Precautions

All reagents should be handled and disposed of as potentially hazardous. Do not eat, drink, or smoke where samples and reagents are handled. Do not pipette by mouth. Some reagents may be harmful if ingested. If ingested, seek medical attention. Do not use expired or contaminated reagents.

Component B, Positive Control, contains sodium azide as a preservative.

Component C, Negative Control, contains sodium azide as a preservative.

Component D, Peroxidase Conjugate, contains Proclin 300, methylisothiazolone and bromonitrodioxane as a preservative.

Component E, Sample Diluting Buffer, contains sodium azide as a preservative.

Version 200519

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vmrd Veterinary Medical Research & Development

FOR VETERINARY USE ONLY

VLN/PCN 332/5064.20

MYCOBACTERIUM AVIUM SUBSPECIES *PARATUBERCULOSIS* ANTIBODY TEST KIT

Assay instructions for catalog number 5064.20-2 and 5064.20-5

General Description

This enzyme-linked immunosorbent assay (ELISA) detects antibodies to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in bovine sera, caprine sera, and bovine milk. Sample serum and milk antibodies bind to MAP antigen attached to the plastic wells of the microtiter plate. Binding of these antibodies is detected by reaction with horseradish peroxidase (HRP)-labeled secondary antibody. Attached HRP-labeled antibodies are detected by addition of enzyme substrate and quantified by subsequent color product development. Strong color development indicates the presence of antibody to MAP in the sample serum or milk sample. Very weak or no color development indicates the absence of antibody to MAP in the sample serum or milk.

Kit Contents

Component	5064.20-2	5064.20-5
A Antigen-Coated Plates	2 plates	5 plates
B Positive Control	4 ml	4 ml
C Negative Control	4 ml	4 ml
D Peroxidase Conjugate	16 ml	40 ml
E Sample Diluting Buffer	60 ml	2 x 60 ml
F 10X Wash Solution Concentrate	120 ml	2 x 120 ml
G Substrate Solution	30 ml	60 ml
H Stop Solution	30 ml	60 ml
Assay Instructions		

Materials Required But Not Included in the Test Kit

Single and multichannel adjustable-volume pipettors and disposable plastic tips, test tubes or non-antigen-coated transfer plate(s), ELISA microplate reader or spectrophotometer with 450 nm filter, deionized or distilled water, paper towels, graduated cylinder, timer, multichannel pipettor reservoirs, wash bottle, manual multichannel washing device or automatic plate washer

Storage and Stability

Store all reagents at 2-8°C. **Do not freeze.** Reagents will remain stable until the expiration date when stored as instructed.

Preparation

a. **Warm reagents:** Bring the samples, reagents and plate(s) to room temperature (23 ± 2°C) prior to starting the test.

b. **Prepare samples:** Test bovine or caprine serum samples must be diluted 1/20 in Sample Diluting Buffer (E) for use in the test. Test bovine milk samples must be diluted 1/2 in Sample Diluting Buffer (E) for use in the test.

To dilute serum, add 190 µl of Sample Diluting Buffer (E) to the appropriate wells of a transfer plate according to your set-up record, leaving empty wells for subsequent addition of controls. Add 10 µl of serum to each well containing the Sample Diluting Buffer. Change tips between samples.

To dilute milk, add 100 µl of Sample Diluting Buffer (E) to the appropriate wells of a transfer plate according to your set-up record, leaving empty wells for subsequent addition of controls. Add 100 µl of milk to each well containing the Sample Diluting Buffer. Change tips between samples.

c. **Prepare plates:** Remove the plate(s) from the pouch(es) (A). If applicable: Return any unused strips to the pouch and securely seal it. Place strips to be used in the frame and number the top of each strip to maintain orientation. Always mark the strips in case they dislodge from the frame during washing.

d. **Prepare wash solution:** Prepare 1X Wash Solution by diluting 1 part of the 10X Wash Solution Concentrate (F) with 9 parts of deionized or distilled water. Approximately 1.5 ml are needed per well. Allow extra quantity for reservoirs, washing equipment, tubing, etc.

Test Procedure

1. **Load controls and serum or milk samples:** Positive and Negative Controls are provided READY-TO-USE. Load Positive Control (B) and Negative Control (C) in duplicate, regardless of the number of samples to be tested. Add the controls to the empty transfer plate wells for Preparation Step b. When whole plate(s) are used, it is best to put the controls on different areas of the plate. The controls must be loaded every time the assay is performed and on each plate if multiple plates are used. Using a pipettor set at 50 µl, mix the samples in the transfer plate by cycling the pipettor at least 3 times. Load controls and samples into the Antigen-Coated Plate (A). Samples and controls should be loaded into the Antigen-Coated Plate (A) as quickly as possible. Change tips between samples. Tap the side of the loaded assay plate several times to make sure the samples coat the bottom of the wells. Use care not to spill samples from well to well. Incubate the plate for 30 minutes at room temperature (23 ± 2°C).
2. **Wash wells:** After the 30-minute incubation, wash the plate 3 times:

   If an automatic washer is used, place the plate on the washing apparatus and wash plate 3 times, filling the wells each time with at least 200 µl of 1X Wash Solution.

   If manual washing is used, dump well contents and remove remaining samples and controls by sharply striking the inverted plate 4 times on a clean paper towel, striking a clean area each time. Immediately fill each well with at least 200 µl of 1X Wash Solution using a multichannel filling device or a wash bottle. Empty the wash solution from the plate and strike the inverted plate sharply on a clean paper towel as above. Fill and empty the plate by the same method 2 additional times for a total of 3 washes.
3. **Add conjugate:** Add 50 µl of Peroxidase Conjugate (D) to each well. Tap the side of the loaded assay plate several times to make sure the conjugate coats the bottom of the wells. Incubate for 30 minutes at room temperature (23 ± 2°C).
4. **Wash wells:** After the 30-minute incubation, wash the plate 3 times as in Step 2.
5. **Add substrate solution:** Add 50 µl of Substrate Solution (G) to each well. Tap the side of the loaded assay plate several times to make sure that the substrate coats the bottom of the wells. Incubate 10 minutes at room temperature (23 ± 2°C). Avoid leaving the plate in direct sunlight. *Do not empty wells.* The plate may be incubated for up to 15 minutes if using an automated ELISA system.
6. **Add stop solution:** Add 50 µl of Stop Solution (H) to each well. Tap the side of the loaded assay plate several times to mix the Substrate Solution and the Stop Solution. *Do not empty wells.*
7. **Read and record the test results:** Immediately after adding the Stop Solution, the plate should be read on a microplate absorbance spectrophotometer. Set the optical density (OD) reading wavelength to 450 nm and read plate(s). Some readers require an empty well on the plate for blanking. In this case, no reagents should be added to this well.
8. Return all remaining kit reagents to 2-8°C for storage.

Calculation of S/P:

$$S/P = \frac{\text{Sample OD - Mean Negative Control OD}}{\text{Mean Positive Control OD - Mean Negative Control OD}}$$

Test Validation

- The mean OD of the **Positive Control** must be ≥ 0.300
- The mean OD of the **Negative Control** must be < 0.200

Interpreting the Results

- Bovine serum samples having an S/P ≥ 0.30 are positive.
- Bovine serum samples having an S/P < 0.30 are negative.
- Caprine serum samples having an S/P ≥ 0.80 are positive.
- Caprine serum samples having an S/P < 0.80 are negative.
- Bovine milk samples having an S/P ≥ 0.13 are positive.
- Bovine milk samples having an S/P < 0.13 are negative.

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