

**United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol**

SAM 513

**Supplemental Assay Method for the Determination of Protein and Phenol in
PPD (Purified Protein Derivative Produced From Cultures of *Mycobacterium
bovis* Strain AN-5) Bovis Tuberculin**

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Contact: Debra L. Owens, (515) 663-7512

Approvals: /s/Patricia A. Meinhardt Date: 26Mar08
Patricia A. Meinhardt, Acting Section Head
Chemistry & Analytical Services

/s/Byron E. Rippke Date: 28Mar08
Byron E. Rippke, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde Date: 03Apr08
Rebecca L.W. Hyde, Section Leader
Quality Management
Center for Veterinary Biologics

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

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Supplemental Assay Method for the Determination of Protein and Phenol in PPD (Purified Protein Derivative Produced From Cultures of *Mycobacterium bovis* Strain AN-5) Bovis Tuberculin

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1. Introduction

The Code of Federal Regulations, Title 9 (9 CFR) (Animals and Animal Products) states that the Animal and Plant Health Inspection Service (APHIS) is responsible for administering the Virus-Serum-Toxin Act. It specifies testing methods for licensed tuberculin products. Protein concentration is determined by classical Kjeldahl digestion, distillation, and titration of the ammonia. Phenol is determined by end-point titration with bromate/bromide. Satisfactory product must contain $1.0 \text{ mg/mL} \pm 0.1 \text{ mg/mL}$ protein. Phenol content must be $0.50\% \pm 0.04\%$.

2. Materials

2.1 Equipment

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

- 2.1.1** Balance, top loading, capable of measuring 0.01 g
- 2.1.2** Digestion unit (Buchi, B-426, with digestion tubes)
- 2.1.3** Distillation unit (Buchi, B-316)
- 2.1.4** Volumetric pipettes, Class A, meets ASTM Standard E969-83
- 2.1.5** Volumetric flasks, Class A, with barrel head glass stopper, meets ASTM E288 requirements
- 2.1.6** Erlenmeyer flasks, 125-mL
- 2.1.7** Buret with PTFE stopcock, 10-mL, precision bore, calibrated to ASTM E-694 accuracy requirements
- 2.1.8** Buret with PTFE stopcock, 50-mL, precision bore, calibrated to ASTM E-694 requirements
- 2.1.9** Graduated cylinders, 50-, 100-, 250-, 500-, and 1,000-mL (PYREX), meeting ASTM D86, D216, and D447 requirements
- 2.1.10** Glass-stoppered Erlenmeyer flasks, 250-mL
- 2.1.11** Heating/stirring plate with stirring bars
- 2.1.12** Fast filter paper, Whatman No. 1

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2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All chemicals are reagent grade. Use distilled or demineralized water or water of equivalent purity.

2.2.1 Protein test

1. Sulfuric acid (H₂SO₄)--Purity: Minimum 95.0%, Maximum 98.0%
2. Mercury tablets (Brinkmann Instruments, Catalog No. 015-00-646-3)
3. Sodium hydroxide (NaOH)--Purity: 98.5%
4. Boric acid (H₃BO₃)--Purity: 99.9%
5. Methyl red--Purity: 98.0%
6. Hydrochloric acid (HCl)--Assay: 36.5%-38.0%
7. Sodium carbonate (Na₂CO₃)--Purity: 99.9%
8. Bromo phenol blue--Purity: 98.0%
9. National Veterinary Services Laboratories (NVSL) Control--Pool of PPD tuberculin products with established protein and phenol values
10. Protein-Standard, National Institute of Standards and Technology, Gaithersburg, MD 20899, Standard Reference Material[®] 927 C, Bovine Serum Albumin, Certified Protein Concentration 71.57 g/L

2.2.2 Phenol test (some reagents same as for protein)

1. Methyl orange--Purity: 98.0%
2. Silicotungstic acid (H₄[Si(W₃O₁₀)₄]*26H₂O)--Purity: 99.0% Store at 4°C.
3. Arsenic trioxide (As₂O₃)--Purity: 99.9%
4. Sodium bicarbonate (NaHCO₃)--Purity: 99.9%
5. Potassium bromate (KBrO₃)--Purity: 98.5%

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6. Potassium bromide (KBr)--Purity: 99.0%

7. Phenol (C₆H₅OH)--Purity: ≥ 99.0%

3. Preparation for the Test

3.1 Personnel qualifications/training

No special test-related training is needed for this testing. Analysts performing this procedure should first conduct 2 trial runs using controls and standards and obtain results within acceptable limits.

3.2 Preparation of equipment/instrumentation

Become familiar with Buchi instruction regarding operation. Turn on water that aspirates fumes from the digestion unit and keeps the water cool in the condenser of the distillation unit. Adjust water flow in the distillation unit to approximately 1 L per minute. Turn on the distillation unit. Set time preselector to “2” (2 minutes) and stopcock for aspiration to “Off.” Make sure that Buchi bottles of NaOH and water are adequately filled.

3.3 Preparation of reagents/control procedures

3.3.1 Protein test (all reagents stable for at least 6 months unless specified)

1. Cut Hg tablets in half.

Caution: Because tablets contain mercury, handle in fume hood and wear gloves, protective glasses, and mask.

2. 32% NaOH: Dissolve 640 g ± 1 g NaOH in 1.4 L H₂O in 2-L volumetric flask on the magnetic stirrer. Cool to room temperature. Dilute to volume with H₂O. Repeat above until Buchi 10-L bottle is full. Store at room temperature.

Caution: NaOH is caustic--Avoid contact with skin.

3. Saturated H₃BO₃: Add 15 g to 100 mL H₂O. Stir, with heat, until all H₃BO₃ dissolves. Some H₃BO₃ recrystallizes when cool. Store at room temperature.

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4. 0.1% bromo phenol blue: Dissolve 0.1 g in 100 mL H₂O. Store at room temperature.
5. 0.5% methyl red: Dissolve 0.5 g in 100 mL ethanol. Store at 4°C.
6. Standardized 0.01 N HCl-0.02 N HCl, 1.7 mL HCl/L H₂O: Titrate approximately 0.0100 g dried sodium carbonate dissolved in 25 mL H₂O. Indicator: 3 drops 0.1% bromo phenol blue; the color of endpoint is green, not bluish green nor yellowish green. Store at room temperature.

Calculation:

$$\underline{N} \text{ HCl} = [(g \text{ Na}_2\text{CO}_3) \times (1000)] / [(Vol \text{ HCl}) \times (52.994)]$$

Caution: Concentrated HCl is corrosive--Handle in fume hood. Avoid contact with skin.

7. Protein Standard: Dilute protein (**Section 2.2.1[10]**) to the range of 0.9-1.1 mg/mL. Prepare sufficient dilution to provide several aliquots of 15-mL portions in 30-mL serum vials. Store at 4°C.

3.3.2 Phenol test (all reagents stable for at least 6 months unless specified)

1. 20% HCl: Slowly add 200 mL HCl to 600 mL H₂O; dilute to 1 L. Store at room temperature.
2. 0.1% methyl orange: Add 0.1 g methyl orange to 100 mL H₂O. Filter if necessary. Store at room temperature.
3. Silicotungstic acid solution (SAS): Dissolve 60 g H₄[Si(W₃O₁₀)₄]*26H₂O in 400 mL H₂O in a 500-mL volumetric flask. Add 50 mL H₂SO₄. When cool, dilute to volume with H₂O. Store at room temperature.
4. Clarifying solution (CS): Add 50 mL SAS and 125 mL 20% HCl to 325 mL H₂O. Prepare fresh prior to each test.
5. "Acid solution" for As₂O₃ standard solution: Add 110 mL HCl and 2.5 mL methyl orange to 100 mL H₂O. Store at room temperature.
6. 0.0500 N As₂O₃: Dissolve 2.4730 g dried As₂O₃ in 25 mL hot 1N NaOH in 1-L volumetric flask. Neutralize with 25 mL 1N H₂SO₄. Cool and dilute to volume with H₂O. Store at room temperature.

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Caution: As₂O₃ is extremely toxic--Avoid contact; handle in fume hood using gloves, mask, and goggles. Consult Material Safety Data Sheet for specific handling instructions.

7. Phenol standard, 0.50%: Dissolve 2.50 g phenol in 500 mL H₂O. Prepare in 500-mL volumetric flask. Store at room temperature.

Critical Control Point: The final diluted volume of the test fluid must be adjusted as described in Step8.

8. Test fluid (TF): Dissolve 0.30 g NaHCO₃, 1.67 g KBrO₃, and 15.00 g KBr in H₂O and Q.S. to 1 L with H₂O. Store at room temperature. The TF volume must be adjusted by adding corrected volume of H₂O to TF. It must take a volume of 21.3 mL to titrate 25 mL 0.050 N As₂O₃ in 10 mL "Acid Solution." A first time titration will require less than 21.3 mL TF.

Adjust as described in the following example:

Example: Assume the first time titration volume is 20.5 mL.

$$(1,000 \text{ mL of TF}) - (20.5 \text{ mL}) = 979.5 \text{ mL}$$

$$\frac{(979.5)(\text{desired vol})}{(\text{actual vol})} \text{ or } \frac{(979.5)(21.3)}{(20.5)} = 1,017.2 \text{ mL}$$

For corrected volume of H₂O: 1017.2 - 979.4 = 37.8 mL to be added to TF.

Note: TF in buret has to be put back into flask.

3.4 Preparation of the sample

3.4.1 Receipt

Follow sample receipt procedures as described by standard operating procedures.

3.4.2 Preparation

PPD tuberculin products are received in sealed serum bottles. They are stored at 4°C in the walk-in refrigerator prior to testing. Before testing, allow sample vials and reagents to warm to room temperature.

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4. Performance of the Test

4.1 Protein

Analyze the control pool and protein standard each time testing is performed. Analyze each in triplicate.

4.1.1 Place 5.0 mL sample, one half Hg tablet, and 3.0 mL H₂SO₄ into a digestion tube

Caution: HgO is poisonous--Use gloves, mask, and goggles.

Caution: Concentrated H₂SO₄ is corrosive--Avoid contact with skin.

4.1.2 Place the tubes in a digestion tube holder. Place the holder into the digestion unit. Turn on the unit and set energy regulator to "5". Fifteen minutes later, set to "7".

4.1.3 Digest until acid comes to true boil or no longer "burned smoke," about 50 to 60 minutes. Set to "9" for 15 more minutes.

4.1.4 Cool, add 6 mL H₂O, mix, and cool again.

4.1.5 Place digestion tube and a flask containing 5 mL H₃BO₃ and 3 drops indicator into the distillation unit. Tilt the flask so the tip of the condenser is immersed in the H₃BO₃.

4.1.6 Press and hold "NaOH" button and count to 3. Then hit "Start" button to start distillation unit. Distill for 2 minutes.

4.1.7 Titrate collected distillate to endpoint color change of yellow to deep rose (pH 5.0) with standardized HCl. Record the volume of HCl on log sheet.

4.2 Phenol

Analyze the control pool and phenol standard each time testing is performed. Analyze each in triplicate.

4.2.1 Add 5 mL sample and 100 mL CS to 250-mL glass-stoppered flask. Shake 2 minutes. Filter through filter paper into 50-mL cylinder.

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4.2.2 Transfer 50 mL filtrate to another flask. Add 1 drop methyl orange, stopper and shake a few seconds. Observe the color; when red, go to **Section 4.2.3**.

4.2.3 Titrate with 2 mL test fluid (TF), stopper and shake a few seconds and observe the color. When red, repeat **Section 4.2.3**. When colorless, go to **Section 4.2.4**.

4.2.4 Shake 30 seconds. Add 1 drop indicator, stopper and shake a few seconds and observe the color. When it does not turn to colorless within 10 seconds, titrate with 1 mL TF, stopper and repeat **Section 4.2.4**. When colorless, go to **Section 4.2.5**.

4.2.5 Shake 1 minute. Add 1 drop indicator, stopper and shake a few seconds. Observe the color. When red stays longer than 10 seconds, titrate with 0.50 mL TF, stopper and repeat **Section 4.2.5**. When colorless, record total volume of TF as the endpoint of titration and use for calculation of percent phenol.

5. Interpretation of the Test Results

5.1 Protein (Report average of triplicates)

$$\text{mg Protein/mL} = (\text{mL HCl})(\text{N HCl})(1.400)(6.25)/(5 \text{ mL PPD})$$

Satisfactory Protein Content: 1.0 mg/mL \pm 0.1 mg/mL

5.2 Phenol (Report average of triplicates)

$$\text{Percent phenol} = (\text{vol of test fluid})(0.04)-(0.04)$$

Satisfactory Phenol Content: 0.50% \pm 0.04%

5.3 Controls

Results for controls and standards must be within acceptable limits; otherwise repeat testing.

6. Report of Test Results

Validate and report results according to the current standard operating procedures.

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7. References

7.1 Code of Federal Regulations, Title 9, Part 113.409, U.S. Government Printing Office, Washington, DC.

7.2 Official Methods of Analysis of AOAC International, Arlington, Virginia, 16th Edition, Pat Cuniff, Editor (1995), Volume I, Chapter 12, page 7.

8. Summary of Revisions

Version .07

- The document number has been changed from TCSAM513 to SAM 513.

Version .06

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the name of the contact person has changed.