Designation: ~~D7818 – 12~~ D7818 – 12 (Reapproved 2016)

Standard Test Method for Enumeration of Proteolytic Bacteria in Fresh (Uncured) Hides and Skins[1]

This standard is issued under the fixed designation D7818; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers the enumeration of bacteria that can hydrolyze protein/collagen in fresh (uncured) hides and skins. This test method is applicable to uncured hides and skins.

1.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D6715 Practice for Sampling and Preparation of Fresh or Salt-Preserved (Cured) Hides and Skins for Chemical and Physical Tests

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E177 Practice for Use of the Terms Precision and Bias in ASTM Test Methods

3. Summary of Test Method

3.1 Samples of uncured hides and skins are serially diluted and plated on agar containing casein from skim milk. The plates are incubated under aerobic conditions at 35°C for 48 h. After incubation, to determine bacteria that can hydrolyze protein (proteolytic), the plates are flooded with dilute acid and the colonies showing a halo are counted.

4. Significance and Use

4.1 This test method enumerates proteolytic bacteria. Proteolytic bacteria have been known to cause damage to hides and skins.

5. Apparatus

5.1 *Incubator,* 35 ± 1°C.

5.2 *Colony counter*—(not mandatory, but highly recommended).

5.3 *Sterile pipets.*

5.4 *Bent glass rods,* sterile.

5.5 *Stomacher,* for mixing initial dilution. (If stomacher is unavailable, hand-mix.)

5.6 *Balance.*

5.7 *Sterile petri dishes.*

5.8 *Autoclave (sterilizer).* (Check the effectiveness of sterilization weekly. For example, place spore suspensions or strips of *Bacillus stearothermophilus* (commercially available) inside glassware for a full autoclave cycle. Follow manufacturer's directions for sterilization of specific media.)

[1] This test method is under the jurisdiction of ASTM Committee D31 on Leather and is the direct responsibility of Subcommittee D31.02 on Wet Blue.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

5.9 *Stomacher bags,* or sterile, sealable quart plastic bag (e.g. food storage type, sterile bag).

5.10 *Cutting tool,* sterile (e.g. scalpel blade and forcep, as needed for cutting fresh hides and skins).

5.11 *Vortex mixer,* for mixing dilution tubes (optional).

5.12 *pH meter.*

5.13 *Waterbath,* 45 ± 1°C.

5.14 *Autoclave thermometer.*

6. Reagents and Materials

6.1 *5 % acetic acid.*

6.2 *Butterfield's Phosphate Stock Solution:* Dissolve 34 g KH_2PO_4 (Potassium Phosphate monobasic) in 500 mL DI water. Adjust the pH to 7.2 ± 0.1 with 1N – 6N NaOH. Bring volume to 1 L with DI water. Sterilize for 15 min at 121°C.

NOTE 1—Typical autoclave setting is ~~120–124°C.~~120 – 124°C. (See 5.8.)

6.3 *Butterfield's Phosphate Diluent (BPD):* Take 1.25 mL of Butterfield's Phosphate Stock solution (6.2) and bring to 1 L with DI water. Dispense into ~~1 L~~ 1-L bottles and ~~9 mL~~ 9-mL dilution tubes. Sterilize for 15 min at 121°C. (See Note 1.)

6.4 *Standard plate count agar* containing 100 mL of 10 % powdered skim milk solution per litre of agar.

6.5 *Alcohol* (for flame sterilizing), e.g. 70 % Isopropyl alcohol.

6.6 *Bent glass rod* ("hockey-stick").

6.7 *Powdered skim milk.*

6.8 *Distilled or deionized water.*

6.9 *Bacillus stearothermophilus* spore suspensions or strips (commercially available), or equivalent.

6.10 *1N – 6N NaOH.*

7. Hazards

7.1 All reagents and chemicals should be handled with care. Before using any chemical, read and follow all safety precautions and instructions on the manufacturer's label or MSDS (Material Safety Data Sheet).

8. Sampling

8.1 The specimen shall be sampled in accordance with Practice D6715, and placed in sterile containers.

9. Preparation of Standard Plate Count Agar

9.1 Prepare the standard plate count agar per manufacturer label directions.

9.2 Autoclave the prepared agar for 15 min at 121°C. (See Note 1.)

9.3 Prepare a 10 % powdered skim milk mixture by adding 10 g powdered skim milk to 100 mL DI water, then stirring the mixture to dissolve it. Autoclave the mixture for 15 min at 121°C. (See Note 1.)

9.4 Cool the agar (9.2) to 45 ± 1°C, then add 100 mL of the sterile 10 % powdered skim milk mixture (9.3) per litre of agar.

NOTE 2—Do not allow agar to solidify prior to pouring (9.5).

9.5 Pour the sterile agar into petri dishes. Replace the cover and swirl to evenly distribute the agar. Allow to solidify at room temperature on a flat surface. When solid, invert the petri dishes, with the cover on the bottom, leaving a slight opening to allow the plates to dry for ½ h.

10. Procedure

10.1 Using a sterile scalpel, aseptically weigh a 20 ± 0.1 g specimen in a sterile bag. Include both flesh and hair side.

10.2 Add 180 g of BPD (6.3) diluent into the same sterile bag (10.1). Stomach or hand-massage for 1 min. This provides a 1:10 dilution.

10.3 Prepare the following sample dilutions using ~~9mL~~ 9-mL dilution tubes (BPD): 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} (see Fig. 1).

10.3.1 *Control Blank*—In 10.9, incubate one of the petri dishes prepared in 9.5 as-is, with the sample plates.

Example: To obtain a 10^{-2} dilution, mix the 10^{-1} dilution and pipet ~~1mL~~ 1 mL of that 10^{-1} dilution into a ~~9 mL~~ 9-mL dilution tube.

NOTE 3—When transferring the aliquots between the tubes, the analyst must use a different pipet or pipet tip for each transfer.

10.4 Pipet an appropriate portion ~~(0.1mL or 0.2mL),~~ (0.1 mL or 0.2 mL), of the 10^{-2} dilution and place the liquid in the middle of a dried, skim milk agar plate.

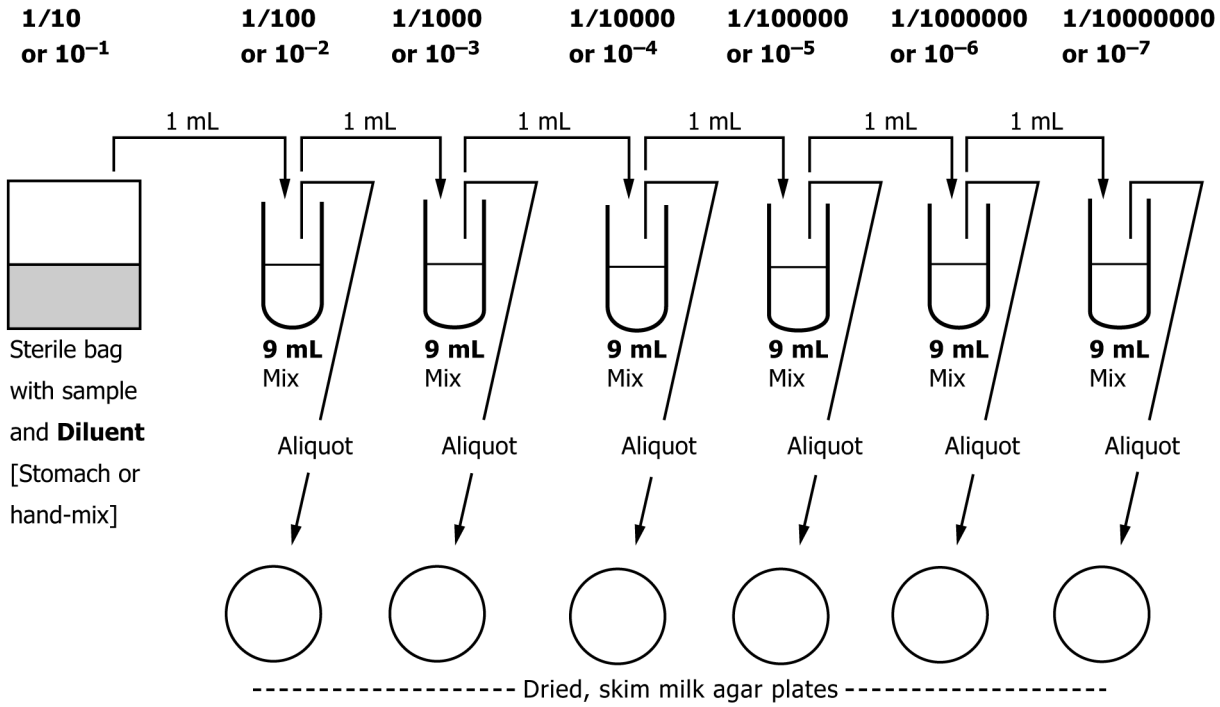

FIG. 1 Plating

10.5 Flame sterilize a bent glass rod, or obtain a sterile, autoclaved bent glass rod.

10.6 Using the glass rod, spread the liquid evenly on the agar surface.

10.7 Replace the cover and allow the plate to dry at room temperature.

10.8 Repeat steps 10.4 – 10.7 for each dilution.

10.9 Invert all plates and incubate at 35 ± 1°C for 48 ± 3 h.

10.10 Following incubation, count only those plates that have ~~25–250~~25 – 250 colonies.

NOTE 4—If a plate shows *confluent growth* (i.e. bacterial growth covers the entire plate, making it impossible to determine the existence of discrete colonies), record that plate's count as TNTC – "Too Numerous To Count"). See Figs. 2 and 3 for diagrams of a countable plate and a TNTC plate, respectively.

NOTE 5—Count all the distinct colonies on the plate. If there are similar-appearing colonies growing in close proximity but not touching, count them as individual colonies, provided the distance between them is at least equal to the diameter of the smallest colony. Colonies that are impinging, and that

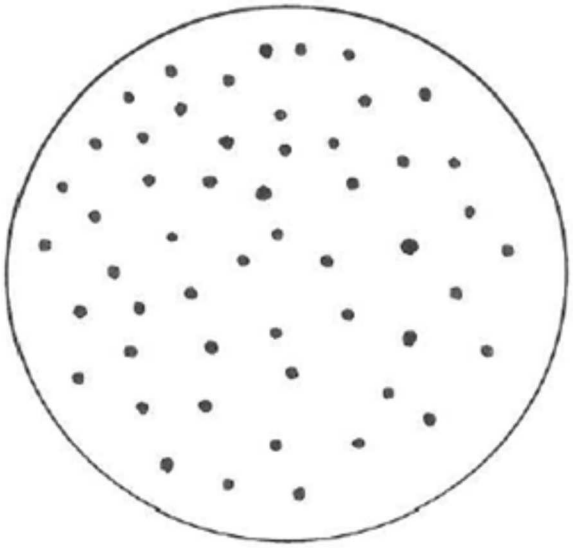

FIG. 2 Diagram of a Countable Plate