

**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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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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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

1. Introduction

This Supplemental Assay Method (SAM) describes the procedures for determination of protein concentration and phenol content in Purified Protein Derivative (PPD) produced from cultures of *Mycobacterium bovis* strain AN-5; as prescribed in title 9, *Code of Federal Regulations*, (9 CFR), part 113.409.

Protein concentration is determined by the Microkjeldahl test for nitrogen. Phenol is determined by direct titration with a standardized bromide-bromate solution.

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 0.001 g
- 2.1.2 Digestion unit (Büchi)
- 2.1.3 Distillation unit (Büchi)
- 2.1.4 Volumetric pipettes, Class A, 5-, 10-, and 25-mL
- 2.1.5 Volumetric flasks with barrel head glass stopper, Class A, 500-mL and 1-L
- 2.1.6 Erlenmeyer flasks, 125-mL
- 2.1.7 Burets with PTFE stopcocks, precision bore, Class A, 10-, 25-, and 50-mL
- 2.1.8 Weigh boats, or equivalent
- 2.1.9 Graduated cylinders, Class A, 50-, 100-, 250-, 500-mL, and 1-L
- 2.1.10 Glass-stoppered Erlenmeyer flasks, 250-mL
- 2.1.11 Heating/stirring plate with stirring bars
- 2.1.12 Filter paper, 11µm particle retention (Whatman No. 1)
- 2.1.13 Timers, 30 seconds to 2 minutes

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2.1.14 Dropper, i.e., transfer pipette, Pasteur pipette, dropper bottle

2.1.15 Pipettor and tips to accurately dispense 100- to 1000- μ L

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All chemicals are reagent grade, unless specified.

2.2.1 Protein test

1. Sulfuric acid (H_2SO_4), CAS# 7664-93-9, Purity: Minimum 95.0%, Maximum 98.0%
2. Kjeldahl Catalyst Tablets, 1.5 g K_2SO_4 + 0.075 g HgO
3. Sodium hydroxide (NaOH), CAS# 1310-73-2, Purity: 98.5%
4. Boric acid (H_3BO_3), CAS# 10043-35-3, Purity: 99.9%
5. Methyl red, CAS# 493-52-7, Purity: 98.0%
6. Hydrochloric acid (HCl), CAS# 7647-01-0, Assay: 36.5-38.0%
7. Sodium carbonate (Na_2CO_3), CAS# 497-19-8, Purity: 99.9%
8. Bromo phenol blue, CAS# 115-39-9, Purity: 98.0%
9. Control Sample – Either a pool of PPD tuberculin products with established protein and phenol values as tested by PL-CAS; or a product produced for use as a control sample and tested by PL-CAS.
10. Protein Standard Reference Material, Bovine Serum Albumin (current lot of SRM 927 from National Institute of Standards and Technology)

2.2.2 Phenol test (some reagents same as for protein)

1. Hydrochloric acid (HCl), CAS# 7647-01-0, Assay: 36.5-38.0%
2. Water (H_2O), Purity: distilled, demineralized, reverse osmosis or equivalent.
3. Methyl orange, CAS# 547-58-0, Purity: 98.0%

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4. Silicotungstic acid hydrate ($H_4[Si(W_3O_{10})_4] \cdot 26H_2O$), CAS# 12027-43-9, Purity: 99.0%. Store at 4°C.
5. Sulfuric acid (H_2SO_4), CAS# 7664-93-9, Purity: Minimum 95.0%, Maximum 98.0%
6. Arsenic trioxide, anhydrous (As_2O_3), CAS# 1327-53-3, Purity: 99.9%
7. Sodium hydroxide (NaOH), CAS# 1310-73-2, Purity: 98.5%
8. Phenol (C_6H_6O), CAS# 108-95-2, Purity: $\geq 99.0\%$
This can be a purchased NIST standard and diluted, if necessary, to the appropriate level.
9. Sodium bicarbonate ($NaHCO_3$), CAS# 144-55-8, Purity: 99.9%
10. Potassium bromate ($KBrO_3$), CAS# 7758-01-2, Purity: 98.5%
11. Potassium bromide (KBr), CAS# 7758-02-3, Purity: 99.0%

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel must have experience or training in this protocol. This includes working knowledge of the use of general laboratory equipment, glassware and chemical safety, and specific training in the operation of the laboratory equipment and reagents listed in **Section 2**.

Analysts performing this procedure should first conduct at least 2 trial runs using controls and standards and obtain results within acceptable limits.

3.2 Preparation of equipment/instrumentation

All equipment must be operated according to manufacturers' recommendations and monitored in compliance with applicable standard operating procedures.

3.2.1 Protein Test

1. Prepare digestion and distillation units according to manufacturer's recommendations.

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2. Check levels of water and sodium hydroxide tanks on the distillation unit, fill if necessary.
3. Prime the buret by rinsing with standardized HCl.

3.2.2 Phenol Test

Prime the buret by rinsing with test fluid.

3.3 Preparation of reagents

Reagents are stable for 6 months from date of preparation and stored at room temperature, unless otherwise noted. Prepare reagents in volumes appropriate to demand to minimize waste due to expiration.

Glassware used for preparation of reagents must meet ASTM requirements; measurements are based on the measurements of uncertainty outlined in those requirements.

All references to “water” indicate distilled, demineralized, reverse osmosis, or water of equivalent purity (**Section 2.2.2.2**).

In the following steps the acronym QS is used. It is defined as quantity sufficient; as much as is sufficient.

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

1. **Standard, 1.0 ± 0.1 mg/mL Protein:** Dilute protein standard reference material (**Section 2.2.1(10)**) to the range of 1.0 ± 0.1 mg/mL protein with water. Prepare sufficient dilution to provide several 15-mL portions. Store at 4°C.
2. **Control Sample:** Either (1) a pool of PPD tuberculin products with established protein and phenol values as tested by PL-CAS; or (2) a product produced for use as a control sample and tested by PL-CAS.
 - (1) Combine any sample volumes remaining after test completion in a pool. Record all identifying information, CAS protein result and expiration date for each sample. Control sample protein concentration is the mean of CAS results for all samples included in the pool.
 - (2) Obtain a product produced for use as a control sample. Analyze the product a total of three times, mean of these trials must be 1.0 ± 0.1 mg/mL protein. Expiration date is as indicated on product. Store at $4^{\circ} \pm 10^{\circ}$ C.

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3. 32% Sodium Hydroxide (NaOH): *Caution!! NaOH is caustic-- Avoid contact with skin.* Dissolve 640 g ± 1 g sodium hydroxide in approximately 1.4 L water in a 2-L volumetric flask on a stir plate. *Solution will be HOT!!* Cool to room temperature. QS with water. Store at room temperature.

4. Saturated Boric Acid (H₃BO₃): *Use a container with at least twice as much volumetric capacity as your final volume.* Add 15.0 ± 0.1 g boric acid to 100 mL water. Stir, with heat, until all boric acid dissolves. Some boric acid recrystallizes when cool. Store at room temperature.

5. 0.1% bromo phenol blue: Dissolve 0.1 ± 0.1 g in 100 mL water. Store at room temperature.

6. 0.5% methyl red: Dissolve 0.5 ± 0.1 g in 100 mL ethanol. Store at 4°C.

7. Standardized 0.01 - 0.02N Hydrochloric acid (HCl): *Caution!! Concentrated HCl is corrosive – Handle in fume hood. Avoid contact with skin. May be prepared and standardized or a standardized solution may be purchased.*

Preparation: Add 1.72 ml hydrochloric acid to approximately 900 mL water in a 1-L volumetric flask. QS with water. Store at room temperature.

Standardization: Weigh approximately 0.010 g dried sodium carbonate. Record weight. Dissolve in 25 mL water. Add three drops 0.1% bromo phenol blue (indicator). Titrate with prepared 0.01 – 0.02N hydrochloric acid to an endpoint color of green, not bluish green nor yellowish green. Calculate the normality of the hydrochloric acid solution as below. Perform three trials and use the calculated mean as the normality of the hydrochloric acid solution.

Calculation:

$$N\ HCl = \frac{[(g\ Na_2CO_3)(1000)]}{[(ml\ HCl)(52.994)]}$$

3.3.2 Phenol test

1. Standard, 0.50% phenol: *Caution!! Handle in fume hood. Avoid contact with skin.* Dissolve 5.0 ± 0.01g phenol in approximately 500 mL water in a 1-L volumetric flask; QS to 1L with water.

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- 2. Control Sample:** Either (1) a pool of PPD tuberculin products with established protein and phenol values as tested by PL-CAS; or (2) a product produced for use as a control sample and tested by PL-CAS.
 - (1) Combine any sample volumes remaining after test completion in a pool. Record all identifying information, CAS phenol result, and expiration date for each sample. Control sample phenol concentration is the mean of CAS results for all samples included in the pool.
 - (2) Obtain a product produced for use as a control sample. Analyze the product a total of three times, mean of these trials must be 0.50 ± 0.04 % phenol. Expiration date is as indicated on product. Store at $4^{\circ} \pm 10^{\circ}$ C.
- 3. 20% Hydrochloric Acid (HCl):** In a 1-L volumetric flask, slowly add 200 mL hydrochloric acid to 600 mL water; QS to 1 L with water.
- 4. 0.1% Methyl Orange:** Dissolve 0.1 ± 0.01 g methyl orange in 100 mL water. Filter if necessary.
- 5. Silicotungstic acid solution (SAS):** Dissolve 60.00 ± 0.5 g silicotungstic acid hydrate in 400 mL water in a 500-mL volumetric flask. Add 50 mL sulfuric acid. When cool, QS to 500 mL with water.
- 6. Clarifying solution (CS):** Add 50 mL SAS and 125 mL 20% hydrochloric acid to 325 mL water. Prepare fresh prior to each test.
- 7. "Acid solution" for As₂O₃ standardization solution:** Add 110 mL hydrochloric acid and 2.5 mL 0.1% methyl orange to 100 mL water.
- 8. 0.050 N Arsenic trioxide (As₂O₃):** *CAUTION!! Arsenic trioxide is extremely toxic. Avoid contact; handle in fume hood using gloves, mask, and goggles. Consult the Safety Data Sheet for specific handling instructions before proceeding.* Dissolve 2.4730 ± 0.001 g anhydrous arsenic trioxide in 25 mL hot 1 N sodium hydroxide in a 1-L volumetric flask. Neutralize solution with 25 mL 1 N sulfuric acid. When cool, QS to 1 L with water.
- 9. 1N Sodium hydroxide:** Dissolve 4.00 ± 0.01 g of sodium hydroxide in 60 mL water in a 100-mL volumetric flask; QS to 100 mL with water.
- 10. 1N Sulfuric acid:** In a 100-mL volumetric flask, slowly add 4.904 mL sulfuric acid to 60 mL water; QS to 100 mL with water.
- 11. Test fluid (TF):** Dissolve 0.30 ± 0.01 g sodium bicarbonate, 1.67 ± 0.01 g potassium bromate, and 15.00 ± 0.01 g potassium bromide in water

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and QS to 1 L with water. ***CRITICAL CONTROL POINT: The test fluid must be standardized as described in Section 3.3.11(1) prior to use.***

1. Standardization

- a.** Prepare standardization solution: Add 25 mL 0.050N arsenic trioxide to 10 mL “Acid Solution.”
- b.** Confirm standardization solution by titrating with previous lot of TF. It should take 21.3 mL TF to titrate the standardization solution.
- c.** Titrate standardization solution with new lot of TF. The required titration volume is 21.3 mL TF. A first time titration may require less than 21.3 mL TF, in which case the TF volume must be adjusted by adding the correct volume of water to the TF, continue to **Step 1d** if this is the case. If the first time titration is 21.3 ± 0.1 mL TF, continue to **Step 2**.
- d.** Adjust the TF volume. For this step the calculations are shown and an example is used to illustrate.

A = Starting volume of TF (mL)

B = Titration volume of TF (mL)

C = Volume of TF left (mL)

D = Required titration volume (21.3 mL)

E = Adjusted volume of TF (mL)

F = Volume of water to be added to volume of TF left to achieve the adjusted volume (mL)

Example: Assume the starting volume of TF is 1000 mL and the titration volume is 20.5 mL.

- $A - B = C$

Example: $(1000 \text{ mL}) - (20.5 \text{ mL}) = 979.5 \text{ mL}$

- $\frac{(C)(D)}{(B)} = E$

Example: $\frac{(979.5 \text{ mL})(21.3 \text{ mL})}{(20.5 \text{ mL})} = 1017.7 \text{ mL}$

- $E - C = F$

Example: $(1017.7 \text{ mL}) - (979.5 \text{ mL}) = 38.2 \text{ mL}$

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- Add the calculated volume of water (F) to the existing TF and put any TF remaining in the buret back into flask. Continue to **Step 2**.

2. Repeat **Step 1c** until three consecutive trials produce an average titration volume of 21.3 mL.

3.4 Preparation of the sample

3.4.1 Receipt

Complete sample receipt as described by standard operating procedures.

3.4.2 Preparation

Licensed or prelicense biologics products are generally received in sealed serum bottles and stored at $4^{\circ}\pm 10^{\circ}\text{C}$ prior to testing. Before testing, allow sample vials and reagents to come to room temperature.

4. Performance of the Test

4.1 Protein

Analyze the control pool, protein standard and a reagent blank sample in duplicate each time testing is performed. Analyze each sample in triplicate.

- 4.1.1 Place one Kjeldahl Catalyst tablet, 5.0 mL sample and 3.0 mL sulfuric acid into a digestion flask

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

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

- 4.1.2 Place the digestion flasks in the digestion unit.

- 4.1.3 Digest in a method that results in a fully digested product, i.e., 250°C for 15 minutes, 410°C for 60 minutes, and 500°C for 15 minutes. Final product should be clear to white-cloudy.

- 4.1.4 Cool in digestion flasks, add 6 mL water, mix (a vortex may be used), and cool again.

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4.1.5 Place digestion flask and a 125-mL Erlenmeyer flask containing 5 mL saturated boric acid solution and 3 drops 0.5% methyl red into the distillation unit. Tilt the Erlenmeyer flask so the tip of the condenser is immersed in the boric acid.

4.1.6 Add a sufficient amount of 32% sodium hydroxide to make the solution in the digestion flask alkaline, i.e., 25 mL. A sodium hydroxide pump may be used for this, if the distillation unit is equipped with one.

4.1.7 Distill for two minutes.

4.1.8 Titrate collected distillate to endpoint color change of yellow to deep rose (pH 5.0) with standardized hydrochloric acid. Record the volume of hydrochloric acid titrated.

4.2 Phenol

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

4.2.1 Combine 5 mL sample/standard/control and 100 mL clarifying solution (CS) to a 250-mL glass-stoppered flask. Shake 2 minutes. Filter through filter paper and collect 50 mL of filtrate.

4.2.2 Transfer 50 mL of filtrate to another 250-mL glass-stoppered flask. Add a stir bar and place flask on stir plate with buret directly above. Add 1 drop 0.1% methyl orange (indicator), stir for a few seconds. Observe the color as pink. *An acceptable alternative to using a stir plate and stir bar would be shaking the flask.*

4.2.3 Titrate with 2 mL test fluid (TF), stir or shake for a few seconds. Observe the color; if pink, repeat. If colorless, go to **Section 4.2.4**.

4.2.4 Stir or shake 30 seconds. Add 1 drop indicator, stir for a few seconds. Observe the color. If colorless for ≤ 10 seconds or if pink, titrate with 1 mL TF, and repeat. If colorless for ≥ 10 seconds, go to **Section 4.2.5**.

4.2.5 Stir or shake 1 minute. Add 1 drop indicator, stir for a few seconds. Observe the color. If pink for ≥ 10 seconds, titrate with 0.50 mL TF, and repeat. When colorless within 10 seconds, record total volume of TF as the endpoint of titration and use this volume for calculation of percent phenol.

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5. Interpretation of the Test Results

5.1 Protein (Report mean of triplicates)

$$\text{mg Protein/mL} = \frac{(\text{mL sample} - \text{mL blank})(N \text{ HCl})(1.4007)(6.25)(10)}{5.0 \text{ mL}}$$

mL sample = Volume of standardized HCl required for sample

mL blank = Volume of standardized HCl required for blank

N HCL = Normality of HCl

1.4007 = Milliequivalent weight of nitrogen x 100

6.25 = % Nitrogen to % Protein conversion factor

10 = Conversion factor for percent protein to mg protein/mL: %P(10)=mgP/mL

5.0 mL = Volume of sample

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

*This value is to be used unless otherwise noted in the approved Outline of Production for the product or 9 CFR 113.409.

5.2 Phenol (Report average of triplicates)

Percent phenol = (vol of test fluid) x (0.04)

Satisfactory Phenol Content*: 0.50% \pm 0.04%

*This value is to be used unless otherwise noted in the approved Outline of Production for the product or 9 CFR 113.409.

5.3 Controls

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

6. Report of Test Results

Report results of the test(s) as described by standard operating procedures.

7. References

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

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7.2 AOAC Official Method 960.52, Official Methods of Analysis of AOAC International, Arlington, Virginia, 16th Edition, Pat Cuniff, Editor (1995), Volume I, Chapter 12, page 7.

7.3 ASTM Standard E969, Standard Specification for Glass Volumetric (Transfer) Pipets.

7.4 ASTM Standard E288, Standard Specification for Laboratory Glass Volumetric Flasks.

7.5 ASTM Standard E694, Standard Specification for Laboratory Glass Volumetric Apparatus.

8. Summary of Revisions

Version .11

Updated the coversheet.

Version .10

- Includes option to purchase a NIST standard phenol.

Version .09

- The document has been revised to provide additional detail.
- **3.3.1:** Standardized 0.01 – 0.02 N Hydrochloric acid may be purchased.
- **3.3.1 and 3.3.2:** Includes an option for obtaining a product produced for use as a control sample.

Version .08

- The document has been revised to reflect changes in instrumentation, personnel, and to provide additional detail.
- **5.1:** Protein, the calculation has been changed to reflect that used in AOAC 960.52 taking into account the blank sample and conversion from percent protein to mg protein/mL. The satisfactory protein content level did not change.

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- **5.2:** Phenol, the calculation has been changed to reflect the 9 CFR, Part 113.409. Percent phenol = (vol of test fluid) (0.04). The satisfactory phenol content level did not change.
- A protein blank sample requirement has been added to the procedure to reflect AOAC Official Method 960.52.

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.

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