



FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM

Standard Operating Procedure

Title: **VSL Microbiological and Chemical Analysis**

SOP #: **7.22**

Version: 02

Revision Date: **4/21/20**

Effective Date: **Date Upload**

Author: **Nate Henderson**

Approved by: **fill in after Martin OK's**

VSL Microbiological and Chemical Analysis

FILE NAME: VSL SOP.doc

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Last Modified on: April 21st, 2020

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EFFECTIVE DATE: Date of Approval

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(date)



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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to set forth standard guidelines for microbiologic and chemical analysis of Voluntary Shelf-Life (VSL) samples.

1.2 Scope

This SOP applies to the Milk Quality Improvement Program (MQIP) Lab

1.3 Definitions

PPC: Post-pasteurization contamination

Stress Test: Test that stresses pasteurized milk at 21°C for 18 hours for rapid detection and characterization of post pasteurization contamination (PPC)

PSC: psychro-tolerant spore count

MSC: mesophilic spore count

TSC: thermophilic spore count

Spore Pasteurization: Raw milk test performed on Day Initial that mimics pasteurization by heating samples to 80°C for 12 minutes, followed by pour plating using molten SPC agar and incubation.

PBC: Psychrotrophic Bacteria Count is a test that is designed to enumerate cold tolerant bacteria present in raw milk. This test is performed by plating raw milk on Standard Plate Count (SPC) media followed by a 10 day incubation at 7°C.

CVTA: Crystal Violet Tetrazolium Agar is a selective media designed to enumerate Gram-negative organisms in raw or pasteurized milk.



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Initial Day: the initial day of testing is not tied to the pasteurization date of the product, it is simply the first day of testing.

1.4 Safety



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SECTION 2 MATERIALS

- **Standard Plate Count (SPC) Agar.** Pre-poured SPC plates must be prepared and dried prior to use with the spiral plater.
- **Crystal Violet Tetrazolium Agar (CVTA).** Pre-poured CVTA plates must be prepared and dried prior to use with the spiral plater.
- **Molten SPC agar. For pour plating Spore Pasteurization test**
- **Sterile 500mL Corning Orange Top Bottles.** Bottles should be clean, sterile and free of condensation.
- **2oz Vials**
- **Coliform Petrifilms**
- **Ethanol**
- **Kimwipes**
- **Digital Thermometers**
- **Sterile 5mL Screw Capped Tubes**
- **Waterbath.** Set at 63°C
- **Incubators.** Set at 32°C, 21°C and 7°C
- **Spiral Plater**
- **Sphere flash**
- **Quebec Colony Counter**



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- **Hand Tallies**
- **Phosphate Buffer Dilution Blanks.** 900 μ L and 99 mL volumes.
- **Variable Volume Pipettes.** 100-1000 μ L volume and corresponding tips, 2mL fixed pipette with corresponding tips.
- **Vortex**



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SECTION 3 PROCEDURES

3.1. Preparing For VSL

3.1.1. *1-2 Days Prior to Arrival of VSL Samples*

3.1.1.1 Calculate the number of bottles, vials and plates you will need by checking the posted schedule. Tier 1 plants (and those with code dates >17 days) (bolded on VSL schedule) will need **3** bottles per core sample, all other plants will need **2** bottles per core sample.

3.1.1.2 Label sample bottles using the labels generated from the VSL Excel Sheet, place the piece of tape at either the 300mL mark (Tier 1 plants (and those with code dates >17 days)) or the 400mL mark (all other plants).

3.1.1.3 White milk samples need three vials: one 4oz for the Stress Test and two 2oz vials. One 2oz vial will go to Dairy One for dairy chemistry and the other will be used for micro testing.

3.1.1.4 Label (in duplicate) pre-poured SPC plates and coliform petrifilms with the sample number, the day of plating (DI for day initial) and the dilution plated. Additionally, label two plates of CVTA, one for micro and another for the Stress Test.



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3.2. VSL Initial Day

3.2.1. *Collecting Coolers and Logging in Plant Information*

3.2.1.1 On the designated Day Initial, likely Thursday, enter room BO2, log incubator temperatures, and sterilize pour off area using 70% Ethanol solution. Then proceed to collect samples from the 6°C incubator.

3.2.1.2 Before pouring off any of the samples check the sample label against the sample record sheet for sample ID, Sample type (confirm % fat on low fat containers), Container size and Type, Label, Code date and Package date and record the plant #.

3.2.1.3 With the **lights turned off and blinds closed** (to minimize opportunity for sample to become light oxidized) in the pour off area, locate the temperature control (TC) associated with plant 1. In accordance with Standard Methods for the Examination of Dairy Products (SMEDP 17th Edition) and using **aseptic technique** fully invert 25 times and wipe down the outside of the TC container and pour 400mLs into a labeled orange top TC bottle with temperature probe cap. Record temperature and time of reading on log sheet and place the control in the first 6°C incubator, noting the location of the TC bottle.

3.2.2. *Pouring off Core Samples*



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- 3.2.2.1 To ensure the temperature of the samples remains cold, set up the bottles and vials for each sample before removing it from the cooler. Pour off one sample at a time.
- 3.2.2.2 In accordance with Standard Methods for the Examination of Dairy Products (SMEDP 17th Edition) and using **aseptic technique** fully invert 25 times and wipe down the outside of the sample container and pour off 1 vial (to the vial line) for DI plating, leaving enough head space for mixing. Then pour enough sample to reach the pre-labeled volume into each of the labeled orange top bottles for that sample. Reserve enough sample to fill the remaining three vials for initial day sampling.
- 3.2.2.3 Check the empty sample container for any off odors, or physical defects (e.g. coagulation, particles, etc).
- 3.2.2.4 After pouring the sample into bottles and vials, move the orange top bottles to the 6°C incubator. Place two of the vials in racks in the bottom of the 6°C incubator, while the Stress Test and the remaining vial (micro vial for DI plating) should be placed in separate racks in the 4°C incubator.
- 3.2.2.5 Continue checking the VSL samples in, pouring off and incubating samples until all samples are poured off.



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3.2.3. Pouring off and Processing Extras and Raw Samples

3.2.3.1 Label two 500mL bottles and three vials for each half-pint Extras sample. For each non half-pint Extras sample, label one 500mL bottle and three vials. Label three vials and a small sterile screw capped tube for each raw sample.

3.2.3.2 Check recorded sample information for each Extras and raw sample. Set aside two of the six half pint Extras containers for sensory.

3.2.3.3 The remaining four half pint Extras containers should be shaken and aseptically co-mingled in accordance with SMEDP. After co-mingling the sample, re-shake the sample and then aseptically pour off the sample into the vials, then split the remaining sample between the two 500 mL bottles. Non half-pint Extras should be poured off in the same manner as core samples.

3.2.3.4 Incubate the Extras sample bottles at 6°C for future sampling. Vials can be placed with the core sample vials for initial day testing.

3.2.3.5 Raw samples should also be shaken (25 times within 7 seconds in a 1-foot arc) and poured off aseptically according to SMEDP into the vials. Measure 5mL of each raw sample into screw cap tubes for the laboratory pasteurized (LP) test. One additional tube should be prepared for use as a temperature control. These tubes should be kept on ice until performing the LP test.



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3.2.4 Spore Pasteurization

3.2.4.1 Roughly 50mL of each raw sample as well as a temperature control should be poured off into 100ml glass black capped tubes

3.2.4.2 Samples are put in an 80°C water bath and incubated for 12 minutes. Timer can start when the temperature of the temperature control reaches 79.5°C

3.2.4.3 Samples should be put on ice after heat treatment and cooled to 6°C or lower before plating. Adding water to the ice bucket will speed up the cooling time

3.2.5 Pour Plating with Molten SPC

3.2.5.1.1.1 Should store the SPC in a water bath that is about 55°C. The media shouldn't be stored at higher temperatures because it could bacteria in the sample.

3.2.5.1.1.2 Each sample: PSC, MSC, and TSC gets 10 plates each per raw sample

3.2.5.1.1.3 Using a pipette dispense 1 ml of the raw sample onto each PSC, MSC, and TSC plate. Then follow with pour plating molten SPC and swirl each plate to mix the inoculum with the agar.

3.2.5.1.1.4 Once this is completed PSC samples get incubated at 6°C for 10 days, MSC samples incubated at 32°C for 48 hours, and TSC samples incubated at 55°C for 48 hours.



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3.2.6 Laboratory Pasteurization:

- 3.2.6.1.1 Turn the waterbath set at 63°C on several hours before beginning pasteurization.
- 3.2.6.1.2 Place all LP samples as well as the temperature control tube in a rack and place all in the waterbath. The level of the water in the bath should be at least 4 cm above the level of sample in the tubes.
- 3.2.6.1.3 Monitor the TC temperature, once the temperature has reached 63°C, set a timer for 30 minutes. During the pasteurization closely monitor the temperature of the samples.
- 3.2.6.1.4 At the end of the pasteurization remove the tubes from the bath and place on ice until TC reads 6°C. Samples may then be plated, or held at refrigeration temperature until plated.
- 3.2.6.2 In duplicate, label pre-poured SPC plates (undiluted and 1:100 dilutions) and coliform petrifilms (undiluted, 1:10 and 1:100 dilutions) for all Extras and raw samples with the sample number, the day of plating (DI for day initial) and the dilution plated.

3.2.7 Psychro-tolerant Bacteria Count



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3.2.7.1.1 Using the raw milk samples and 900 μ L Phosphate Buffer Dilution Blanks dilute the sample log 0 and log 2.

3.2.7.1.2 Plate the raw milk samples in duplicated on SPC(spiral plater 50 μ L exp) and store plates at 6°C for 10 days.

3.2.8 Plating Day Initial Samples

3.2.8.1 Remove cover plate from spiral plater. Rinse the Spiral plater and the surrounding area with 70% ethanol solution.

3.2.8.2 Turn the spiral plater and once the home screen appears, select the blue pipet button. Make sure that you have selected the correct number of replicates and plating method for your inoculum

3.2.8.3 Using a sample that has either been vortexed or shaken (SMEDP), carefully immerse the tip of the stylus into the sample in an area that is free from foam. Press either green play button, being careful not to introduce air into the tubing by keeping the tip of the stylus always immersed in the sample.

3.2.8.4 Once the sample has been taken up, position the pre-poured plate on the turntable, ensuring that the plate is firmly and centrally located. Be sure to line up the Plate correctly on the turntable, so not make errors while plating



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- 3.2.8.5 Press the green arrow button. While the sample is being distributed on the plate pay attention to the delivery. If any abnormalities or problems arise be sure to address them before continuing. After the sample has been completely plated the stylus will raise. Remove the plate from the turntable and replace the lid.
- 3.2.8.6 The stylist will then discard the tip in the bin to the left of the turntable. Once this is done the process can be repeated.
- 3.2.8.7 After all the replicates for the sample are completed, whip the stylist with 70% ethanol on a Kimwipe before continuing to the next sample. This reduces chances of contamination between samples
- 3.2.8.8 Once all samples have been plated using this method, whip down the Spiral platter and surrounding area with 70% ethanol, turn the power and replace the cover over the plating area.
- 3.2.8.9 Incubate all LP SPC plates, all control plates and coliform petrifilms at 32°C for 48 hours and 24 hours respectively. Incubate PBC plates at 7°C for 10 days. CVTA plates will be incubated at 21°C for 48 hours. Additionally, TSC plates are incubated at 55°C for 48 hours

3.2.9 Day Initial Chemistry

- 3.2.9.1 All sample vials for cryoscope and butterfat analysis should be labeled and taken to DairyOne for analysis.



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3.2.10 Paperwork

3.2.10.1 After the original VSL log sheet has been checked and the information is correct and up to date, make a schedule of remaining test days. This is done by calculating days 7, 10, 14, 17 and 21 (if applicable) from the date the milk was processed (day 0).

3.2.10.2 Make copies of the schedule and the log sheet for all team members involved in VSL. Attach original to the back of the core worksheets.

3.3 VSL Day 1

3.3.1 Counting

3.3.1.1 Count coliform petrifilms after 24 hours (+/- 1 hour) of incubation at 32°C.

3.3.1.2 Only count colonies that are red and are associated with gas production

3.3.1.3 If films are Too Numerous To Count (TNTC) (i.e., dark red/purple color develops on the film, but no individual colonies with gas production are obvious), a Brilliant Green Bile Broth (BG) tube may be preformed to confirm the presence of coliform. BG tubes should be incubated at 32°C for 48 hours and checked for acid and gas production.

3.3.1.4 If a TNTC plate is confirmed (either by gas production, but no individual colonies, or by BG confirmation), the final count is >150 estimate (E). This count is raised to the power of the highest dilution plated that was TNTC.



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3.3.1.5 Coliform petrifilms with no colonies present should be recorded and reported as <1 cfu/mL (detection limit), and those that are TNTC should be recorded and reported as >150 cfu/mL.

3.3.1.6 An estimate may be done of the film that is very crowded by counting the confirmed colonies in one square and multiplying that number by 20 to obtain the estimated number of colonies on the entire film.

3.4 VSL Day 2

3.4.1 Counting

3.4.1.1 Remove all SPC (LP, Controls) and CVTA plates from the 32°C and 21°C incubators, respectively and TSC plates from 55°C.

3.4.1.2 Count all plates using the Sphere Flash machine.

3.4.1.3 Count plates in sequential order by group (core, schools, raws). When naming the sample in the program include the sample ID, the day of plating and the test type (ie, 11 SPC DI or 52 CVTA D14).

3.4.1.4 For more detailed procedures for counting plates using the Sphere flash consult the manual located near the colony counters.

3.4.1.5 Record the dilutions used for each test and the plate counts on the VSL worksheets. Plates with a SPC count of less than 400 should be recorded as estimate (E) on the worksheet final count. SPC plates that are TNTC (spiral plate won't give an estimate), should be recorded as >400,000 E to the power of the dilution used. SPC plates with no colonies present should be recorded as less than the detection limit (<10 cfu/mL).



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Raw SPC	X						
Raw PBC	X						
Raw LP	X						
Raw TSC	X						
Raw MSC	X						
Raw PSC				X			
Raw Coliform	X						
Core SPC	X		X	X	X	X	X
Core Coliform	X						
Core Sensory	X				X		X
Core CVTA	X		X	X	X	X	X
Extra SPC	X		X	X	X	X	X
Extra Coliform	X						



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Extra Sensory**		X			X		
Extra CVTA	X		X	X	X	X	X

* Only Tier 1 plants and Tier 2 plants with >17d code dates will be tested at day 17 and day 21

** Sensory only performed on 2% and chocolate half-pint extras

3.5.1.2 Samples must be ready for sensory by 9am on VSL testing days 10, 14, 17 and 21 (only NYS tier 1 plants and those plants with >17d code dates will be tested for sensory at day 21).

3.5.1.2.1 Day 7 micro sample should be poured off from the day 10 Sensory bottle.

3.5.1.3 With the lights turned off gently invert the bottles 25 times before aseptically filling a labeled 2oz vial with sample.

3.5.1.4 When all samples are poured off into vials, place the remaining sample in the bottles on the door of the 6°C refrigerator for sensory.

3.5.1.5 Vials should be refrigerated until used for micro testing.

3.5.1.6 Perform SPC and CVTA testing as described above at appropriate dilutions (on days 7 and beyond, plate two different dilutions that are at least one dilution apart to ensure plates will be countable; i.e., -1 and -3 dilution) in duplicate to ensure countable plates (i.e., a 1:10 and 1:1,000 dilution done to cover a larger number of dilutions).

3.5.1.7 Counting and data entry as described above.



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SECTION 4

TROUBLESHOOTING

- In the case that a sample arrives frozen leave the sample in the 6°C incubator until the sample is completely free of ice before pouring off.



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SECTION 5

REFERENCES

Wehr, H. M. and J. F. Frank eds. 2004. Standard Methods for the Examination of Dairy Products. 17th ed. American Public Health Association, Washington, DC.



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SECTION 6

METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	10/15/2015	Nicole Martin	Unsure when the original was composed
Version 2	4/21/2020	Nate Henderson	Section 1.3: Added the definitions: PPC, Stress Test, Spore Pasteurization, MSC, TSC, PSC Section 2: Added materials molten SPC agar and Sphere Flash Updated the Table of Contents with new page numbers and sections Section 6: Created Method Version and Changes Section 7: Added Appendix Section 3.1: Updated changes in number of bottles used, bottle labeling, and label generating Section 3.2.1.1: Updated the Day initial procedures of gathering samples Section 3.2.4: Added the Spore Pasteurization test to the DI procedure Section 3.2.5: Added the Pour Plating with SPC section Section 3.2.7: Added Psycho- Tolerant Bacteria section



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			<p>Section 3.2.8: Updated the plating instructions due to the new Spiral plating system.</p> <p>Section 3.3.1: Added replace air bubble with gas production when count coliform petrifilms</p> <p>Section 3.4.1: Replaced Q count with Sphere flash</p> <p>Section 3.5.1: Added the Spore Pasteurization tests MSC, PSC, TSC to the table</p>
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Appendix A:

For a quick comprehensive look at the entire month of VSL sampling please refer to the document titled VSL Prep Checklist