This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.

Designation: D7816 – 23

Standard Test Method for Enumeration of Halophilic and Proteolytic Bacteria in Raceway Brine, Brine-Cured Hides and Skins[1]

This standard is issued under the fixed designation D7816; 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 tolerate high salt concentrations or can hydrolyze protein/collagen, or both. This test method is applicable to raceway brine, brine-cured hides and skins, and pre-charge raceway liquor.

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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.4 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

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

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

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

3. Summary of Test Method

3.1 Samples of brine-cured hides and skins, raceway brine, or pre-charge raceway liquor are serially diluted and plated on agar containing 7 % NaCl and casein from skim milk. The plates are incubated under aerobic conditions at 35 °C for 48 h. After incubation, to determine salt tolerant (halophilic) bacteria, all colonies are counted. To determine bacteria that can hydrolyze protein (proteolytic), the plates are flooded with dilute acid and the colonies showing a "halo" (semi-clear zones) are counted.

4. Significance and Use

4.1 This test method enumerates salt tolerant (halophilic) bacteria, and proteolytic bacteria that are also salt tolerant. Under the conditions of this test method those bacteria are equated as halophilic organisms. Salt tolerant proteolytic bacteria have been known to cause damage to hides and skins in raceway brine.

5. Apparatus

5.1 *Incubator,* 35 °C ± 1 °C.

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

5.3 *Sterile pipets.*

5.4 *Bent glass rods* ("hockey-stick"), sterile. (If non-sterile, will need to flame sterilize.)

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.)

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

5.10 *Cutting tool,* sterile (for example, scalpel blade and forcep, as needed for cutting cured hides and skins).

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

5.12 *pH meter.*

5.13 *Waterbath,* 45 °C ± 1 °C.

[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.

Current edition approved Sept. 1, 2023. Published September 2023. Originally approved in 2012. Last previous edition approved in 2021 as D7816 – 12 (2021). DOI: 10.1520/D7816-23.

[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.

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5.14 *Autoclave thermometer,* or equivalent for monitoring autoclave temperature.

6. Reagents and Materials

6.1 *5 % Acetic Acid.*

6.2 1N – 6N NaOH.

6.3 *Standard Plate Count Agar* containing 100 mL of 10 % powdered skim milk solution and 77 g of salt per litre of agar.

6.4 *Ready-To-Use Plate,* (optional)[3]: Plate Count Agar + 10 % skim milk + 7.7 % NaCl.

6.5 *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 °C to 124 °C at 15 psi. (See 5.8.)

6.6 *Butterfield's Phosphate Diluent with salt (BPD w/salt)*—Take 1.25 mL of Butterfield's Phosphate Stock solution (6.5) and bring to 1 L with DI water, then add 77 g of salt (NaCl) per litre prior to autoclaving. Dispense into 1 L bottles and 9 mL dilution tubes. Sterilize for 15 min at 121 °C. (See Note 1.)

6.7 *Alcohol* (for flame sterilizing), for example, 70 % Isopropyl alcohol.

6.8 *Powdered skim milk.*

6.9 *Distilled or deionized water.*

6.10 *Salt (NaCl),* Sodium chloride – reagent grade.

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

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

NOTE 2—Omit steps 9.1 – 9.5 if using Reagent 6.4.

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

9.2 Add 77 g of salt per litre of agar and autoclave for 15 min at 121 °C.

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.

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

NOTE 3—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.

NOTE 4—The prepared agar plates may be used immediately after the ½ h drying or sealed for later use. Store the prepared agar plates upside down in a sealed plastic bag, in the refrigerator or cold room at 4 °C. Use the stored prepared agar plates within 2 weeks of preparation.

10. Procedure

10.1 Using a sterile scalpel, aseptically weigh a 20 g ± 0.1 g specimen in a sterile bag. For brine-cured hides and skins, include both flesh and hair side.

10.2 Add 180 g of BPD w/salt (6.6) 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 9 mL dilution tubes (BPD w/salt): 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 1 mL of that 10^{-1} dilution into a 9 mL dilution tube.

NOTE 5—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 (0.1 mL or 0.2 mL), of the 10^{-2} dilution and place the liquid in the middle of a dried, skim milk agar plate. (If you expect a high bacteria load use 0.1 mL aliquot, otherwise use 0.2 mL aliquot. If approximate range of bacteria load is unknown then use both 0.1 and 0.2 aliquots. Refer to Table 1 for Plating Factor.)

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 °C ± 1 °C for 48 h ± 3 h.

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

NOTE 6—If a plate shows *confluent growth* (that is, 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 Fig. 2 and Fig. 3 for diagrams of a countable plate and a TNTC plate, respectively.

NOTE 7—*Halophilic Colonies*—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 differ in appearance, such as morphology or color, are counted as individual colonies, provided the distance between

[3] The sole source of supply of the reagent known to the committee at this time is Edge Biologicals, Inc., Memphis, TN, Cat.# 4P-468. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend.

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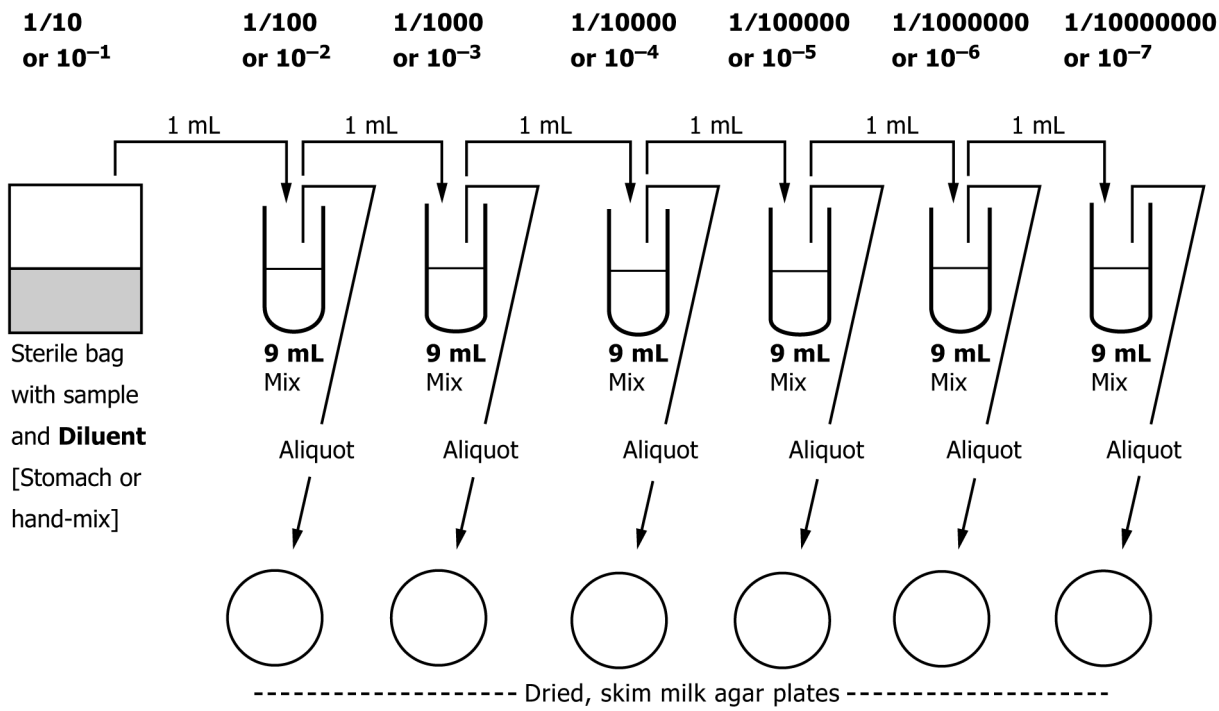

FIG. 1 Plating

TABLE 1 Plating Factor

mL Plated (10.4)	Plating Factor
0.1 mL	10
0.2 mL	5

TABLE 2 Dilution Factor

Test Tube Plated (10.4)	Dilution Factor
10^{-2}	100
10^{-3}	1,000
10^{-4}	10,000
10^{-5}	100,000
10^{-6}	1,000,000
10^{-7}	10,000,000

a cluster, and are similar in appearance, such as morphology or color, are counted as one colony (see arrow Fig. 4 – "7:00" position). There may also be "spreaders": a chain of colonies not distinctly separated. Count as one colony if a chain of colonies appears to be caused by disintegration of a bacterial clump as agar and sample were mixed. Count as one colony if a spreader developed as a film of growth between the agar and bottom of petri dish. Count as one colony if a colony forms in a film of water at the edge or over the agar surface.

Estimated counts can be made on plates with >250 colonies: report as estimated counts. In making such counts, the standard 15 × 100 mm petri dish is considered to have an area of about 56 cm^2, therefore, use a factor of 56 when estimating the count. Example: 0.1 mL of a 10^{-4} dilution was plated and the plate has an average count of 10 colonies per cm^2. Therefore, the estimated count for that plate is 10 × 56 = 560, and the estimated count for that dilution is 560 × 10 × 10,000 = 56,000,000. Estimated counts can also be made on plates with <25 colonies: report as estimated counts.

10.11 Record each plate's dilution and count on the worksheet. This initial count is the halophilic count (A).

10.12 For the same plate(s) counted in 10.10, flood the plate(s) with 5 % acetic acid.

NOTE 8—Use enough of the 5 % acetic acid to fully cover the surface of the plate.

10.13 Pour off the acetic acid and immediately count only those colonies surrounded by a semi-clear zone.

NOTE 9—*Proteolytic Colonies*—Do not count all the colonies inside the zone; only count the colonies that cause/affect the shape of the zone. When identifying the semi-clear zone(s) it is sometimes helpful to pick up the plate and change the angle of view slightly to determine if it is a true circle or if it has another protruberance off its edge. (For weak semi-clear zones, it is helpful to rotate the plate in the light to be sure about the clearing.) Look for a circle of clearing around a centrally located colony. If the clearing is an elongated shape such as in the bottom right quadrant of Fig. 5 ("5:30" position), break the cleared zone into separate circles of clearing (circles can be of varying sizes) and count the colonies located in the centers of each of the circles. When there are multiple colonies in the same clear zone, look to see if the zone is a uniform circle (caused by one centrally located colony), or if it appears to have circular bumps protruding from the edges of the central circle, each bump being caused by a separate colony.

NOTE 10—Colonies on the edge of the zone that are not fully encompassed or do not affect the shape of the zone should not be counted.

10.14 Record each plate's dilution and count on the worksheet. This second count is the proteolytic count (B).

11. Calculation of Results for Halophilic and Proteolytic Bacteria

11.1 Calculate the result for halophilic bacteria by using the following formula:

$$\text{Halophilic (salt tolerant) bacteria} = A \times D \times E \quad (1)$$

where:

A = number of colonies counted in step 10.10,
D = plating factor (see Table 1), and
E = dilution factor (see Table 2).

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