

United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 636

Supplemental Assay Method for the Evaluation of Tuberculin Purified
Protein Derivative (PPD)

Date: May 27, 2016
Number: SAM 636.04
Supersedes: SAM 636.03, July 25, 2011
Standard Requirement: 9 CFR 113.409
Contact: Janet M. Wilson, (515) 337-7245

Approvals: /s/Larry R. Ludemann Date: 15Jun16
Larry R. Ludemann, Section Leader
Bacteriology

/s/Paul J. Hauer Date: 20Jun16
Paul J. Hauer, Director
Policy, Evaluation, and Licensing
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 Evaluation of Tuberculin Purified Protein Derivative (PPD)

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Supplemental Assay Method for the Evaluation of Tuberculin Purified Protein Derivative (PPD)

1. Introduction

This is a Supplemental Assay Method (SAM) for the evaluation of production lots of tuberculin purified protein derivative (PPD) in accordance with title 9, *Code of Federal Regulations*, (9 CFR), part 113.409.

2. Materials

2.1 Reagents/supplies

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

2.1.1 Reference PPD tuberculin, current lot. This reference is obtained from the United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, National Centers for Animal Health (NCAH).

2.1.2 *Mycobacterium bovis* sensitizing agent, current lot. This reagent is available from the NCAH.

2.1.3 *M. avium* sensitizing agent, current lot. This reagent is available from the NCAH.

2.1.4 Phosphate buffer, PPB II

2.1.5 Validated digital calipers or metric ruler made of clear plastic

2.1.6 Needles, 20-gauge x 1-inch and 26-gauge x 3/8-inch

2.1.7 Disposable luer-locking syringes, 1-mL and 3-mL

2.1.8 Pipettes, 1-mL, 2-mL, 5-mL, 10-mL and 25-mL

2.1.9 Glass serum bottles, 20-mL and 30-mL

2.1.10 Rubber seals and metal caps for serum bottles

2.1.11 Crimper for aluminum seals

2.1.12 Depilatory cream, Nair[®]

2.1.13 Animal clippers, equipped with a sharpened #40 or #50 blade

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2.1.14 Ear tags for small animals

2.1.15 Ear tag applicator

2.1.16 Sprayola animal paint and applicator

2.1.17 Cage cards

2.2 Animals

Guinea pigs, 500-700 g, white-haired, nonpregnant females. Twenty-three guinea pigs are required for each lot to be tested. For each group of unknowns to be tested simultaneously, 20 guinea pigs are required for the reference PPD. All guinea pigs used for a test must be from the same source and housed and fed in the same manner.

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel must have working knowledge of the use of general laboratory chemicals, equipment, and glassware and have specific training and experience in the safe handling of laboratory animals. They must have experience in the performance of this assay.

3.2 Selection and handling of test animals

3.2.1 Select guinea pigs that are healthy and free of external parasites and have an unblemished hair coat.

3.2.2 Examine guinea pigs the day they are received, and house according to the current standard operating procedures.

3.2.3 When the test is concluded, instruct the animal caretakers to euthanize the guinea pigs, unless they can be used as second use animals for additional testing or blood collection.

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3.3 Preparation of reagents

Phenolized Phosphate Buffer Number 2 (PPB II) – NCAH Media #10528

| | |
|----------------------------------|--------|
| Na ₂ HPO ₄ | 1.89 g |
| KH ₂ PO ₄ | 0.36 g |
| NaCl | 8 g |
| Distilled water | 900 mL |

Adjust pH to 7.2 ± 0.1. Autoclave at ≥121°C for 20 minutes. When cool, add:

| | |
|----------------------|--------|
| 5% phenol (in water) | 100 mL |
|----------------------|--------|

3.4 Preparation of supplies

3.4.1 Sterilize all glassware before use.

3.4.2 Use only sterile supplies (syringes, needles, rubber seals, metal caps, etc.).

3.5 Test animal sensitization

3.5.1 Sensitize 20 guinea pigs per lot of PPD to be evaluated. Sensitize 10 of the animals with *M. bovis* sensitinogen, and sensitize the remaining 10 animals with *M. avium* sensitinogen. For each group of PPD lots that will be tested simultaneously, similarly sensitize 20 additional guinea pigs to be used for testing the *M. bovis* reference PPD. Wait **35 days** before performing the potency portion of the assay.

3.5.2 Administer 0.5 mL of the respective sensitinogen intramuscularly to each guinea pig. Split the dose, administering 0.25 mL into each rear leg. Use 3-mL syringes fitted with 20-gauge x 1-inch needles. Identify the sensitized guinea pigs by cage cards or equivalent.

3.5.3 Retain 3 guinea pigs as nonsensitized controls for each unknown lot of PPD to be tested. Unsensitized controls are not necessary for the reference PPD.

4. Performance of the Potency Test

4.1 Preparation of guinea pigs for the potency assay

Clip the entire abdomen of each guinea pig with animal clippers. Generously apply a depilatory cream to the clipped abdomen. Wait at least 3 minutes. Wash off the depilatory cream with warm water within 10 minutes of application and dry the abdomen with a soft

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towel. Allow the guinea pigs to rest for at least 4 hours before administering the tuberculin injections.

4.2 Preparation of tuberculin dilutions

Preparation of assay dilutions:

4.2.1 Determine the protein concentration for the serial using the current version of **SAM 513**. The concentration must be 1 mg/mL, so a predilution may need to be made before preparing the following dilutions. Make 4 dilutions of the test lot of PPD, using sterile PPB II as the diluent. Dilute to achieve final concentrations of 0.6, 1.2, 2.4, and 4.8 µg protein per 0.1-mL dose, respectively as indicated below. Place each dilution in a serum bottle. Cap and label the bottles.

| Final concentration (µg protein per 0.1-mL dose) | Contents of serum vial |
|--|--|
| 4.8 | 1.2 mL of 1 mg/mL aliquot + 23.8 mL PPB II |
| 2.4 | 10 mL of 4.8 µg protein per 0.1-mL dose dilution + 10 mL PPB II |
| 1.2 | 5 mL of 4.8 µg protein per 0.1-mL dose dilution + 15 mL PPB II |
| 0.6 | 2.5 mL of 4.8 µg protein per 0.1-mL dose Dilution + 17.5 mL PPB II |

4.2.2 Repeat Step 1 with each additional serial of PPD to be tested and with the *M. bovis* reference PPD. Determine the protein concentration for the *M. bovis* reference. The concentration must be 1 mg/mL, so a predilution may need to be made before preparing the 4 dilutions listed above.

4.3 Intradermal injection of test animals

4.3.1 Identify 4 injection sites on the abdomen of each guinea pig, with 2 sites on each side, equidistant from the midline (see the **Appendix**). Sites must be spaced sufficiently far apart to avoid overlapping of subsequent skin reactions. Do not mark the sites on the abdomen with ink. Randomly assign and record which dilution (0.6, 1.2, 2.4, or 4.8 µg protein per 0.1-mL dose) will be injected into each site.

4.3.2 Inject each sensitized guinea pig at the 4 defined injection sites inserting the length of the bevel on a 3/8-inch needle. Inject individual guinea pigs with only a single lot of PPD, 1 site per dilution, as prepared in **Section 4.2.1**. Needles must be sharp to perform ideal intradermal injections and it is recommended that needles be replaced after insertion through a rubber stopper or after injecting 10 guinea pigs.

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1. Insert ear tags in each guinea pig for identification purposes. Alternatively, mark guinea pigs with Sprayola to differentiate them. Animals may be marked with different colors on the head, back, rear, or foot for identification. Administer each preparation intradermally using 1-mL tuberculin syringes fitted with 26-gauge x 3/8-inch needles.
2. For each serial of PPD to be tested, inject 10 guinea pigs sensitized with *M. bovis* and 10 guinea pigs sensitized with *M. avium*. Similarly inject 3 nonsensitized guinea pigs.
3. Inject the *M. bovis* reference PPD into each of 10 guinea pigs sensitized with *M. bovis* and 10 guinea pigs sensitized with *M. avium*.

5. Interpretation of the Test Results

5.1 Recording of test results

- 5.1.1 Measure the test reactions in good lighting at 24 hours following injection.
- 5.1.2 Measure the greater and lesser diameters of erythema and/or swelling to the closest mm at each injection site. Gently palpate the lesion to determine the margin of the swelling, which may or may not extend beyond the margin of erythema. Record the results.
- 5.1.3 Calculate the area of erythema and/or swelling (in mm²) by multiplying the greater and lesser diameter measurements.
- 5.1.4 Determine the total area of erythema and/or swelling for each guinea pig by adding the areas of the 4 injection sites.
- 5.1.5 Add together the totals from **Section 5.1.4** for all guinea pigs with the same sensitization and same PPD tuberculin injection. Then divide by the number of guinea pigs in that treatment group to determine the average response per guinea pig to the given PPD tuberculin for the given type of sensitization.
- 5.1.6 Calculate the specificity index of each lot of PPD tuberculin by subtracting the average response obtained on *M. avium*-sensitized guinea pigs injected with that lot from the average response of *M. bovis*-sensitized guinea pigs injected with that lot. Record all calculations.

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5.2 Criteria for a valid test

5.2.1 The specificity index of the *M. bovis* reference PPD must be at least 400 mm² for a valid test.

5.2.2 The test lot of PPD tuberculin is unsatisfactory if erythema and/or swelling is noted at the injection site(s) of 1 or more of the 3 unsensitized guinea pigs injected with that lot.

5.2.3 If the test is valid and no reactions are observed in unsensitized guinea pigs, each lot of PPD tuberculin is classified according to the following table:

| Specificity Index | Classification |
|--------------------------------|----------------|
| 440 mm ² or greater | Satisfactory |
| 360-440 mm ² | Inconclusive |
| Less than 360 mm ² | Unsatisfactory |

5.2.4 If a lot of PPD tuberculin is inconclusive, perform a second-stage test. If a second-stage test is not performed, the lot is unsatisfactory.

1. Conduct the second-stage test in a manner identical to the first, except omit the unsensitized control guinea pigs.
2. Combine the results obtained on all guinea pigs in stages 1 and 2. Calculate the average response on the 20 guinea pigs sensitized with each antigen and calculate the specificity index.
3. If the cumulative specificity index is ≥ 400 mm², the lot of PPD tuberculin is satisfactory; if it is < 400 mm², the lot is unsatisfactory.

6. Reporting of Test Results

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

7. References

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

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8. Summary of Revisions

Version .04

- The Section Leader and Director information has been updated.
- Alternative guinea pig identification method added.

Version .03

- The Contact information has been updated.
- **2.1.7:** Disposable syringes, 5-mL and 10-mL have been removed to reflect the syringe size used for sensitizing guinea pigs.
- **2.1.8:** Pipettes, 2- mL and 5- mL, have been added to the list of reagents/supplies to reflect the other volumes measured while preparing the tuberculin dilutions.
- **2.1.13:** Animal clippers equipped with a sharpened #50 blade have been added since it is also used for better hair removal on the guinea pigs.
- **4.3.2:** The entire needle length is no longer being used for each injection as inserting the needle just past the beveled edge is the correct depth into the dermis.
- All references to the National Veterinary Services Laboratories (NVSL) have been changed to the National Centers for Animal Health (NCAH) throughout the document.

Version .02

- The Contact has been changed from Charles Egemo to Janet Wilson.
- **2.1.1/2.1.2/2.1.3:** The source of *M. bovis* Reference PPD tuberculin, *M. bovis* sensitizing agent, and *M. avium* sensitizing agent has been changed to reflect the current supplier.
- **2.1.5:** Validated digital calipers have been added.
- **2.1.7:** Five-mL and 10-mL disposable syringes have been added.
- **2.1.8:** One-mL and 10-mL pipettes have been added.
- **2.1.9:** Thirty-mL glass serum bottles have been added.

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- **2.1.13:** The phrase “with a sharpened” has been added.
- **2.1.14/2.1.15/2.1.16:** Ear tags for small animals, ear tag applicator, and cage cards have been added to the list of reagents/supplies.
- **3.2.2:** The phrase “Animal Users’ Manual” has been replaced by “standard operating procedures”.
- **3.2.3:** This section has been changed to reflect more current procedures.
- **3.3.1:** The phrases “Phenolized” and “Buffer Number 2 (PPB-2)” have been added. The NVSL Media number has been changed to #10528.
- **3.5.1:** The phrase “*M. bovis*” has been added.
- **3.5.2:** The phrase “Identify the sensitized guinea pigs by cage cards or equivalent” has been added.
- **4.1:** The phrase “wait at least 10 minutes” has been changed to “wait at least 3 minutes”. The phrase “within 10 minutes of application” has been added.
- **4.2/4.3:** These sections have been updated to reflect current procedures.
- **4.3.1** Removed worksheet BBFRM0002 and added “the current” worksheet.
- **5.1.1:** The phrase “in good lighting” has been added and the section has been updated to reflect current procedures.
- **5.2.1:** The phrase “*M. bovis*” has been added.

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Appendix

Tuberculin Purified Protein Derivative (PPD) Test Record

Tuberculin ID _____ Date read _____ Initials _____
 Dilution 1 = 4.8 µg/0.1 mL dose Dilution 2 = 2.4 µg/0.1 mL dose Dilution 3 = 1.2 µg/0.1 mL dose Dilution 4 = 0.6 µg/0.1 mL dose

Mycobacterium avium Sensitized

| Guinea Pig Tag# | Site A | Site B | Site C | Site D |
|-----------------|--------|--------|--------|--------|
| | 1 | 2 | 3 | 4 |
| | 1 | 4 | 2 | 3 |
| | 2 | 3 | 1 | 4 |
| | 2 | 4 | 3 | 1 |
| | 3 | 1 | 2 | 4 |
| | 3 | 2 | 4 | 1 |
| | 4 | 1 | 3 | 2 |
| | 4 | 3 | 2 | 1 |
| | 2 | 1 | 4 | 3 |
| | 3 | 4 | 1 | 2 |

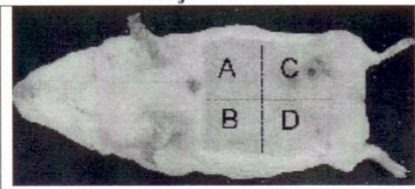
Mycobacterium bovis Sensitized

| Guinea Pig Tag# | Site A | Site B | Site C | Site D |
|-----------------|--------|--------|--------|--------|
| | 1 | 2 | 4 | 3 |
| | 1 | 4 | 3 | 2 |
| | 2 | 3 | 4 | 1 |
| | 2 | 4 | 1 | 3 |
| | 3 | 1 | 4 | 2 |
| | 3 | 2 | 1 | 4 |
| | 4 | 1 | 2 | 3 |
| | 4 | 3 | 1 | 2 |
| | 1 | 3 | 2 | 4 |
| | 4 | 2 | 3 | 1 |

Nonsensitized Control Guinea Pigs

| Guinea pig Tag# | Site A | Site B | Site C | Site D |
|-----------------|--------|--------|--------|--------|
| | 1 | 2 | 3 | 4 |
| | 1 | 4 | 2 | 3 |
| | 2 | 3 | 1 | 4 |

Injection Sites



Total area for *Mycobacterium avium* sensitization _____ mm²
 Total area for *Mycobacterium bovis* sensitization _____ mm²
M. bovis total – *M. avium* total _____ mm²
 ÷ Number of guinea pigs per group: 10
 Specificity index: _____ mm²

NR= No reaction

Initials _____ Date _____
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