



Standard Test Method for Enumeration of Halophilic and Proteolytic Bacteria in Raceway Brine, Brine-Cured Hides and Skins¹

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 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 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 ± 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 (e.g. food storage type, sterile bag).

5.10 *Cutting tool*, sterile (e.g. 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 ± 1°C.

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

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.

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 120–124°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), e.g. 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 $45 \pm 1^\circ\text{C}$, 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.

10. Procedure

10.1 Using a sterile scalpel, aseptically weigh a $20 \pm 0.1\text{g}$ 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 9mL 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 1mL of that 10^{-1} dilution into a 9 mL dilution tube.

NOTE 4—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 $35 \pm 1^\circ\text{C}$ for 48 ± 3 h.

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

NOTE 5—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 6—*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. Colonies that are 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.

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.

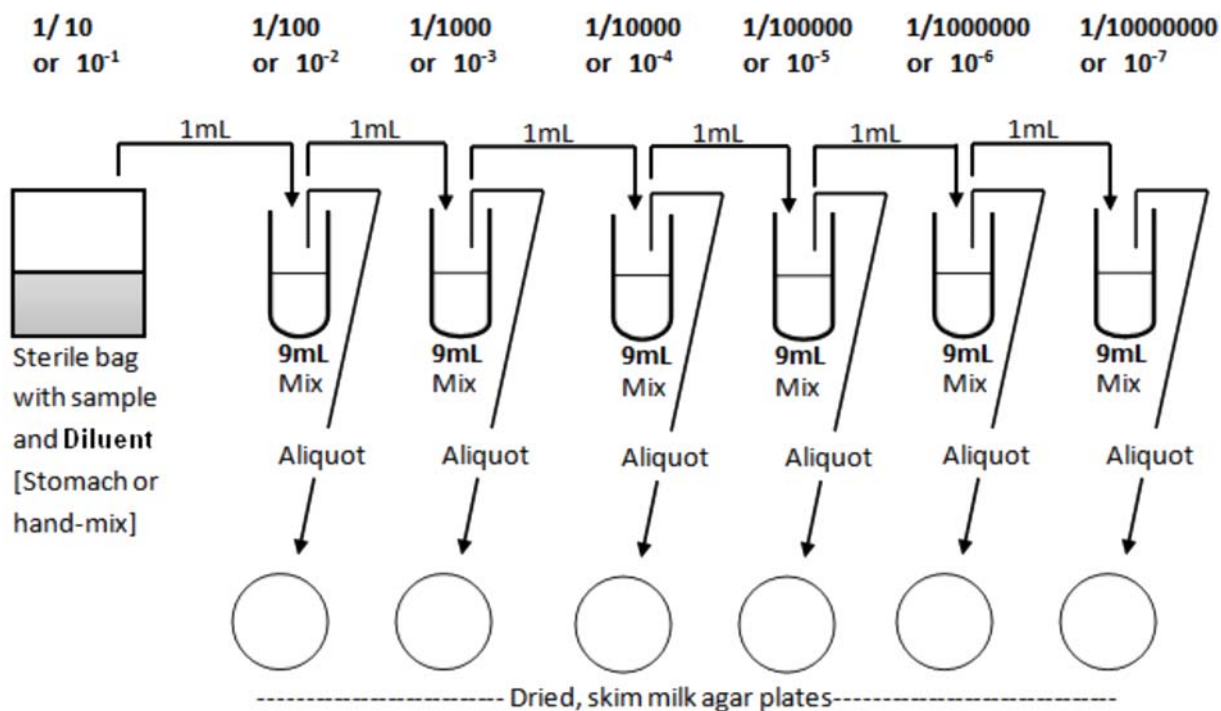


FIG. 1 Plating

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

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 cm², therefore, use a factor of 56 when estimating the count. Example: 0.1mL of a 10^{-4} dilution was plated and the plate has an average count of 10 colonies per cm². 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 7—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 8—*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 it's 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 9—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 plates'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).

11.2 Calculate the result for proteolytic bacteria by using the following formula:

$$\text{Proteolytic bacteria} = B \times D \times E \quad (2)$$

where:

B = number of colonies counted in step 10.13,
D = plating factor (see Table 1), and
E = dilution factor (see Table 2).

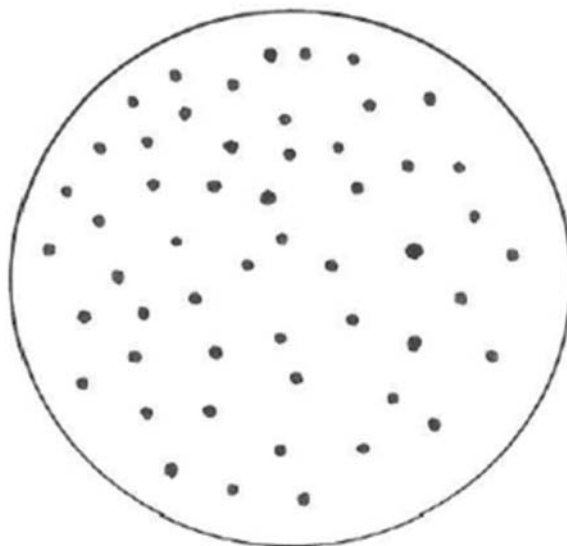


FIG. 2 Diagram of a Countable Plate

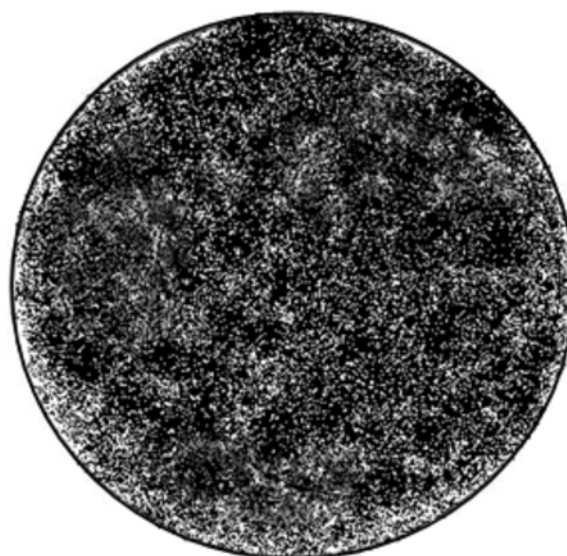


FIG. 3 Diagram of Confluent Growth (TNTC)

12. Report

12.1 Report the result from 11.1 as Halophilic (salt tolerant) bacteria per gram of *sample* and the result from 11.2 as Proteolytic bacteria per gram of *sample*.

12.2 Report the condition of the specimen (hair-on or dehaired).

13. Precision and Bias

13.1 The precision of this test method is based on an interlaboratory study of Test Method D7816 conducted in 2009. One laboratory participated in this study. The laboratory reported five replicate test results for six different specimen types. Every “test result” reported represents an individual

determination. Except for the use of only one laboratory, Practice E691 was followed for the design and analysis of the data.⁴

13.1.1 *Repeatability Limit (r)*—Two test results obtained within one laboratory shall be judged not equivalent if they differ by more than the “*r*” value for that material; “*r*” is the interval representing the critical difference between two test results for the same material, obtained by the same operator using the same equipment on the same day in the same laboratory.

13.1.1.1 Repeatability limits are listed in Tables 3 and 4.

⁴ Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:D31-1015.

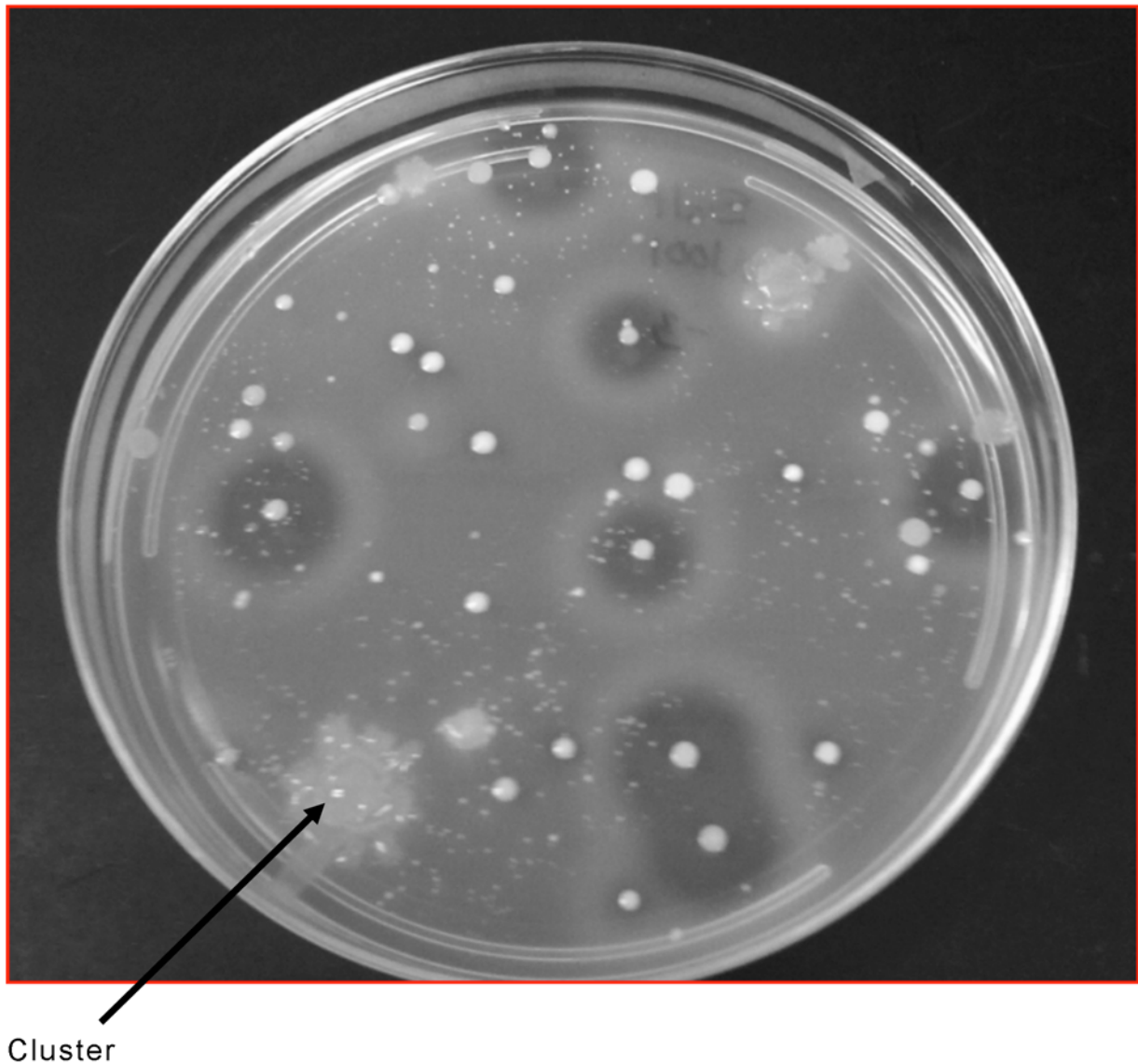


FIG. 4 Example of a Plate Before Flooding It with the Acid Solution

13.1.2 *Reproducibility Limit (R)*—Two test results shall be judged not equivalent if they differ by more than the “*R*” value for that material; “*R*” is the interval representing the critical difference between two test results for the same material, obtained by different operators using different equipment in different laboratories.

13.1.2.1 Reproducibility limits cannot be determined with data from only one reporting laboratory.

13.1.3 The above terms (repeatability limit and reproducibility limit) are used as specified in Practice E177.

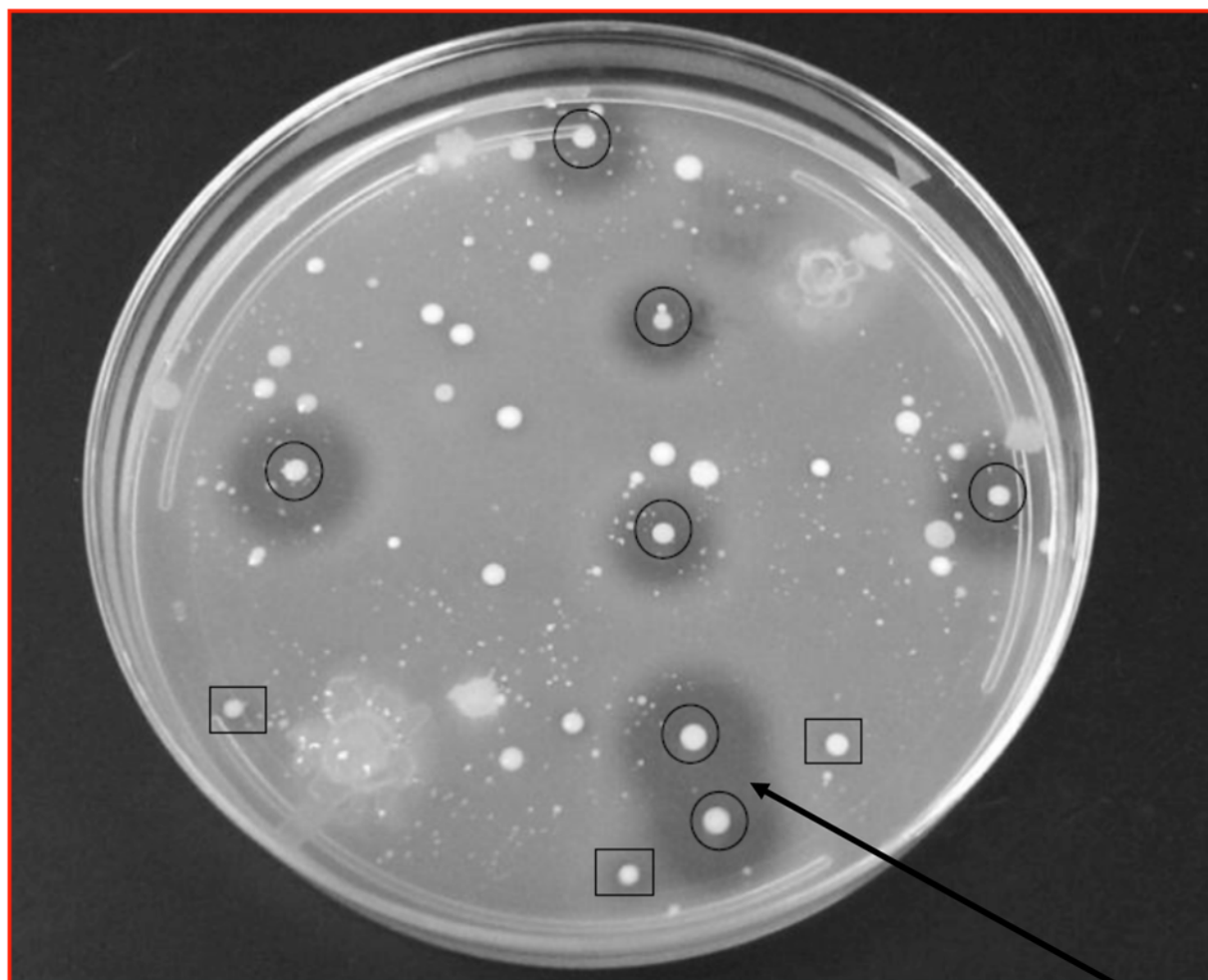
13.1.4 Any judgment in accordance with 13.1.1 would normally have an approximate 95 % probability of being correct, however the precision statistics obtained in this ILS must not be treated as exact mathematical quantities which are applicable to all circumstances and uses. The limited number of locations tested and laboratories reporting results guarantees that there will be times when differences greater than predicted

by the ILS results will arise, sometimes with considerably greater or smaller frequency than the 95 % probability limit would imply. Consider the repeatability limit as a general guide, and the associated probability of 95 % as only a rough indicator of what can be expected.

13.2 *Bias*—At the time of the study, no accepted reference material suitable for determining the bias for this test method was utilized, therefore no statement on bias is being made.

13.3 The precision statement was determined through statistical examination of 60 results, from one laboratory, on six materials. These materials were described as the following:

- Material 1: HGS Flesh – Flesh side – uncured (green)
- Material 2: HGS Hair – Hair side – uncured (green)
- Material 3: IPHS Flesh – Flesh side – in process ~ 6 h
- Material 4: IPHS Hair – Hair side – in process ~ 6 h
- Material 5: CHS Flesh – Flesh side – cured ~16 h
- Material 6: CHS Hair – Hair side – cured ~ 16 h



Elongated Shape

NOTE 1—The colonies noted with black circles are considered proteolytic colonies. The colonies noted with black squares are also considered proteolytic colonies, although their semi-clear zones are weak compared to those noted with black circles.

FIG. 5 Example of the Same Plate After Flooding It with the Acid Solution

TABLE 3 Halophilic Bacteria (log count)

Material	Average	Repeatability Standard Deviation	Repeatability Limit
	$\bar{x}$	s_r	r
IPHS Flesh	7.41	0.18	0.51
HGS Flesh	7.02	0.26	0.73
HGS Hair	7.10	0.38	1.06
IPHS Hair	6.70	0.31	0.88
CHS Flesh	7.11	0.26	0.73
CHS Hair	5.95	0.41	1.16

TABLE 4 Proteolytic Bacteria (log count)

Material	Average	Repeatability Standard Deviation	Repeatability Limit
	$\bar{x}$	s_r	r
IPHS Flesh	4.75	0.30	0.84
HGS Flesh	3.37	1.33	3.71
HGS Hair	3.91	0.39	1.10
IPHS Hair	4.11	0.63	1.77
CHS Flesh	4.87	0.91	2.54
CHS Hair	3.54	0.67	1.87

13.3.1 To judge the equivalency of two test results, it is recommended to choose the material described as closest in characteristics and concentration to the test material.

14. Keywords

14.1 bacteria; brine; collagen; halophilic; hides; proteolytic; raceway; salt tolerant; skins

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