United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 500

Supplemental Assay Method for the Determination of Phenol in Veterinary Biologics (pullorum antigen, *Mycoplasma synoviae* antigen, and *Mycoplasma gallisepticum* antigen)

Date:	**June 16, 2022**
Number:	SAM 500.06
Supersedes:	SAM 500.05, February 5, 2016
Standard Requirement:	9 CFR 113.407-408
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1. Introduction

This Supplemental Assay Method (SAM) describes the procedures for determination of phenol in the following veterinary biologics: pullorum tube antigen and avian mycoplasma antigens; *Mycoplasma synoviae* and *Mycoplasma gallisepticum*; as prescribed in title 9, *Code of Federal Regulations* (9 CFR), parts 113.407 and 113.408, respectively.

Phenol concentration is determined by a direct titration with a standardized bromide-bromate solution.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Balance, analytical, capable of measuring 0.0001 g

2.1.2 Balance, top loading, capable of measuring 0.01 g

2.1.3 Volumetric pipettes, Class A, 5-mL

2.1.4 Volumetric flasks with barrel head glass stopper, Class A, 500-mL and 1-L

2.1.5 Burets with PTFE stopcocks, precision bore, Class A, 25- and 50-mL

2.1.6 Graduated cylinders, Class A, 10-, 50-, 100-, 250-, 500-, and 1,000-mL

2.1.7 Glass-stoppered Erlenmeyer flasks, 250-mL

2.1.8 Heating/stirring plate with stirring bars

2.1.9 Fast filter paper, Whatman No. 1

2.1.10 Weigh boats, or equivalent

2.1.11 Timers, 30 seconds to 1 minute

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

2.1.13 Pipettor and tips to accurately dispense 100- to 1000-µL

2.1.14 Stir plate

2.1.15 Stir bars

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 Hydrochloric acid (HCl), CAS# 7647-01-0, Assay: 36.5-38.0%

2.2.2 Water (H_2O), Purity: distilled, demineralized, reverse osmosis or equivalent.

2.2.3 Methyl orange, CAS#547-58-0, Purity: 98.0%

2.2.4 Silicotungstic acid hydrate ($H_4[Si(W_3O_{10})_4]*26H_2O$), CAS# 12027-43-9, Purity: 99.0%, store at 4°C.

2.2.5 Sulfuric acid (H_2SO_4), CAS# 7664-93-9, Purity: Minimum 95.0%, Maximum 98.0%

2.2.6 Arsenic trioxide, anhydrous (As_2O_3), CAS# 1327-53-3, Purity: 99.9%

2.2.7 Sodium hydroxide (NaOH), CAS# 1310-73-2, Purity: 98.5%

2.2.8 Phenol (C_6H_6O), CAS#108-95-2, Purity: ≥ 99.0%
This can be a purchased NIST standard and diluted, if necessary, to the appropriate level.

2.2.9 Sodium bicarbonate ($NaHCO_3$), CAS#144-55-8, Purity: 99.9%

2.2.10 Potassium bromate ($KBrO_3$), CAS#7758-01-2, Purity: 98.5%

2.2.11 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 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 policies/procedures.

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.

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

3.3.1 Standard, 0.25% phenol: Dissolve 2.50 ± 0.01g phenol in approximately 500 mL water in a 1L volumetric flask; QS to 1L with water.

3.3.2 Control Sample:

Either (1) a pool of PPD tuberculin products with established protein and phenol values as tested by Pathobiology Laboratory-Chemistry and Analytical Services (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°± 10° C.

3.3.3 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.

3.3.4 0.1% Methyl Orange: Dissolve 0.1 ± 0.01 g methyl orange in 100 mL water. Filter if necessary.

3.3.5 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.

3.3.6 Clarifying solution (CS): Add 50 mL SAS and 125 mL 20% hydrochloric acid to 325 mL water. Prepare fresh prior to each test.

3.3.7 "Acid solution" for As_2O_3 standardization solution: Add 110 mL hydrochloric acid and 2.5 mL 0.1% methyl orange to 100 mL water.

3.3.8 0.050 N Arsenic trioxide (As_2O_3): ***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.

3.3.9 1 N 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.

3.3.10 1 N 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.

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

1. Standardization

a. Prepare standardization solution: Add 25 mL 0.050 N arsenic trioxide to 10 mL “Acid Solution.”

b. Confirm standardization solution by titrating with previous lot of TF. It should take 21.3 mL test fluid (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, **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 mL) - (20.5 mL) = 979.5 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 mL) - (979.5 mL) = 38.2 mL

- 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°± 10°C prior to testing. Before testing, allow sample vials and reagents to come to room temperature.

4. Performance of the Test

4.1 Pullorum tube antigen

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

4.1.1 Add 5 mL sample and 50 mL 20% HCl to a 250-mL glass-stoppered flask. Shake until the solution decolorizes (final appearance will be white-cloudy, typically takes 2 to 3 minutes). Add 50 mL H_2O and mix. Filter through filter paper and collect 50 mL of filtrate.

4.1.2 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.*

4.1.3 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 4.1.4**.

4.1.4 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 4.1.5**.

4.1.5 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.

4.2 *Mycoplasma gallisepticum* and *Mycoplasma synoviae* antigen

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

4.2.1 Combine 5 mL sample 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 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.

5. Interpretation of the Test Results

5.1 Pullorum tube antigen (report average of triplicates)

% Phenol = (volume of test fluid) x (0.04)

Satisfactory Phenol Content according to 9 CFR 113.407: 0.55 ± 0.05%.

5.2 *Mycoplasma gallisepticum* and *Mycoplasma synoviae* antigen (report average of triplicates)

% Phenol = (volume of test fluid) x (0.04)

Satisfactory Phenol Content according to 9 CFR 113.408: 0.25 ± 0.05%.

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*, parts 113.407-408, U.S. Government Printing Office, Washington, DC.

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

7.3 ASTM Standard E288, Standard Specification for Laboratory Glass Volumetric Flasks

7.4 ASTM Standard E694, Standard Specification for Laboratory Glass Volumetric Apparatus

8. Summary of Revisions

Version .06

- Updated coversheet and contact information.

Version .05

- Updated Contact information.
- Includes option to purchase a NIST standard phenol.
- Updated control sample information.

Version .04

- The Contact information has been updated.
- **3.1:** Personnel qualifications/training has been revised.
- **7:** ASTM Standards E969, E288 and E694 have been added as references.
- All references to chemicals in procedural steps have been changed from the chemical formula to the chemical name

- The entire document has been revised to reflect current practices.

Version .03

- The document number has been changed from TCSAM0500 to SAM 500.