Title: Quantitation of Tobacco Specific Bacteria	Control #: ST-TM-950-104	Revision #: 1.0
	Approval Date: 02/23/2015	Effective Date: 02/23/2015
Document Owner: J. A. Sampson, ALCS, RD&E, Analytical Technical Services		

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NOTICE
This method may involve the use of hazardous substances and/or equipment. The user must not assume that all of the safety issues associated with its use have been described. Prior to use of this method, the user is responsible for establishing appropriate safety and health practices and determining the applicability of regulatory requirements.
The employee performing this method must be trained according to the safety guidelines specific to the job task and area of assignment. The employee must use all appropriate safety equipment referenced by the facility's safety guidelines. Copies of Material Safety Data Sheets (MSDS) are available from the Altria Safety Health and Environmental intranet site, facility safety department or the area supervisor.

A. Scope

1. This test method defines the process for the quantitation and identification of tobacco specific bacteria (TSB) in fermented and non-fermented tobacco samples. This method applies to all stages of moist smokeless tobacco (MST) analysis for both quality control evaluations and experimental applications.
2. This method may be used by personnel at U.S. Smokeless Tobacco Company (USSTC) manufacturing facilities and at Altria Client Services (ALCS) Research, Development and Engineering.

B. Definitions

1. Analyte - Component measured by the method of analysis. In the case of microbiological methods, it is the microorganism or associated by-products.
2. Aseptic technique - The method of performing tasks while ensuring that the sterile or present microbiological integrity of the equipment, product, or process is not compromised by environmental contaminants.
3. (b) (4)
4. Colony forming unit (CFU) - In reference to a circular or irregular shaped formation observed on a solid or semi-solid nutrient media which indicates visible growth of microorganisms arising from presumably one single cell. CFU is a quantifiable unit of measure estimating viable cells under the premise that one visible colony arose from significant growth of one initial cell plated.
5. Culture - A particular strain or kind of microorganism growing on or in a nutritive medium.
6. Culture Media - Any liquid or solid preparation made specifically for the growth, promotion, storage, or transport of microorganisms.

7. Diluent - Sterile liquid used to dilute a sample.
8. Gram Staining - A method for the differential staining of bacteria that involves fixing bacterial cells to a slide and staining with a series of dyes and decolorizing agents.
9. Inoculum - A microbial culture added to a substrate to initiate a biological growth response.
10. Log_{10} value - The exponential representation of actual bacterial cell counts in a sample. Example: 42,000 CFU/g = Log_{10} 4.62. Log_{10} values are used in microbiological graphical representation of data and can be converted back the original count by computing the inverse $\log(10^x)$.
11. (b) (4)
12. (b) (4)
13. Sterile - Free of living microorganisms.
14. Water activity (a_w) - The intensity with which water associates with various non-aqueous constituents and solids and the measure of energy status of water in a system. It is defined as the vapor pressure of water in a substance divided by that of pure water at the same temperature, and it is the measure of water that is biologically available for microorganism metabolism.

C. Responsibilities

1. Laboratory management shall ensure that personnel performing this method have demonstrated competence and documented proficiency.
2. Designated, trained, and qualified microbiology laboratory personnel are responsible for performing testing and documenting information as defined in this method. Any significant deviations from this method are to be documented and reported to laboratory management.

D. Equipment and Apparatus

1. Instrumentation
   a. Mechanical Diluter, Gravimat Interscience, Dilumat 3mk2, or equivalent.
   b. Mechanical Circulator Stomacher, Seward 400, or equivalent.
   c. Spiral Plating System, Spiral Biotech Autoplate AP5000, Autoplate 4000, Wasp 2, Eddy-Jet CE 99, or equivalent.
   d. Top load balance, accurate to 0.01g, Mettler Toledo, P/N XS6002S, or equivalent.
   e. Analytical balance, accurate to 0.0001g, A&D, P/N HR-200, or equivalent.

f. Incubator, capable of maintaining 25°C or 32°C, Fisher, P/N 6500, or equivalent.

g. Microscope, with 100x magnification.

h. Autoclave, Amsco, P/N 2011 Gravity, or equivalent.

i. Vortex mixer, Fisher Vortex Genie 2, P/N 12-812, or equivalent.

2. Equipment and Apparatus

a. Aluminum foil.

b. Bag holder.

c. Cell spreader, l-shaped, sterile, Fisher, P/N 14-665-230.

d. (b) (4)

e. Diluent jug, tubing, nozzle, filter unit, pre-sterilized.

f. Diluent test tubes with caps, pre-sterilized.

g. Disinfectant bottles, sterile.

h. Dropper.

i. Flame source.

j. Glass microscopic slides.

k. Gloves, cotton and disposable nitrile.

l. Graduated cylinders.

m. Hand tally counter.

n. Hot plate.

o. Immersion oil.

p. Inoculation loop.

q. Manual plate counter.

r. Metal spatula, small.

s. Milk dilution bottles.

t. Pasteur pipettes and bulbs.

u. Pipettor with matching pipette tips, 1000 uL.

v. Sample cups, 5 mL.

w. Sampling scoop or stick, sterile.

x. Slide rack.

y. Stomacher dilution filter bag, sterile, 400 mL, Microbiology International P/N 36-C0001.

3. Instrument Setup

a. Mechanical Diluter

1) Per Work Instruction NW63.0021, power on diluter at least 10 minutes prior to use to ensure temperature and weigh cell stabilization. Once set-up is finished, the Gravimat will run through its initialization cycle.

2) Verify 10g and 30g weights on the platform before use and record on form N63.0049FM. If verification weights are outside of the +/-0.05g tolerance after tarring, proceed to the calibration step.

3) (b) (4)

4) Aseptically pour the diluent into the jar, tighten jar cap and remove foil from filter.

5) Place jar containing diluent beside the Gravimat.

6) Slide tubing into the peristaltic pump (from the front to the back).

7) Immediately prior to use, remove foil from dispensing nozzle and slide it into the slot of the lever bar.

8) For Dilumat 3mk2 or other model dilutor operation, consult work instruction FW46.0012 and/or user's manual.

b. Spiral Biotech Autoplate AP5000 Spiral Plater

1) Per work instruction NW63.0058, turn on Spiral Plater and wait for the Autoplate to initialize.

2) Use the Home Screen on the electronic touch pad to program the following settings. If alternate settings are necessary, consult the Spiral Biotech Autoplate 5000 owner's manual for descriptions.

a) Sample volume to deposit: 50 microliter aliquots

b) Cleaning Mode: "Power Clean 2X" or "Expel Power Clean 2X"

c) Deposition Mode: "Slow/50" or "/50"

d) Replicates: "2 Replicates"

e) Fill Volume: "Max 500µL : Min 100µL"

f) Plate Size: "100mm"

g) Sample Container Type: sample cup symbol

3) Prepare cleaning station and bottles.

a) Insert the emptied cleaning station waste container into the compartment below the turntable.

b) Connect a sterile water bottle and accompanying sterile disinfectant bottle both with compatible connection port lid to the appropriately labeled tubing line.

c) Aseptically add 100% Ethanol to the round disinfectant bottle and sterile deionized water to the square water bottle, being careful not to contaminate the sterile interior of the bottles and caps.

d) If a plater is being used for the first time of the day, perform a “Power Clean 2X” cycle to ensure delivery line and stylus are sanitized and line is primed with liquid prior to use by pressing the cleaning logo.

4) Verify the volume being dispensed by the Autoplate 5000 once per day if in use.

a) Using a verified and calibrated analytical balance, weigh an unused media plate (including lid) and record the "BEFORE" weight on form N63.0052FM.

b) Dispense a 0.05 mL (50µL) onto the surface of the media plate.

c) Quickly return the plate and lid to the analytical balance and record the "AFTER" weight on form N63.0052FM.

d) The difference between the BEFORE and AFTER weights should be 0.05g ± 0.01g. If outside the range, repeat the plating step. If the retest values are outside of the tolerance range, proceed to the calibration step.

5) For alternate model spiral platers, refer to applicable work instructions for Autoplate 4000 (NW63.0026), Wasp 2 (FW46.0015), or Eddy-Jet CE 99, and/or user’s manual.

4. Instrument Maintenance

a. All product contact surfaces of equipment should be aseptically sanitized prior to and after microbiological analysis.

b. Analysis area should be free of dust and away from heavy ventilation and busy human traffic to avoid contamination.

c. Temperature of testing area should be near ambient unless otherwise noted.

d. The Mechanical Spiral Plater should be subjected to a “Power Clean 2X” sanitization cycle prior to and after each use.

E. Chemicals and Reagents

1. Chemicals Required
   a. Hydrogen Peroxide, 30%, Certified ACS, Fisher, P/N H325-500.
   b. Ethanol, 70% Fisher P/N HC-1000, or 100%, Fisher, P/N BP2818.
   c. Deionized water, No specific purity specs. Millipak, P/N Direct 8/16 System, equivalent, or house deionized water.
   d. (b) (4)
   e. (b) (4)
   f. BD Difco BBL Gram Stain Kit, P/N 212539.
      1) Crystal Violet
      2) Decolorizing solution
      3) Safranin stain
      4) Stabilized Iodine solution
   g. Nitrate A Reagent dropper, Sulfanilic acid, Becton Dickinson, P/N# 261197.
   h. Nitrate B Reagent dropper, N,N-Dimethyl-alpha-naphthylamine, Becton Dickinson, P/N# 261197.
   i. Water (sterilized).
   j. Zinc powder Fisher, P/N Z5-500.
2. Reagent Preparation
   a. Record all reagent lots on form F46.0006FM when reagent is initially opened. Record all media preparations on form N63.0060FM.
   b. Gram stain identification of specimen
      1) Gather gram stain kit, deionized water, and timer.
   c. Nitrate reduction test
      1) Gather Nitrate A reagent dropper, Nitrate B reagent dropper, and Zinc powder under the fume hood.
   d. Catalase detection
      1) Prepare 3% hydrogen peroxide reagent by adding 5 mL of 30% hydrogen peroxide to 45 mL deionized water in a graduated cylinder. Mix well and store reagent in a tight container under refrigeration. Label container with % solution, date, initials, and expiration date (3 months).
   e. Diluent media
      1) Prepare TN5 diluent according to UTS formula #CM-0080 and work instructions NW63.0006/FW46.0009.

2) For serial dilutions, dispense 9.0 mL of diluent into test tubes. Lightly finger-tighten tube caps.

3) Pour large volumes of diluent for diluter jug into large flasks and cover flask mouth with thick foil.

4) Sterilize flasks and tubes at 121°C for 15 minutes.

3. Standard Preparation

a. N/A

F. Sample Requirements

1. Whenever possible, it is preferred that sample processing and plating be performed within 24 hours upon receipt. Otherwise, samples should be stored tightly sealed and secured against moisture ingress/egress in an approximately 4°C refrigerator to ensure minimal change/shift in current bacterial population.

2. If samples are being received from an alternate facility, samples should be overnight shipped to be received the next day. Otherwise, samples should be shipped as quickly as possible and packed well in frozen ice packs and marked “urgent delivery”.

3. Sample shall be a well representative composite version of the entity from which it was taken. If from a full cure or cure bin process stage (i.e. Into-cure, turn, out-of-cure) tobacco should be taken from multiple parts during/throughout that process stage and shaken well.

G. Test Procedure

1. Sample Handling

a. Aseptically collect composite tobacco samples for analysis per work instructions NW63.0023/ FW46.0010, NW64.0127/FW42.0105, and NW63.0008/FW42.0082 and refer to aseptic technique document N63.0027RF.

b. Using a calibrated top loading balance, aseptically dispense 30g +/- 0.75g (or 2.5%) of each well mixed composite sample into a sterile filter bag for the initial dilution step. Ensure the sample integrity is not compromised by rolling up filter bag and securing it with tape or a rubber band.

NOTE: If testing packed/canned samples, dispense approximate equal portions of tobacco from two (2) cans to equal 30g total. If composite sample volume is limited (i.e. mini-cure jar samples), it is acceptable to sample 15g +/- 0.35g.

c. Diluting the initial composite samples per work instruction NW63.0021/FW46.0012.

1) Mechanical Diluting:

a) On the HOME screen select DILUTION 1/10.

b) Enter SAMPLE NUMBER if necessary and/or press OK.

c) When prompted, place an empty filter bag in the bag holder to tare its weight and select CONTINUE then TARE. Remove empty bag.

d) Place the previously weighed filter bag sample into the sample bag holder.

e) Lower the lever arm into locked position to begin diluent dispensing. Based on the mechanically assessed weight, the pump will deliver a 1/10 w/v ratio dilution. This sample is labeled as "10".

f) When dilution is complete and when prompted, raise the lever arm.

g) The digital output screen will indicate the %Precision. Precision shall be >99%. For additional initial sample dilution, select SAME OPERATION and repeat steps above.

2) Manual Dilution:

a) Using a calibrated and verified top loading balance, per work instructions NW63.0040/FW42.0055, place the sterile filter bag into a bag holder and tare the balance.

b) Aseptically weigh 30g of sample into the filter bag.

c) (b) (4)

d. Mixing the initial composite samples:

1) Mix the sample utilizing the mechanical circulator stomacher.

2) Raise the stomacher lid opening the door completely to give access to the paddle compartment.

3) Place the diluted sample filter bag into the paddle compartment, pat out excess air and fold a small amount of the bag over the clamp lip to seal it.

4) Briskly close door.

5) On the Seward model stomacher, select PROG.

6) Select the setting for 200 RPM for 3 minutes. Select green START.

7) If an alternate stomacher model is used, ensure sample is pummeled for 3 minutes and that the speed setting is not excessive enough to puncture or bust the filter bag.

e. Prepare serial dilutions:

1) (b) (4)

2) Aseptically pipette 1000µl (1 mL) of liquid sample from the mechanically pummeled initial composite "10" filter bag. Ensure sample is pipetted from the filtrate side of the bag to avoid clogging the spiral plater line.

3) Dispense pipetted liquid sample into test tube blank. This sample is labeled "100" for the 1/100 dilution.

4) Mix the test tube on a vortexer for a minimum of 8 seconds.

5) Create the 1/1,000 ("1T") dilution by pipetting 1000µl (1 mL) of vortexed liquid from "100" test tube into next test tube blank. Vortex minimum 8 seconds.

6) Repeat vortexed transfers up to dilution 1/100,000 ("100T"). If a larger microbial load is anticipated, repeat transfers up to 1/1,000,000 ("1M").

f. Mechanically spiral plate the sample dilutions per work instruction NW63.0058/FW46.0015/NW63.0026.

1) Sanitize working area with 70% or 100% ethanol or disinfectant spray.

2) Starting with the highest dilution ("100T"), pour diluted tobacco sample into 5 mL sample cup filling cup ¾ full.

3) Place the sample cup in the cup holder on the right side of the turntable.

4) From the Home screen, select NORMAL CLEAN or EXPEL NORMAL. If plating from the highest dilution to the lowest dilution, it is only necessary to use "NORMAL" mode between dilutions. The "POWER" CLEAN mode is only necessary between the "10" dilution and the next tobacco sample to be plated.

5) (b) (4)

6) Place media plate securely on the plate turntable and aseptically remove the media plate lid. Hold lid close to the plate, yet be sure not to obstruct stylus motion.

7) Select the "All" button (right arrow button) on the keypad. Stylus will uptake inoculum and deposit it on plate surface.

8) Replace plate lid and carefully remove the plate from turn table avoiding tilting.

9) Plate a duplicate plate by selecting PLATE NEXT.

10) Stack plates (lid side up) until sample is absorbed (minimum of 30 minutes).

11) After the duplicate sample is plated, the cleaning cycle will automatically initialize. Once cleaned, plate one control blank tube of diluent to ensure the machine has been properly sanitized.

12) For successive dilutions, repeat steps until all dilutions/samples are completed.

    NOTE: Remember to perform "POWER CLEAN" or "EXPEL POWER CLEAN" in between samples.

13) Once all samples are completed, perform a "Power Clean 2X" or "Expel Power Clean 2X" before shutting down the spiral plating machine.

14) [redacted] (b) (4)

g. Manually spread plate the sample dilutions (Alternate method)

1) Prepare dilutions and label plates as stated above but prepare an extra dilution ("1M").

2) Starting with the highest dilution ("1M"), aseptically pipet 100µl (0.10 mL) of sample onto the middle surface of each duplicate plate. These plates will be labeled as 1/1,000,000 ("1M").

3) For the "10" composite filter sample, you may opt to plate a 1/1 plate by adding 1000µl in equal portions to 3 plates (333µl + 333µl + 334µl) and repeat this in order to obtain duplicate readings. This will render a limit of detection of Log 1.0.

4) Using a sterile L-shaped cell spreader, aseptically lift the plate lid of the highest dilution plate and hold the spreader to the surface at a 45 degree angle.

5) Manually spread the inoculum over the entire surface of the agar but avoid applying excessive pressure.

6) Repeat the spreading process for the duplicate plate.

7) You may use the same cell spreader as long as you are spreading from the highest dilution to the lowest dilution.

8) Stack, bag, label, and incubate the plates according to Attachment 1.

2. Calibration

a. Mechanical Dilutor

1) Per Work Instruction NW63.0021, power on the diluter at least 10 minutes prior to use to ensure temperature and weigh cell stabilization. Once set-up is finished, the Gravimat will run through its initialization cycle.

2) Remove the "bag open support" from the platform.

3) Select ADVANCED FUNCTIONS from the menu.

4) Select CALIBRATION.

5) The date/time of the last calibration will be displayed, select CONTINUE.

6) The next screen will list conditions needed for calibration, select CONTINUE to begin calibration or CANCEL to exit.

   NOTE: Ensure the weighing platform is empty and clean, before selecting CONTINUE.

7) Place the 500g calibrated weight on the platform and select CONTINUE.

   NOTE: ONLY a 500g weight may be used for calibration.

8) The screen will read "calibration completed" and the dilutor is ready to be used.

9) Record calibration on form N63.0049FM.

10) For Dilumat 3mk2 or other model dilutor operation, consult work instruction FW46.0012 and/or user's manual.

b. Spiral Biotech Autoplate AP5000 Spiral Plater

1) Per work instruction NW63.0058, using the Validation Test Fixture, validate the Autoplate 5000 once per quarter or as needed based on verification process above.

2) Turn on Spiral Plater and wait for the Autoplate to initialize.

3) From the Home screen, select SETUP.

4) Select CALIBRATE, and then select VALIDATION.

5) When prompted, select "Remove the plate centering adapter from the turntable, and remove the right side cover". When complete, select NEXT.

6) When prompted, select "Check the stylus tip" and confirm proper positioning. Refer to owner's manual. Select NEXT.

7) Carefully unwind the tubing stored under the Validation Test Fixture.

8) Lift the tubing tip end of the Validation Fixture and place it into an empty sample cup. Follow the prompt on the Validation Setup screen to "Use a syringe to flush the validation test fixture with acid cleaner, then water, then air". When complete, select NEXT.

9) When prompted, unscrew the tubing Luer-lock from the top of the Autoplate syringe and attach the Luer-lock from the tubing on the end of the Validation Fixture to the top of the Autoplate syringe. Ensure reinstallation of the O-ring in the Validation Fixture tube Luer-lock before connecting it to the syringe. Place the tubing tip of the Validation Fixture into a sample cup of water.

10) Select FLUSH until there are no bubbles in the sample cup of water. Tubing fills with water. When filling, observe syringe to ensure no large air bubbles are entering the space below the plunger. Select NEXT.

11) When prompted, place the tubing tip into a sample cup of water. Select NEXT.

12) When prompted, press and hold the syringe "UP" arrow button until water reaches about halfway down the Validation Fixture. Select NEXT.

13) When prompted, lift the tubing tip to expose it to air. Wipe off any excessive water on the outside of the tubing tip with a paper towel. Select NEXT. The system will then automatically introduce a bubble of air, creating a water/air interface.

14) When prompted, place tubing tip into a sample cup of water, then select NEXT.

15) When prompted, use the "UP" arrow button to move the air bubble within 10mm of the zero mark on the scale. Use the "Down" arrow button if you go too far. Observe the bubble for half a minute, making sure it does not drift. When complete, select NEXT.

16) The Validation Procedure screen will appear with the prompt, "Checking Backlash: click the ‘Down' arrow until bubble just begins to move to the left". When complete, select NEXT.

17) The prompt "Checking Backlash: click the 'UP' arrow until bubble just begins to move to the right" will appear. When complete, select NEXT.

18) Slide the scale, if necessary, to locate the bubble in the center of the tolerance band at the zero mark. Select NEXT and wait for bubble movement to stop.

19) When prompted, check that bubble is within the 20µL mark on the scale, and the stylus is at the next test line (± 0.5mm) on the turntable. If bubble does not end within the black tolerance bands during the validation refer to manual for further direction. Select NEXT.

20) Slide scale if necessary to realign the zero mark.

21) Select NEXT. This will cause 20µL of water to be taken in, and bubble will return to the zero mark. Slide scale if necessary to realign the zero mark.

22) Select NEXT again to advance the bubble consecutively to the 35, 45, and 50µL marks and the stylus to the corresponding lines. Readjust the scale each time, as necessary, to start the bubble at zero. Check that the positions are within the tolerances given above. Select NEXT.

23) Disconnect the Validation Fixture tube from the syringe. Replace sample cup of water with an empty sample cup, then use the syringe to rinse the validation tubing with acid cleaner, water, and then air.

24) Insert the O-ring into the Luer-lock on the autoplate tube. Reattach the tube to the syringe and select NEXT.

25) The Validation Cleanup screen appears and a clean sequence is performed. Select NEXT.

26) The Validation Complete screen appears. Replace the right side cover and centering adapter and select the “check-mark” button. This will return the operator to the Home screen.

27) For alternate model spiral platers, refer to applicable work instructions Autoplate 4000 (NW63.0026), Wasp 2 (FW46.0015), or Eddy-Jet CE 99, and/or user’s manual.

3. Analysis

a. Remove incubated media plates from bag and spread out in order of dilution series.

b. Confirm that the dilution series has worked by looking at the number of colonies of each plate. There should visually appear to be a 10-fold decrease in ascending order of dilution.

c. (b) (4)

Attachment 2 and UTS research report USSTMC #080006 for descriptions.

d. Halophilic Cocci:

1) Using the manual plate counter or hand tally counter, count each colony that is present on the countable duplicate plates and record the results on form N63.0035FM.

2) (b) (4)

(b) (4)

e. (b) (4)

1) Using the manual plate counter or hand tally counter, count each colony that is present on the countable duplicate plates and record the results on form N63.0035FM.

NOTE: Due to the subjectivity of colony appearance, it may be necessary to perform Gram staining for microscopic morphology.

Gram Staining: per work instructions NW63.0015, FW46.0004.

(1) Flame sterilize inoculation loop for approximately 10 seconds.

(2) Add one drop of sterile water to slide.

(3) Allow loop to cool next to flame then aseptically touch loop to single bacterial colony and place it in drop of water on slide.

(4) Mix colony with water to create smear.

(5) Allow smear to dry.

(6) Heat-fix dried stain to slide by passing it quickly over open flame 5-6 times or by placing it on a paper towel and warm hot plate (low heat/100°C) for 3 minutes.

(7) Place slide on slide rack in sink.

(8) Alternate flooding the slide with stain, rinsing well with deionized water, and shaking off excess water as follows:

(a) Crystal violet stain: 1 minute

(b) Iodine solution: 1 minute

(c) Decolorizing solution: a few seconds

(d) Safranin stain: 1 minute

NOTE: Overexposure of the decolorizing solution can cause a false result.

(9) Allow the slide to air dry. Do not wipe or blot stain.

(10) View stain using highest microscope magnification (100x) under immersion oil for identification and classification. Refer to Attachment 2 for descriptions and record results on form

b (b) (4)

(4) Distribute the reagents over the entire agar plate by gently tilting and rocking the agar plate back and forth until the reagents have mixed and covered and majority of the inoculated surface.

(5) [redacted] (b) (4)

(6) A "presumptive" negative test result is indicated if there is no visible color change. A further confirmation test is required.

(7) Confirmation of negative test:

(a) Use a small metal spatula to sprinkle a small amount of zinc powder over the plate and add 2-5 additional drops of Nitrate B dropper to the zinc. Mix well by rocking the agar plate back and forth.

(b) [redacted] (b) (4)

(c) [redacted] (b) (4)

[redacted] (b) (4)

(8) [redacted] (b) (4)

4. Calculations and Reporting

a. Calculation of bacterial counts by Mechanical Spiral Plating Method per work instructions NW63.0073/FW46.0020.

1) Obtain the data collection form NW63.0035FM that contains the duplicate colony counts for the four classifications of TSB.

2) For duplicate plates, add the counts and divide by 2 to obtain the average plate count.

3) Divide the average plate count by 0.05 which is identified as the spiral plater total plate volume constant of 50µL dispensed onto the agar plate.

4) Multiply this number by the dilution of the plate being read. For example:

a) Duplicate plates rendered 50 and 30 CFUs → average 40 CFUs.

b) The countable plate was the 1/100,000 ("100T").

c) 40/0.05 = 800 x 100,000 = 80,000,000 CFU/gram.

5) Find the log of the total colony forming units.

6) [redacted] (b) (4)

7) [redacted] (b) (4)

8) Record all counts on N63.0035FM.

b. Calculation of bacterial counts by Manual Spread Plating Method

1) For duplicate plates, add the counts and divide by 2 to obtain the average plate count. NOTE: If the 1/1 plate was plated, add the CFUs for all six plates and divide by 2 to obtain the average plate count.

2) Divide the average plate count by 0.10 which is identified as the spread plated volume constant of 100µL dispensed onto the agar plate.

3) Multiply this number by the dilution of the plate being read. For example:

a) Duplicate plates rendered 50 and 30 CFUs → average 40 CFUs.

b) The countable plate was the 1/100,000 ("100T").

c) 40/0.10 = 400 x 100,000 = 40,000,000 CFU/gram.

NOTE: To calculate the 1/1 plate, multiply the average CFUs x 10.

4) Find the log of the total colony forming units.

5. Quality Control and Acceptance Criteria

a. Ensure that Gram stain kit, nitrate reagents, and 3% peroxide have not expired.

b. Visually inspect all culture media for any contaminant growth, large bubbles, and ensure media is used before expiration date (TN5 and TN18 agar media: 16 days, TN5 and TN18 diluent: 24 hours). Refer to UTS formulas CM #0076-81.

c. Identify that all instrumentation used during microbiological analysis have been verified and/or calibrated prior to use.

d. A sterile diluent control used to dilute the samples (TN5, TN18, water, etc.) shall be plated at each sample session on selective media and incubated to ensure sterility.

H. Related Documents

1. Aseptic Manual Sampling of Cures NW64.0127, FW42.0105.
2. Aseptic Technique, N63.0027RF.
3. Autoplate Validation/Verification Log Sheet, N63.0052FM.
4. Balance Calibration and Verification, NW63.0040, FW42.0055.
5. [redacted] (b) (4)
6. Dilution Preparation of Tobacco Samples, NW63.0021.
7. Dilutor Balance Calibration/Verification Log, N63.0049FM.
8. Fermented Tobacco for Seed Cures Sampling For Shelf Life Stability, NW63.0008, FW42.0082.
9. [redacted] (b) (4)
10. Media Batch Log, N63.0060FM.
11. Media Formulas CM #0076 – 0081, UTS Firewall.
12. Microbiological Gram Staining, NW63.0015, FW46.0004.
13. Microbiological Lab Reagent Log, F46.0006FM.
14. Microbiological Plating Results for Tobacco Samples, N63.0035FM.
15. Microbiological Spiral Plating Using the Autoplate 4000, NW63.0026.
16. Microbiological Spiral Plating Using the Autoplate 5000, NW63.0058.
17. Microbiological Spiral Plating Using the Wasp 2 Spiral Plater, FW46.0015.
18. Operation of the Dilumat 3mk2 Volumetric Dilutor, FW46.0012.
19. Preparation of Various Culture Media for Microbiological Analysis, NW63.0006, FW46.0009.
20. Preparing A Tobacco Sample For Chemical and/or Microbiological Analysis NW63.0023, FW46.0010.
21. [redacted] (b) (4)
22. [redacted] (b) (4)

I. Attachments

Attachment 1- Selective Media Incubation Conditions

Selective Agar Media	Incubation Temp (°C)	Incubation Period	Special Instructions	Indications

(b) (4)

Attachment 2- General Microbiological Descriptions of Tobacco Specific Bacteria

Category of TSB	Differentiations	Gram Stain Reaction	Catalase Reaction	Nitrate Reduction	Physical Colony Appearance	Microscopic Morphology

(b) (4)

(b) (4)