



Animal and Plant
Health Inspection
Service

Veterinary Services

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Standard Bacterial Plate Count

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Standard Bacterial Plate Count

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1. Purpose

This document describes the titration methods used by the Bacteriology Section of the Center for Veterinary Biologics (CVB) to determine the colony-forming units (CFU) in final container samples. These methods use agar for determining CFUs and broths or solutions (see **Appendices**) as a diluent.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

- 2.1.1 Vortex mixer
- 2.1.2 Colony counter
- 2.1.3 Pipetting aid
- 2.1.4 Incubator (Temperature and atmospheric conditions determined by bacterial species being cultured. See **Appendices**)
- 2.1.5 Laminar-flow Class II biosafety cabinet (BSC)

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

- 2.2.1 Diluent (see **Appendices**)
- 2.2.2 Plated agar media (see **Appendices**)
- 2.2.3 Reference culture(s)
- 2.2.4 70% ethanol
- 2.2.5 Sterile water in serum vials
- 2.2.6 Inoculum spreader
- 2.2.7 Disposable syringes and needles
- 2.2.8 Sterile disposable pipettes
- 2.2.9 Sterile screw-capped culture tubes

- 2.2.10 Gloves and lab coat
- 2.2.11 4 x 4-inch sterile gauze pads
- 2.2.12 Test tube rack
- 2.2.13 Sharps container
- 2.2.14 Micropipettors, 100- μ L to 1.0-mL
- 2.2.15 Pipette tips, 100- μ L to 1-mL
- 2.2.16 Pipetting aid
- 2.2.17 Candle jar or appropriate anaerobic container

3. Preparation for the Procedure

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment, supplies, and reagents

- 3.2.1 Sterilize all glassware before use.
- 3.2.2 Use only sterile bacteriological supplies (pipettes, syringes, needles, diluents, etc.).
- 3.2.3 Operate all equipment and instrumentation according to manufacturer's instructions and maintain according to standard operating procedures (SOPs).
- 3.2.4 Turn on the BSC at least 30 minutes prior to use.
- 3.2.5 Monitor temperature of incubators and freezers according to SOPs.
- 3.2.6 Label all plates with sample number or name, vial number, and dilution series. Label 2 or 3 (refer to the firm's Outline of Production (OP)) plates per dilution series for each serial.
- 3.2.7 Warm the samples and reference culture to 20°-25°C before rehydrating to the appropriate volume.

3.2.8 Store plates used for making counts at refrigerator temperature. Place plates to be used for counts in a $35^{\circ}\pm 2^{\circ}\text{C}$ incubator overnight prior to use or dry in a BSC before use. At the time of use, plates are no more than 3 months old.

3.2.9 Prepare reference or positive controls according to the manufacturer's instructions.

3.3 Critical Control Points

3.3.1 All bacterial suspensions must be mixed well prior to drawing an aliquot for each subsequent serial dilution.

3.3.2 All bacterial suspensions must be mixed well prior to placing an aliquot on an agar plate.

3.3.3 Inoculum must be spread evenly on the surface of the agar plates and not allowed to pool around the edges.

3.3.4 Do not use plates with fewer than 30 colonies (unless specified in the firm's OP) as estimates for calculating CFU/dose or CFU/mL.

3.3.5 All dilutions should be plated ≤ 1 hour of sample resuspension or thawing.

4. Performance of the Procedure

4.1 Remove 2 vials (or the number of vials stated in the OP for testing) of product to be tested and 1 vial of reference stock culture from the freezer or cooler storage and allow to warm to 20° - 25°C .

4.2 Disinfect the cap with 70% ethanol. Rehydrate the vials and allow the contents to reconstitute for at least 5 minutes. Shake the vials by inversion until thoroughly mixed.

4.3 Prepare a tenfold dilution series of the product by setting up a rack of 20 x 150-mm screw-capped tubes and pipetting 9.0 mL of the diluent into each tube using a 10-mL pipette. Label the tubes 10^{-1} to 10^{-x} as needed.

4.4 Transfer 1.0 mL of the first sample from **Section 4.2** into the first tube of diluent by using a pipette or micropipettor with pipet tip. Cap the tube and vortex. The dilution series is continued by using a new pipette to transfer a 1.0 mL sample from this tube to the tube labeled 10^{-2} . Repeat this method using a sterile pipette for each transfer until the required number of serial tenfold dilutions (refer to the firm's OP) is attained.

4.5 Deposit 0.1 mL* of the sample from the last 3 dilution points of the dilution series for the product onto the surface of media (see **Appendices**) using a sterile pipette or micropipettor with pipet tip.

***Sometimes it is acceptable to plate larger amounts of samples onto the surface of the agar. This is dependent on the test and the firm's OP.**

4.6 Use a sterile inoculum spreader to evenly distribute the inoculum over the surface of the agar medium.

4.6.1. The same inoculum spreader may be used on plates containing the same dilution. Change spreaders for each dilution plated.

4.6.2. Avoid spreading inoculum completely to the edges of the plate. Inoculum may pool at the edges, resulting in colony growth that is difficult to quantitate.

4.7 Repeat **Sections 4.4** through **4.6** with each of the rest of the vials of product.

4.8 Prepare 3 plates of media as in **Sections 4.5** through **4.6** from each of 3 reference control dilutions as determined from **Section 3.2.9**.

4.9 Invert all plates and incubate according to the specifications in **Appendix I**. After incubation, count plates from each series that contains 30 to 300 CFUs**. Determine the mean CFU/mL or CFU per dose for the number of vials tested using the calculation(s) listed below.

****Exceptions to the dilution that yields 30-300 colonies per plate can be found in the firm's OP.**

$$\frac{(\text{Average count})}{(\text{Dilution plated}) (\text{mL plated})} = \text{CFU/mL}$$

or

$$\frac{(\text{Average Count}) \times (\text{mL used to rehydrate})}{(\text{Dilution used}) \times (\text{mL plated}) \times (\text{Number of doses})} = \text{CFU/dose}$$

or

$$\frac{(\text{Average count}) (\text{mL per animal}) (\text{Challenge dilution})}{(\text{Dilution plated}) (\text{mL plated}) (\text{Number of doses})} = \text{CFU/dose}$$

Example: Triplicate plates inoculated with 0.1 mL of a 10⁻⁵ dilution of culture yielded counts of 82, 79, and 88 colonies, respectively.

$$\text{Average number of colonies} = \frac{82+79+88}{3} = 83 \text{ CFU}$$

$$83 \text{ CFU} \times \frac{1}{10^{-5}} \times \frac{1}{0.1 \text{ mL}} = 8.3 \times 10^7 \text{ CFU/mL}$$

- 4.10** If more than one dilution series has valid CFUs, determine the geometric mean CFU per dose for the number of vials tested using the calculation(s) listed below.

Note: Because of the logarithm and exponentials, rounding error can get easily magnified. For the final result to be accurate to two decimal places, use at least three decimal places during the calculation.

Example:

In vial 1, triplicate plates inoculated with 0.1 mL of a 10^{-5} dilution of culture yielded counts of 82, 79, and 88 colonies, respectively.

$$\text{Average number of colonies} = \frac{82+79+88}{3} = 83 \text{ CFU}$$

$$\frac{(83 \text{ CFU}) \times (20 \text{ mL})}{(10^{-5}) \times (0.1 \text{ mL}) \times (20 \text{ doses})} = \frac{1660 \text{ CFU/mL}}{2 \times 10^{-5} \text{ mL/dose}} = 8.3 \times 10^7 \text{ CFU/dose}$$

In vial 2, triplicate plates inoculated with 0.1 mL of a 10^{-6} dilution of culture yielded counts of 102, 109, and 108 colonies, respectively.

$$\text{Average number of colonies} = \frac{102+109+108}{3} = 106 \text{ CFU}$$

$$\frac{(106 \text{ CFU}) \times (20 \text{ mL})}{(10^{-6}) \times (0.1 \text{ mL}) \times (20 \text{ doses})} = \frac{2120 \text{ CFU/mL}}{2 \times 10^{-6} \text{ mL/dose}} = 1.06 \times 10^9 \text{ CFU/dose}$$

$$\begin{aligned} &\text{Geometric mean CFU/dose of 2 vials} \\ &= 10^{\left(\frac{1}{2}(\log_{10}(8.3 \times 10^7 \text{ CFU/dose}) + \log_{10}(1.06 \times 10^9 \text{ CFU/dose}))\right)} \\ &= 2.97 \times 10^8 \text{ CFU/dose} \end{aligned}$$

5. Interpretation of Test Results

5.1 If on the initial test the CFU per dose is equal to or exceeds the required minimum as written in the firm's OP, the serial or subserial is satisfactory (SAT) for bacterial count without additional testing.

5.2 If on the initial test the CFU per dose is less than the required minimum release titer as written in the firm's OP, the serial or subserial may be retested (RT) using double the amount of new vaccine samples used in the initial test, provided that if the retest is not done, the serial or subserial is unsatisfactory (UNSAT). Compare the firm's OP method to this SOP when retesting with the new vaccine samples. If on the RT, the average count of the new vaccine samples with the firm's OP method is less than the required minimum, the serial or subserial is UNSAT.

5.3 If on the retest with the new vaccine samples, the average using the firm's OP method count is equal to or exceeds the required minimum, the serial is SAT.

5.4 If on the initial test, the reference culture or positive control culture is not within the titer range determined in **Section 3.2.9**, but the serial being tested has a SAT result, the serial or subserial is a no test (NT) for bacterial count without additional testing and the product is released on the results of the firm's tests. If the reference culture is not within the titer and the serial being tested is below the minimum release titer, the serial is retested without bias using the same number of new vaccine samples as the initial test. If on the initial test there is growth on the negative control plates, the serial or subserial is a NT for bacterial count without additional testing.

6. Record and Report of Test Results

Record and report results of the test(s) according to SOPs.

7. Summary of Revisions

Version CVB-SOP-0104.02

- Alphanumeric number changed from BBSOP0019.04 to CVB-SOP-0104.02.
- The contact has been updated.
- **4.10:** Added to clarify calculation practices for multiple dilution series with valid CFUs.
- Updated room temperature to 20°-25°C in Sections 3.7 and 4.1.

Version BBSOP0019.04

- The Bacteriology Section Leader has been updated.
- **Sections 1-7:** These sections have been rewritten for clarification and updated to reflect current practices.
- **Appendix II:** Media storage conditions have been updated.

Version BBSOP0019.03

- **Appendix II.6:** Ethyl alcohol has been changed to QH₂O.

Version BBSOP0019.02

- The Contact has been changed from Nancy Clough to Sophia Campbell and Amanda Byersdorfer.
- **4.1:** Equipment and supplies have been added.
- **4.2:** This section has been updated to reflect current practices.
- **4.3:** This section has been updated to reflect current practices.
- **7:** This section has been added to explain interpretation of test results for firms' vaccines.
- **Appendix I:** This has been added to list media, media numbers, incubation temperature, incubation time, and media formulations.
- **Appendix II:** Media storage information has been added.

Appendix I
Media, Incubation Temps/Duration

(Or as stated in the Manufacturer's Outline of Production)

¹for the National Centers for Animal Health (NCAH) media formulations, see Appendix II

Organism	Agar	*NCAH Media Number	Diluent	¹NCAH Media Number	Incubation Temperature	Duration of Incubation
<i>Salmonella cholerasuis</i>	Trypticase Soy Agar w/5% Bovine Blood	10205	Tryptose Phosphate Broth	10426	35°± 2°C	Up to 48 hours
<i>Escherichia coli</i>	Trypticase Soy Agar	10487	Tryptose Phosphate Broth	10426	35°± 2°C	24 hours
<i>Pasteurella haemolytica-multocida</i>	Trypticase Soy Agar w/5% Sheep Blood & Additives	10596	Mueller-Hinton Broth	10225	35°± 2°C	Up to 96 hours
<i>Pasteurella anatipestifer</i>	Trypticase Soy Agar w / 0.05% Yeast Extract	10225	Trypticase Soy Broth	10423	35°± 2°C	Up to 48 hours anaerobically
<i>Bordetella bronchiseptica</i>	Bordet-Gengou agar with 15% ovine blood	10157	1% Peptone Saline-Solution	10138	35°± 2°C	Up to 72 hours
<i>Bordetella avium</i>	Tryptose Agar w/ 5% Fetal Bovine Serum	10218	Tryptose Broth	10404	35°± 2°C	Up to 72 hours
<i>Streptococcus equi</i>	Columbia CNA Agar w/5% Sheep Blood	10671	1% Peptone Saline Solution	10138	35°± 2°C	Up to 48 hours
<i>Salmonella dublin</i>	Trypticase Soy Agar	10487	1% Peptone Saline Solution	10138	35°± 2°C	Up to 48 hours
<i>Salmonella typhimurium</i>	Blood Agar Base with 5% Bovine Blood	10006	Trypticase Soy Broth	10423	35°± 2°C	Up to 48 hours
<i>Edwardsiella ictaluri</i>	Trypticase Soy Agar w/5% Sheep Blood	10210	1% Peptone Saline Solution	10138	25°± 2°C	48 hours in humidified atmosphere
<i>Actinobacillus pleuropneumoniae</i>	BHIA with 5% NAD	10697	BHI broth with NAD and dextrose	10347	35°± 2°C	48 hours

<i>Haemophilus parasuis</i>	Chocolate Agar II w/ Hemoglobin and Isovitalex	10598	PPLO Broth w/o CV w/ Additives + 400 µg/mL Streptomycin sulfate	10600	36°± 3°C	24-72 hours anaerobically or in 4-6% CO ₂
<i>Flavobacterium columnare</i>	Modified Cytophaga Agar	11012	Tryptone Water w/1% Peptone	10365	27°- 29°C	60-120 hours humidified atmosphere
<i>Arthrobacter</i> spp.	Modified Tryptic Soy Agar	10278	0.9% Sodium Chloride	30192	22°C	5-7 days

Appendix II Media Formulations

1. Tryptose phosphate broth, NCAH Media #10426

Tryptose phosphate broth (Difco)	29.5 g
H ₂ O	1000.0 mL

Autoclave 20 minutes at 121°C. Store at 2°- 7°C for up to 3 months.

2. Trypticase soy agar with 5% bovine blood (defibrinated), NCAH Media #10205

Trypticase soy agar (BBL)	40.0 g
QH ₂ O	950.0 mL

Autoclave 20 minutes. Cool to 47°C in waterbath. Add: defibrinated bovine blood, 50.0 mL.
Store at 2°- 7°C for up to 3 months.

3. Trypticase soy agar (TSA), NCAH Media #10487

Trypticase soy agar (BBL)	40.0 g
QH ₂ O	1000.0 mL

Autoclave 20 minutes at 121°C. Store at 2°- 7°C for up to 3 months.

4. Mueller-Hinton broth, NCAH Media #10225

Mueller Hinton broth	21.0 g
QH ₂ O	1000.0 mL

Gently warm to dissolve and autoclave 15 minutes at 121°C. Store at 2°- 7°C for up to 3 months.
Add 2.5 mL each of 1% yeast, supplement B, and 10% streptomycin solution before using in a test. Dispose of unused complete broth after use.

5. 1% Yeast Solution, NCAH Media #10999

Yeast extract (Difco)	1.0 g
QH ₂ O	99.0 mL

Gently warm to dissolve and autoclave 15 minutes at 121°C. Store at 2°- 7°C for up to 3 months.

6. 10% Streptomycin Solution, NCAH Media #10999

Dihydrostreptomycin	10.0 g
QH ₂ O	90.0 mL

Filter and dispense.

7. Bacto-Supplement B with Bacto-Supplement reconstituting fluid, 6 x 10 mL, cat. #0276-60-4, Difco Laboratories, Detroit, MI 48232**8. Trypticase soy agar w/ 5% sheep blood w/ additives, NCAH Media #10596**

Trypticase soy agar	40.0 g
QH ₂ O	939.0 mL

QS for blood and additives. Mix and autoclave 20 minutes at 121°C. Cool to 56°C in waterbath.

Add:

Defibrinated sheep blood	50.0 mL
1% yeast extract (Difco) powder	6.25 mL
10% streptomycin	5.0 mL

Yeast and streptomycin can be made in stock solutions and stored in refrigerator. Store completed agar at 2°- 7°C for up to 3 months.

9. Trypticase soy broth (TSB), NCAH Media #10423

Trypticase soy broth	30.0 g
H ₂ O	1000.0 mL

Autoclave 20 minutes at ≥ 121°C. Store at 2°- 7°C for up to 3 months.

10. Trypticase soy agar w/ 0.05% yeast extract, NCAH Media #10543

Trypticase soy agar	40.0 g
Yeast extract	0.5 g
QH ₂ O	1000.0 mL

Autoclave 20 minutes at ≥ 121°C. Cool in 46°- 50°C water bath. Store at 2°- 7°C for up to 3 months.

11. Peptone solution 1% + 0.5% NaCl, NCAH Media #10138

Bacto peptone	10.0 gm
NaCl	5.0 gm
QH ₂ O	1000.0 mL

Autoclave for 20 minutes at $\geq 121^{\circ}\text{C}$. Store at 2° - 7°C for up to 3 months.

12. Bordet-Gengou agar with 15% sheep blood (defibrinated), NCAH Media #10157

Bordet-Gengou agar	30.0 gm
Glycerol	10.0 mL
H ₂ O	QS to 840.0 mL

Heat to boiling. Autoclave for 15 minutes. Cool. Add 150 mL of defibrinated sheep blood. Store at 2° - 7°C for up to 3 months.

13. Tryptose broth, NCAH Media #10404

Tryptose broth	26.0 g
H ₂ O	1000.0 mL

Autoclave 20 minutes at $\geq 121^{\circ}\text{C}$. Store at 2° - 7°C for up to 3 months.

14. Tryptose agar with 5% fetal bovine serum, NCAH Media #10218

Tryptose agar	41.0 gm
QH ₂ O	950.0 mL

Autoclave for 25 minutes. Cool in waterbath at 56°C and add 50 mL fetal bovine serum. Store at 2° - 7°C for up to 3 months.

15. Columbia CNA agar, NCAH Media #10671

Columbia CNA agar	42.5 g
QH ₂ O	950.0 mL

Heat to boil for 1 minute. Autoclave 12 minutes at $\geq 121^{\circ}\text{C}$. Cool to 46° - 50°C . Add defibrinated sheep blood - 50.0 mL. Store at 2° - 7°C for up to 3 months.

16. Blood agar base with 5% bovine blood, NCAH Media #10006

Blood agar base (Difco)	40.0 g
H ₂ O	950.0 mL

Autoclave 20 minutes. Cool to 47°C. Add defibrinated bovine blood - 50.0 mL. Store at 2°- 7°C for up to 3 months.

17. Trypticase soy agar with 5% sheep blood (defibrinated), NCAH Media #10210

Trypticase soy agar	40.0 g
QH ₂ O	950.0 mL

Mix well and autoclave 20 minutes at $\geq 121^{\circ}\text{C}$. Cool to 46°- 50°C in water bath. Add defibrinated sheep blood - 50.0 mL. Store at 2°- 7°C for up to 3 months.

18. Brain heart infusion broth with NAD and dextrose, NCAH Media #10347

Brain heart infusion broth	37.0 g
QH ₂ O	1000.0 mL

Autoclave for 20 minutes at 121°C.

Aseptically add:

NAD, 5% solution	0.4 mL
Dextrose solution, 50%	10.0 mL

Mix and dispense as requested. Store at 2°- 7°C for up to 3 months.

19. Brain heart infusion agar with 5% NAD, NCAH Media #10697

Brain heart infusion agar	52.0 g
NAD 5% solution (0.5 g/10.0 mL)	10.0 mL
QH ₂ O	990.0 mL

Mix brain heart infusion agar in aspirator bottle and autoclave at $\geq 121^{\circ}\text{C}$ for 20 minutes per L. Place in water bath and let cool to 50°C. **This is critical. NAD will break down if agar is warmer than 50°C.**

Aseptically add 10.0 mL of filtered NAD and dispense as requested. Store at 2°- 7°C for up to 3 months.

20. Chocolate Agar II w/ Hemoglobin and Isovitalex, NCAH Media #10598

GC agar base (BBL)	21.6 g
Bacto agar	2.0 g
QH ₂ O	600.0 mL

Mix in an aspirator jug. Set aside.

Hemoglobin	10.0 g
QH ₂ O	400.0 mL

Mix in a screw-capped flask. Autoclave both 20 minutes at $\geq 121^{\circ}\text{C}$. Cool in a 56° water bath. Aseptically combine the two solutions, and add:

Isovitalex	10.0mL
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Dispense as requested. Store at 2° - 7°C for up to 3 months.

21. PPLO Broth w/o CV w/ Additives + 400 $\mu\text{g}/\text{mL}$ Streptomycin sulfate, NCAH Media #10600

PPLO broth w/o CV	21.0 g
QH ₂ O	1000.0 mL

Mix together. Autoclave 15 minutes at $\geq 121^{\circ}\text{C}$. Cool to 56°C . Aseptically add:

Horse serum (heat inactivated)	50.0 mL
PPLO yeast extract (NCAH Media #40069)	100.0 mL
Streptomycin sulfate	400.0 $\mu\text{g}/\text{mL}$

Mix well. Dispense as requested. Store at 2° - 7°C for up to 3 months.

22. Modified Cytophaga Agar, NCAH Media #11012

Typtone	10.0 g
Yeast extract	0.5 g
Beef extract	0.2 g
Sodium acetate	0.2 g
Super Q water	100.0 mL

Combine ingredients, mix well. Adjust pH to 7.3. Add:

Bacto agar	9.0 g
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Autoclave at $\geq 121^{\circ}\text{C}$. Dispense as requested. May also be autoclaved after dispensing. Store at 2° - 7°C for up to 3 months.

23. Tryptone Water w/ 1% Peptone, NCAH Media #10365

Tryptone	10.0 g
Sodium chloride	5.0 g
Peptone	10.0 g
QH ₂ O	1000.0mL

Mix all ingredients and dispense as requested. Autoclave 15 minutes at $\geq 121^{\circ}\text{C}$. Store at 2° - 7°C for up to 3 months.

24. Modified Tryptic Soy Agar, NCAH Media #10278

Tryptic soy broth	30.0 g
Yeast extract	15.0 g
Antifoam	1.5 mL/L
RO water	Q.S. to 1000.0 mL
Agar	20.0 g

Combine, mix well. Autoclave at $\geq 121^{\circ}\text{C}$ for 15 minutes. Store at 2° - 7°C for up to 3 months.

25. 0.9% Sodium Chloride, NCAH Media #30192

Sodium chloride	9.0 g
Q H ₂ O	1000.0 mL

Mix and autoclave 20 minutes at $\geq 121^{\circ}\text{C}$. Store at 2° - 7°C for up to 3 months.