	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*	MQIP Milk Quality Improvement Program

Title: Direct Microscopic Count for Bacteria or Somatic Cells			
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Direct Microscopic Count for Bacteria or Somatic Cells

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SECTION 1 INTRODUCTION

1.1 Purpose

The direct microscopic method makes it possible for milk or certain milk products to be examined for numbers of bacterial clumps or somatic cells. In the former application, the direct microscopic clump (DMC) count, bacterial clumps may be counted, and, at the same time, an evaluation made of the distinctive morphology and arrangement of bacteria. To a limited extent, cell morphology and configuration of clumps allow the analyst to assess the cause(s) of quality problems. In the latter instance, large numbers of somatic cells indicate mastitis or other abnormal conditions of the udder. Accordingly, the microscopic method as regards somatic cells is referred to as the direct microscopic somatic cell count (DMSCC). As such, it reflects the research findings of several investigators and is applied as one of the officially recognized procedures for confirming somatic cell counts, which were previously estimated by one of several screening tests. Test results are reported in actual counts of bacterial clumps or individual somatic cells.

The microscopic method has limited, but possibly beneficial, application for determining the extent of bacterial contamination in pasteurized fluid milk and cream. Because dead cells lose some of their ability to take up stain and because numbers of bacteria are generally low in pasteurized milk products, this method is not used to determine compliance with finished product standards. But because large numbers of bacteria in uncultured products give evidence of unsanitary conditions, no matter what type of organisms are present or whether they are viable (living) cells, this method does have value as a quality control procedure.

1.2 Scope

This SOP applies to the Milk Quality Improvement Program Laboratory, and is taken in full from Standard Methods for the Examination of Dairy Products, 17th ed.

This method is applicable to raw milk and to dry milk products, with limited application to processed fluid milk products.

1.3 Definitions

- **Direct microscopic counts** and **direct microscopic somatic cell counts** are, with certain limitations, the number of bacterial and somatic cells, respectively, per milliliter or per gram of dairy product.
- **L-W (Levowitz-Weber) stain** is the stain used for both DMC and DMSCC. It contains solvents to dissolve the butterfat from the milk smears and a dye to stain cells.

- The hydrophobic coating on **Angstadt-Weber Milk Smear Slides** delineates the circles on these slides to permit rapid smearing of 0.01 mL of milk over an exact 1 cm^2 area. The slides have 4 hash marks per circle, indicating the exact beginning and ending points for true horizontal and vertical diameter strip counts. These indicator marks eliminate the need to search for your beginning point – the horizontal or vertical apex of each circle. Simply locate the indicator mark and begin counting.
- **Microscopic factors (MFs)** are values by which the average number of bacterial clumps or somatic cells per field is multiplied to calculate the respective count. The area of a single field determines the amount of milk film that can be seen at any one time.

1.4 Safety

Hazard statements for L-W stain:

Highly flammable liquid and vapor. Harmful if swallowed. Harmful in contact with skin. Causes skin irritation. Causes serious eye damage. Harmful if inhaled. May cause allergy or asthma symptoms or breathing difficulties if inhaled. May cause genetic defects. May cause cancer. May damage fertility or the unborn child. Causes damage to organs. Causes damage to organs through prolonged or repeated exposure.

Here is the link to the full SDS.

Only use L-W stain in the fume hood. Cover the stain jar while staining the slide and return the cover to the stain jar as soon as you have removed the stained slide.

SECTION 2 MATERIALS

- Milk sample(s)
  - Raw samples are typical
  - Pasteurized samples will work if they are creamline, i.e., not homogenized.
  - Homogenized samples will not work for this test.
- Angstadt-Weber Milk Smear Slides
- A 10 or 20 µL micropipette and pipette tips
- A bent-point biological dissecting needle
- A level heating block set to 40-45°C
- Forceps
- L-W stain in a stain jar
- Paper towels

- A water bath (DMSCC only)
- 500 mL beaker, glass or plastic
- Glass thermometer
- A compound light microscope with a 100× oil immersion lens
- Immersion oil
- A stage micrometer slide
- A hand tally (or download a phone app)

SECTION 3 PROCEDURES

This section is reproduced faithfully from Chapter 10 of Standard Methods for the Examination of Dairy Products, 17th ed. Notes from the author of this document are in blue, bold text.

1. Preparation of test sample and milk smear:
    a. Warm samples to be tested for a DSMCC to 40°C immediately before transferring them to slides. Do not warm samples to be used for a DMC of bacteria, or for any other purpose.
    b. Mix sample by shaking 25 times in 7 seconds with a 1-foot (30 cm) movement. Allow it to stand until the foam disperses (but no more than 3 minutes) to obtain a virtually foamless test portion.
    c. Legibly and indelibly identify each film as it is prepared; that is, place a number or other symbol on the edged margin of the slide.
    d. Use a separate (new) tip for each sample. Insert tip not more than 1 cm below surface of the sample, avoiding foam, and then depress the plunger to the first stop. Slowly release the plunger completely. With the tip still below dilution surface, depress plunger to the first stop again and slowly and completely release the plunger and then remove the tip from the sample.
    e. Hold the pipeter horizontally and carefully remove the excess milk from exterior of tip by wiping away from the tip. Do not wipe over the tip.
    f. Hold the pipeter vertically and discharge the test portion near the center of the test area **on the Angstadt-Weber Milk Smear Slide** and touch off on a dry spot. Discharge used tips into a biohazard bag.
    g. Spread the milk film with the tip of a bent-point needle (hold the needle as vertically as possible, do not lay down, as a hockey stick).
    h. Dry the milk film on a level surface at 40° to 45°C within 5 minutes, but do not heat rapidly. Protect from contamination by dust, etc. during drying.
2. Staining the milk smear:
    a. Place the stain in a container that can be covered to prevent evaporation while the slides are in the stain solution. Repeated use of stain in an uncovered vessel may result in the formation of precipitate.
    b. Submerge slides of the fixed, dried films, singly or in multiples, into the stain for 2 minutes. Remove and drain off the excess stain by resting the edge of the slide in a near vertical position on absorbent paper.
    c. Dry the slides thoroughly. **i.e., Allow the slides to dry thoroughly while resting on absorbent paper.**
    d. Rinse the dried, stained slides in 3 changes of tap water at 35° to 45°C. **Use a thermometer.**
    e. Drain the slides and allow them to air dry completely before examining the films under the microscope. **Do NOT try to cheat and use bibulous paper like you would for a Gram stain slide. These smears are much more delicate, and you will ruin them and have to start over again from the very beginning.**

Notes:

Proceed as above in preparing and staining cream films. When small numbers of raw milk films are to be stained, flooding the slides may be more practical than submerging them. Care must be taken to limit flooding exposure so that evaporation does not progress to the point where precipitation of dye occurs. Always work with L-W stain in the fume hood.

Discard used stain whenever the solution becomes contaminated or otherwise unsuitable. When solution is not in use, keep containers tightly closed to prevent evaporation. This applies to surplus supplies as well as to working batches of stain. Avoid using containers or closures that may dissolve or disintegrate and thus contaminate staining solutions. Store solutions in a relatively dark, cool place but do not refrigerate.

3. Examining films for bacteria or somatic cells:
    a. **See DMC and DMSCC interpretation guides with numerous example photomicrographs appended to the end of this document. These incredibly helpful guides were prepared by Cornell Dairy Extension Senior Extension Associate Emeritus Steve Murphy.**
    b. To obtain estimates of the bacteria or somatic cell count per milliliter, examine each film with an oil-immersion objective after placing 1 drop of immersion oil on the film.
    c. When counting bacteria **(DMC)**, count as separate clumps any 2 single cells or clumps of cells (apparently of the same type) that are separated by a distance equal to or greater than twice the smallest diameter of the 2 cells nearest each other. Regardless of the proximity to each other of the cells of different types, count each type as a separate unit.
    d. When making DMSCCs, count only those somatic cells with an identifiable stained nucleus. The nucleus is stained dark blue (bovine) or blue or blue-green (caprine). For polymorphonucleated cells, count as a single cell any that has two or more discernible nuclear lobes; for other somatic cells, count any that has a nucleus that appears to be essentially intact. Do not count if the nucleus is less than 8 microns in diameter. If in doubt about a cell, which may in fact be only a fragment, do not count it.
    e. Determining the counting factor: This counting method uses as boundaries a single strip that runs the width of the microscopic field and across the diameter of the milk film. A single strip factor (SSF) must first be calculated:
    $Area\ of\ a\ single\ strip\ (mm^2) = 11.28\ \ mm \times D\ in\ mm$
    where D is the diameter of the field. The field diameter is measured using a stage micrometer calibrated in 0.01-mm divisions.
    f. Determine the number of single strips in the 0.01-mL milk film by dividing 100 mm^2 (area of the 0.01-mL milk film) by the strip area:
    $$Number\ of\ single\ strips = 100\ mm^2 / area\ of\ single\ strip$$
    g. Convert the number of single strips in 0.01 mL of milk to a 1-mL basis by multiplying the number of single strips in 0.01 mL by 100:

$SSF = Number\ of\ strips\ in\ 0.01\ mL \times 100$

h. Example:
$Diameter\ of\ a\ microscopic\ field = 0.160\ mm$
$Length\ of\ strip\ (diameter\ of\ a\ 1 - cm^2\ circle = 11.28\ mm$
$Number\ of\ single\ strips\ in\ area\ of\ milk\ film\ (0.01\ mL\ of\ milk): 100 \div 1.80 = 55.6$
$SSF = 55.6 \times 100 = 5{,}560$

i. Counting procedure: To make a single-strip count, focus on the film edge in the oil-immersion field that appears to be at the maximum horizontal or vertical excursion. Traverse the entire diameter of the milk film, counting those cells within the strip and also those cells touching one edge of the strip (top or bottom if reading horizontally, or left or right if reading vertically). Do not count bacteria or somatic cells that touch the other edge. During scanning of the strip, continually make fine focusing adjustments.

j. Expression of Results:
$Count\ per\ milliliter = Number\ of\ somatic\ cells\ and/or\ bacterial\ clumps\ in\ a\ single\ strip \times SSF$

k. Example:
Assuming an SSF of 5,560, if an analyst counts 84 cells or clumps in a field-wide strip, the count is computed as follows:
$Count\ per\ milliliter = 84 \times 5{,}560 = 467{,}040$
Round off the result to 2 significant figures. The reported count becomes 470,000 per milliliter.

4. Test Report and Interpretation:
   a. Report counts only to the first 2 left hand digits of the estimate, followed by the appropriate number of zeroes. If the third digit is 5, round according to the following rules:
      i. If the second digit is odd, round up, and raise the second digit by 1.
         1. For example, 235 becomes 240.
      ii. If the second digit is even, round down, and delete the 5 and report the second digit as is.
         1. For example, 225 becomes 220.
      iii. A way to summarize these rules is to round the second number to the nearest even digit when the third digit is 5.
   b. **We're not a reference lab, so this isn't really important. For research purposes, you can round (or not round) the results however you choose.**

A gentle note on interpretation: Listen, milk has a lot going on. There's a bunch of stuff in there that kinda sorta looks like it might be bacterial cells. It isn't. It never is. Bacterial cells in a direct microscopic bacterial count look exactly like bacterial cells in a Gram stain – that is, they are strongly pigmented, have sharply defined edges, and are clearly either rods or cocci beyond any possible twist of the imagination. If you see real bacterial cells in a direct microscopic bacterial count, you will know right away beyond any shadow of doubt. If there's any question in your mind about what some random clump of stuff is, then it's not bacteria. That's all.

A second note on interpretation: The volume of milk that actually gets counted in a direct microscopic bacterial count is so infinitesimally small that you are unlikely to find a single bacterial cell or clump in any milk sample of good quality or even of marginal quality. This estimation technique, with emphasis on the word estimation, is effective only for the most egregiously, disgustingly contaminated samples. And even in those, there may still only be a few cells visible in the fields that you count.

SECTION 4 TROUBLESHOOTING

As in other methods of enumerating bacteria or somatic cells, results of the direct microscopic method are to be considered as estimates only. In general, the most important factors in determining accuracy, precision, and reproducibility are the training and skill of the analysts. Even with exacting techniques, however, replicate estimates may vary appreciably. The extent of variations in counts made by different analysts as well as by the same analyst has been reported for certain screening and confirmatory methods. Among factors responsible for such variation are inaccuracy in measuring 0.01-mL quantities of sample, faulty preparation and staining of slides, failure of some bacteria to stain, the minute amount of milk actually examined in counting, irregularity in the distribution of bacteria or somatic cells in the films, failure to count a sufficient number of cells, poor microscopy due to inadequate or excessive illumination of the microscope, poor focusing or improper use of colored filters, failure to dry the films on a level surface, eye fatigue, analyst inexperience, and errors in observation and calculation. **Just do the best you can, it will be ok.**

SECTION 5 REFERENCES

Fitts, J. E., D. Laird, and R. T. Marshall. 2004. Direct Microscopic Methods for Bacteria or Somatic Cells. Pages 269-280 in Standard methods for the examination of dairy products. 17th ed. H. M. Wehr and J. F. Frank, ed. American Public Health Association, Washington, DC.

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	2020-12-10	sjr267	Original SOP
Version 2			
Version 3			