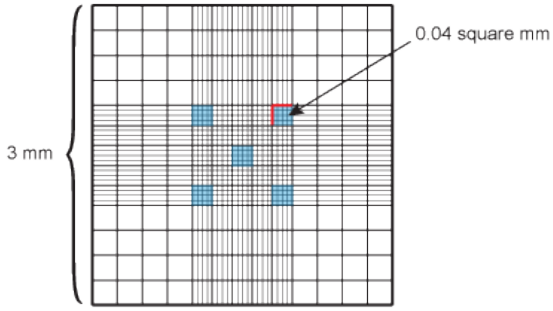

Illustration #3 For the image above the 5 4x4 squares that are highlighted blue are the grids to be counted for quantification when cell concentration is 1.0E7 or greater.

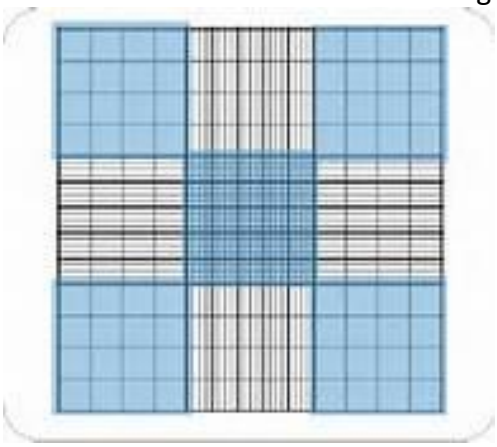

Illustration #4 In the image above the 5 4x4 squares that are highlighted blue are the grids to be counted for quantification when cell concentration is 1.0E6 or smaller.

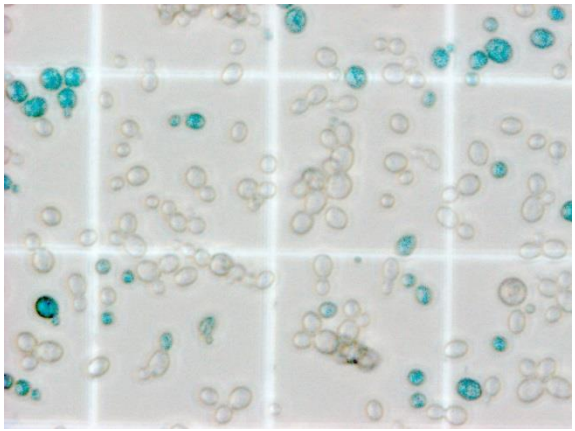

Illustration #5 Viable cells appear transparent, non-viable cells stain blue.

7. Tools and Equipment

Tool/Equipment	Use (if explanation is needed)
Hemocytometer slide and cover slips	Used to count cells in a known volume for quantification. NOTE: The cover slip is specific to the hemocytometer**. A normal glass cover slip cannot be used.**
Compound Microscope	To view the cells in the hemocytometer for counting under magnification
2 Counters	Keeps track of cells being counted (one for viable and one for non-viable cells)
1mL pipet tips	Used to make dilutions
200µL and 20µL pipet tips	Used to make dilutions and load sample into hemocytometer
2 mL Tubes	To hold the sample, reagent, and dilutions
1% Methylene Blue Stain	Used to stain the cells to determine cellular viability
diH_2O	Used for dilutions and to clean the hemocytometer

8. Sample preparation, counting, calculations, and clean-up

8.1. Collect a sample to be counted

8.1.1 Sample can be taken from yeast slurry, newly inoculated wort, fermenting beer, brite tank, etc. Maintain sterility of the sample port.

8.2. Dilute and stain the cells

8.2.1 In order to accurately count live and dead cells adequate sample dilutions are necessary. Amount of dilution required depends on anticipated cell density in the medium.

8.2.2 First dilute the sample 1:2 in water with a final volume of 1 mL. Use a 1 mL pipette to first dispense 500 µL of water into a 2 mL tube. Then use the same tip to take a 500 µL aliquot of the sample that is to be counted and add it to the 500 µL of water, wash out tip in water by pipetting up and down several times.

8.2.3 Dilutions can then be performed using the above 1:2 diluted sample. Final dilution amount will change based on the total cells in the solution. Final dilution

will also need to take into consideration the 20 uL of stock dye solution that needs to be added to 980 uL of final diluted sample. The total amount of cells counted should range from 80-200. Dilutions will need to be re-performed if cell count is out of this range.

8.3. Sample preparation example – Fermenting beer with a cell concentration of 1.0E8

8.3.1. The sample preparation outline below is a step-by-step method of preparing a corn mash medium sample containing 1.0E7 – 1.0E8 cell concentration.

8.3.2. The anticipated cell concentration is 1.0E7 – 1.0E8, hence a 1:20 dilution series is chosen as well as counting the 5 x 0.04 mm^3 squares in the center square of the hemocytometer. These parameters are chosen because in order to get useful results, a cellular count of 80 – 200 cells must be achieved.

8.3.3. <u>Step 1 – Serial Dilutions</u>

8.3.3.1. First, perform the 1:2 dilution in water as done above.

8.3.3.2. Perform a 1:10 dilution of the previously diluted sample by adding 880 uL of water to a 2 mL microfuge tube and 100 uL of the 1:2 diluted sample.

8.3.3.5 Add 20 uL of stock methylene blue to 2 mL microfuge tube and vortex 15 seconds (total volume is now 1 mL).

8.3.4. <u>Step 2 – Load the sample on the hemocytometer slide</u>

8.3.4.1. Using a 20 µL pipette, slowly add 15 µL of the dyed solution to each of the hemocytometer loading chambers.

8.3.4.2. The liquid will fill the chamber via capillary action.

8.3.4.3. The entire chamber needs to be loaded, avoid overfilling or underfilling the chamber as this could produce bad results.

8.3.4.4. If air bubbles are observed or if the chamber is underfilled then the chamber will need to be cleaned and reloaded.

8.3.5. <u>Step 5 – Preparing to count the hemocytometer sample</u>

8.3.5.1. Place the hemocytometer slide on the microscope's stage.

8.3.5.2. Using the coarse and fine focus knobs, bring the hemocytometer counting grid into focus using the 10X objective. A series of lines making up the grid as well as the yeast cells should be visible.

8.3.5.3. Locate the center of the counting grid which is made up of a 5x5 grid of 4x4 grid squares (See illustration #3).

8.3.5.4. Count the boxes highlighted in illustration #3 using the two counters, 1 for living cells (unstained and light blue) and 1 for dead cells (dark blue), see illustration #6.

8.3.5.5. Budding yeast cells should be counted as one cell if the bud is less than half the size of the mother cell. If the bud is ≥ ½ the size of the mother cell then both cells are counted. (See Illustration #7)

8.3.5.6. Two of the outer lines of the 4x4 box will be considered "In Bounds" and two of the lines will be considered "Out of Bounds". If any part of a cell touches the "In Bounds" line it is considered in the grid. If any part of a cell touches the "Out of Bounds" line it is considered out of the grid and should not be counted. The boundary lines are just the inner most of the 3 stripe "triple" line. See the appendix for questions.

8.3.5.7. If the total number of counted living cells and dead cells (for all 5 of the 4x4 squares) is outside the range 80 – 225, then a new appropriate dilution must be prepared for viewing on the hemocytometer.

8.3.5.8. Ensure that all 5 of the designated 4x4 squares are counted.

8.3.6. <u>Step 7 – Calculations</u>

8.3.6.1.1. Cells/ mL = number of cells counted*dilution factor * 5 * 10,000

8.3.6.1.2. The number of cells counted is the total number of living and dead cells counted from all 5 squares on one side of the hemocytometer slide, assume that 100 live cells were counted and 5 dead cells where counted, that gives us a total cell count of 105 cells.

8.3.6.1.3. Cells/ mL = 105 *25 * 5 * 10,000
Cells/ mL = **131,250,000** or **1.31E8**

8.3.6.1.4. To calculate the viability divide the number of living cells by the total number of cells and then multiply that number by 100.

8.3.6.1.5. % Viability = (Total # of cells counted/# of living cells counted) * 100
% Viability = (105/100)*100 = **95.2%**

8.3.7. **In the event too few cells are counted despite a low dilution you will need to count a different grid on the slide. See the Trouble Shooting Guide for further clarification.**

8.4. **Cleaning the Hemocytometer Slide**

8.4.1. Do not allow the sample on the slide to dry.

8.4.2. Spray the slide with 70% EtOH to clean off the sample, kimwipes may be used if the stain and sample do not wash away.

8.4.3. Use diH_2O to rinse away any additional sample or remaining EtOH.

8.4.4. Allow the slide to air dry, if in a hurry a kimwipe may be used carefully to speed the drying process.

9. Original Approval

Written By: Matthew I Peetz	Date: 11/21/2018
Approved By:	Date: MO/DY/20YR

10.Revision History

Revised By: NA	Date: MO/DY/20YR
Approved By:	Date: MO/DY/20YR
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