INSTRUCTIONS FOR USE

BOVIGAM™ TB Kit
An in vitro diagnostic test kit for detection of bovine tuberculosis infection in cattle
Catalog Number 63320, 63326

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from thermofisher.com/support.

WARNING! POTENTIAL BIOHAZARD. Read the biological hazard safety information at this product’s page at thermofisher.com. Wear appropriate protective eyewear, clothing, and gloves.

Introduction
Tuberculosis, a disease caused by Mycobacterium bovis infection of cattle, occurs in every country of the world and is of major importance to the dairy cattle industry. In some countries, the overall incidence of disease in individual dairy herds may approach a morbidity rate of 60–70%.

Description
Applied Biosystems™ BOVIGAM™ TB Kit is a rapid in vitro blood-based assay of cell mediated response to M. bovis PPD tuberculin for the diagnosis of bovine tuberculosis infection in cattle. Tuberculin PPD antigens are presented to lymphocytes in whole blood culture. The production of IFN-γ from the cells is then detected using a monoclonal antibody-based sandwich enzyme immunoassay (EIA). Lymphocytes from cattle not infected with M. bovis do not produce IFN-γ. Therefore, detection of IFN-γ correlates to M. bovis infection.

Field studies
Field trials in over 13,000 head of cattle in Australia, USA, Ireland, New Zealand, Italy and Spain have shown that BOVIGAM™ is more sensitive than the intra-dermal tuberculin test for the diagnosis of bovine tuberculosis and may even detect M. bovis-infected cattle at an earlier stage. A controlled laboratory study was conducted at the USDA/ARS/National Animal Disease Center, Bacterial Diseases of Livestock Research Unit, Ames, IA, USA. The study was carried out in 20 head of Hereford steers sensitized with killed M. bovis and compared BOVIGAM™ responses to USA sourced PPD and Pfizer, Australia PPD. Essentially the study showed that positive diagnosis of sensitized cattle occurred in all cattle stimulated with either USA’s PPD or Pfizer, Australia’s PPD. In addition, studies in New Zealand indicate that the specificity of the assay was not affected by skin testing and it is more sensitive than the Comparative Cervical Skin Test (CCT) when used between 3 and 30 days after the Caudal Fold Skin Test (CFT).

Kit components
Store kit at 35° to 46°F (5±3°C). Bring all reagents except Conjugate Concentrate to room temperature (22±3°C) before use. Return to 35° to 46°F (5±3°C) immediately after use.

<table>
<thead>
<tr>
<th>Component</th>
<th>Ten (10) Microplate Test Kit (63320)</th>
<th>Thirty (30) Microplate Test Kit (63326)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: Microplates coated with antibody to IFN-γ</td>
<td>10 × 96 well plates with lids</td>
<td>30 × 96 well plates with lids</td>
<td>Ready for use.</td>
</tr>
<tr>
<td>2: Positive Bovine IFN-γ Control</td>
<td>2 × 1 mL</td>
<td>3 × 2 mL</td>
<td>Contains 0.01% w/v thimerosal. Freeze dried. Reconstitute with deionized or distilled water.</td>
</tr>
<tr>
<td>3: Negative Bovine IFN-γ Control</td>
<td>2 × 1 mL</td>
<td>3 × 2 mL</td>
<td>Contains 0.01% w/v thimerosal. Freeze dried. Reconstitute with deionized or distilled water.</td>
</tr>
<tr>
<td>4: Green Diluent (Plasma diluent buffer)</td>
<td>1 × 60 mL</td>
<td>1 × 175 mL</td>
<td>Contains 0.01% w/v thimerosal. Ready for use.</td>
</tr>
<tr>
<td>5: Wash Buffer – 20x Concentrate</td>
<td>3 × 125 mL</td>
<td>2 × 500 mL</td>
<td>Contains 0.01% w/v thimerosal. Dilute with deionized or distilled water.</td>
</tr>
<tr>
<td>6: Conjugate – 100x Concentrate. (Horseradish peroxidase-labeled anti-bovine IFN-γ)</td>
<td>1 × 1.5 mL</td>
<td>2 × 2 mL</td>
<td>Contains 0.01% w/v thimerosal. Freeze dried. Reconstitute with deionized or distilled water.</td>
</tr>
<tr>
<td>7A: Blue Diluent (Conjugate diluent buffer – 5x Concentrate)</td>
<td>1 × 25 mL</td>
<td>-</td>
<td>Contains 0.05% w/v thimerosal. Dilute with deionized or distilled water.</td>
</tr>
<tr>
<td>7B: Blue Diluent (Conjugate diluent buffer)</td>
<td>-</td>
<td>2 × 175 mL</td>
<td>Contains 0.01% w/v thimerosal. Ready for use.</td>
</tr>
<tr>
<td>8: Enzyme Substrate Buffer</td>
<td>1 × 125 mL</td>
<td>2 × 175 mL</td>
<td>Contains H2O2. Ready for use.</td>
</tr>
<tr>
<td>9: Chromogen Solution – 100x Concentrate</td>
<td>1 × 1.5 mL</td>
<td>2 × 2 mL</td>
<td>Contains TMB in DMSO. Dilute in Enzyme Substrate Buffer.</td>
</tr>
<tr>
<td>10: Enzyme Stopping Solution (0.5M H2SO4)</td>
<td>1 × 75 mL</td>
<td>1 × 175 mL</td>
<td>Ready for use.</td>
</tr>
</tbody>
</table>

For Veterinary Use Only. For In Vitro Use Only.
Material required but not provided

Unless otherwise indicated, all materials are available through thermofisher.com.

<table>
<thead>
<tr>
<th>Use</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood collection</td>
<td>• Lithium heparin Vacutainers: 1/animal</td>
</tr>
<tr>
<td></td>
<td>• 180 Vacutainer needles — 1 inch: 1/animal</td>
</tr>
<tr>
<td></td>
<td>• Needle holders: 2–3/blood collector</td>
</tr>
<tr>
<td>Blood culture</td>
<td>• Sterile graduated 5 or 10 mL pipettes: 1/animal</td>
</tr>
<tr>
<td></td>
<td>• Sterile 24-well tissue culture trays: 1/8 animals</td>
</tr>
<tr>
<td></td>
<td>• Tips for “Combitip” dispenser (5 mL): 3/herd</td>
</tr>
<tr>
<td></td>
<td>• Sterile phosphate buffered saline: 100 µL/animal</td>
</tr>
<tr>
<td></td>
<td>[0.01M, pH 7.2]</td>
</tr>
<tr>
<td></td>
<td>• Bovine Tuberculin PPD 3000 (Cat. No. 7600060): 1/150 animals</td>
</tr>
<tr>
<td></td>
<td>• Avian Tuberculin PPD 2500 (Cat. No. 7600065): 1/150 animals</td>
</tr>
<tr>
<td></td>
<td>• RPMI-1640 Medium with sodium bicarbonate, without L-glutamine, liquid,</td>
</tr>
<tr>
<td></td>
<td>sterile-filtered, suitable for cell culture</td>
</tr>
<tr>
<td>Plasma harvesting</td>
<td>• Tips to fit 100–1000 µL pipette: 3/animal</td>
</tr>
<tr>
<td></td>
<td>• 1 mL microtubes in 96-well format racks and caps for plasma storage:</td>
</tr>
<tr>
<td></td>
<td>1 rack/30 animals</td>
</tr>
<tr>
<td>Bovine IFN-γ EIA</td>
<td>• Tips to fit 12-channel pipette: 3/animal</td>
</tr>
<tr>
<td></td>
<td>• Various polypropylene tubes, EIA reagent troughs, and tips</td>
</tr>
</tbody>
</table>

Equipment required but not provided

• 37°C humidified incubator.
• Accurate, replaceable-tip variable-volume pipettes (to deliver up to 1 mL)
• Graduated 1, 5 and 10 mL pipettes
• Measuring cylinders - 100 mL, 1 L and 2 L
• Deionized or distilled water - 6 L
• 12-channel pipette (to deliver 50 µL and 100 µL)
• Microplate shaker
• Microplate/strip washer
• Microplate reader. This reader MUST be fitted with a 450 nm and 620-650 nm filters

General precautions

Laboratory safety

National Safety Regulations must be strictly followed.

Preparation of reagents

1. Antigens
   • BOVIGAM™ Avian Tuberculin PPD Stimulating Ag (Cat. No. 7600065)
   • BOVIGAM™ Bovine Tuberculin PPD Stimulating Ag (Cat. No. 7600060)
   Dilute 160 µL of BOVIGAM™ Avian Tuberculin PPD Stimulating Ag or BOVIGAM™ Bovine Tuberculin PPD Stimulating Ag in 840 µL of RPMI medium or PBS. Mix blood samples evenly immediately before use.
   Bovine Tuberculin PPD will be at final concentration of 300 IU/mL. Avian Tuberculin PPD will be at 250 IU/mL.

   For the description of a 96-well antigen preparation please review the package inserts provided with the BOVIGAM™ Avian Tuberculin PPD Stimulating Ag and the BOVIGAM™ Bovine Tuberculin PPD Stimulating Ag.

2. Plates
   Allow plate(s) to equilibrate to room temperature before unsealing plastic pouch. Allow at least 30 minutes.

3. Positive and negative controls
   Reconstitute appropriate vials with 1 mL (10 Plate) or 2 mL (30 Plate) of deionized or distilled water.
   Ensure complete resolubilisation. Reconstituted controls may be stored at 35° to 46°F (5±3°C) for up to 3 months, but must be brought to room temperature and mixed thoroughly before used again.

4. Green Diluent
   Bring to room temperature and mix thoroughly. Use undiluted as plasma diluent buffer.

5. Conjugate
   Reconstitute freeze dried Conjugate 100x Concentrate with 1.5 mL (10 Plate) or 2 mL (30 Plate) of deionized or distilled water.
   Ensure complete resolubilisation. Mixing should be performed with a minimum of frothing.
   Conjugate 100x Concentrate must be kept at 35° to 46°F (5±3°C) at all times and used within 3 months of reconstitution.
   Note: Excessive frothing of conjugate may cause denaturation and reduce its performance in the EIA.

For the 10 plate BOVIGAM™ kit only
   Bring Blue Diluent (Conjugate diluent buffer) 5x Concentrate to room temperature and mix thoroughly. Prepare working strength Blue Diluent (Conjugate diluent buffer) by mixing one part 5x Concentrate with 4 parts deionized or distilled water. Working strength Blue Diluent may be stored at 35° to 46°F (5±3°C) for up to 3 months but must be brought to room temperature and mixed thoroughly before being used again.

For 30 plate BOVIGAM™ kits only
   The Blue Diluent is supplied pre-diluted and ready for use. Prepare working strength Conjugate Reagent combining appropriate volumes of working strength Blue Diluent and reconstituted Conjugate 100x Concentrate as set out in the Reagent Preparation Table (Table 1). Mix thoroughly but gently. Avoid frothing. The working strength Conjugate Reagent should be used within 5 minutes of preparation and unused reagent immediately discarded. Return any unused Conjugate 100x Concentrate to 35° to 46°F (5±3°C) immediately after use.

6. Wash Buffer
   Prepare working strength wash buffer by adding one part 20x Concentrate with 19 parts deionized or distilled water. Mix thoroughly. Working strength wash buffer may be stored at room temperature for up to 2 weeks. Unused Wash Buffer 20x Concentrate should be returned to 35° to 46°F (5±3°C) after use.
   Note: Wash Buffer 20x Concentrate may contain salt crystals. Re-dissolve crystals by warming to 37°C. Mix thoroughly before dilution.

7. Enzyme substrate solution
   Bring the Enzyme Substrate Buffer and Chromogen Solution 100x Concentrate to room temperature and ensure each is thoroughly mixed before dilution.
   Prepare enzyme substrate solution just prior to use by combining appropriate volumes of Chromogen Solution Concentrate and Enzyme Substrate Buffer as shown in the Reagent Preparation Table (Table 1). Enzyme substrate solution must be completely mixed and should be colorless. Discard if blue coloration occurs. Use within 10 minutes of preparation.
   Note: If possible use plastic polypropylene disposable containers sterilized by irradiation to prepare the enzyme substrate solution. Do not use polyethylene containers or pipettes. Any glassware used with the enzyme substrate reagents should be rinsed thoroughly with 1N H2SO4 or HCl followed by at least three washes of deionized or distilled water, ensuring no acid residue remains on the glassware.

8. Safe disposal of reagents
   All waste and unused portions of prepared reagents should be disposed of in accordance with all applicable requirements.

Table 1 Reagent preparation table for diluting conjugate and Chromogen

<table>
<thead>
<tr>
<th>Number of Plates</th>
<th>Volume of Conjugate Concentrate (100x) or Chromogen Solution Concentrate (100x)</th>
<th>Volume of working strength Blue Diluent or Enzyme Substrate Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.12 mL</td>
<td>12 mL</td>
</tr>
<tr>
<td>2</td>
<td>0.24 mL</td>
<td>24 mL</td>
</tr>
<tr>
<td>3</td>
<td>0.35 mL</td>
<td>35 mL</td>
</tr>
<tr>
<td>4</td>
<td>0.45 mL</td>
<td>45 mL</td>
</tr>
<tr>
<td>5</td>
<td>0.55 mL</td>
<td>55 mL</td>
</tr>
<tr>
<td>6</td>
<td>0.65 mL</td>
<td>65 mL</td>
</tr>
<tr>
<td>7</td>
<td>0.75 mL</td>
<td>75 mL</td>
</tr>
<tr>
<td>8</td>
<td>0.85 mL</td>
<td>85 mL</td>
</tr>
<tr>
<td>9</td>
<td>0.95 mL</td>
<td>95 mL</td>
</tr>
<tr>
<td>10</td>
<td>1.05 mL</td>
<td>105 mL</td>
</tr>
<tr>
<td>20</td>
<td>2.0 mL</td>
<td>200 mL</td>
</tr>
<tr>
<td>30</td>
<td>3.0 mL</td>
<td>300 mL</td>
</tr>
</tbody>
</table>

Procedural notes

1. All test plasmas and reagents except the Conjugate 100x Concentrate must be brought to room temperature (22±3°C) before use. Thawed test samples should be mixed thoroughly by carefully vortexing each tube. Do not warm above 37°C.
   Note: Several hours may be required to ensure a full bottle of reagent has reached room temperature. If a shorter equilibration time is desired, an ambient temperature water bath must be used.

2. All kit components are to be stored at 35° to 46°F (5±3°C). Return to 35° to 46°F (5±3°C) immediately after preparation (Table 1). Working strength Blue Diluent Buffer may be stored at room temperature (22±3°C) for up to 2 weeks.

3. The Conjugate 100x Concentrate must be left at 35° to 46°F (5±3°C) at all times, even during reconstitution.

4. Complete reconstitution of freeze dried components is essential for valid performance of the assay. To ensure this, reconstitute reagents and allow vials to sit for at least 15 minutes, then mix by gently inverting each vial 4 or 5 times. A roller-rocketer apparatus may be used. Mix again just prior to use.

BOVIGAM™ TB Kit Instructions for Use
Note: It is important that high quality deionized or distilled water is used to reconstitute and dilute reagents as horseradish peroxidase is readily inactivated by pollutants common in laboratory water supplies.

5. Once the assay has been started it should be completed without interruption.

6. Use a separate disposable tip for each sample to prevent cross contamination.

7. Test plasmas from individual animals should be added simultaneously to EIA wells using a 12-channel pipette.

8. EIA plates should be incubated on a plate shaker at a setting of 600 rpm±50 rpm to minimize inter-well variations. If EIA plates are not incubated on a plate shaker, plates should not be incubated directly on the bench, but elevated on an inverted test-tube rack (or similar). The cold solid surface may act as a heat sink and lead to the phenomenon commonly known as ‘edge effect’.

9. Each test plasma should be assayed in duplicate in adjacent wells (e.g. rows A and B) starting at the top of column 1 of the microplate.

10. Positive and Negative Bovine IFN-γ Controls should be assayed in triplicate in serial wells of Columns 4, 5 and 6 (e.g. row F for positive and row E for negative controls).

Following these recommendations allows 45 test plasmas from 15 animals to be assayed in duplicate per EIA microplate.

Test procedure

Stage one – Whole blood culture method

1. Blood Collection
Collect a minimum volume of 5 mL of blood from each animal into a blood collection tube containing heparin as anti-coagulant and gently mix blood by inversion several times to dissolve the heparin. Blood samples should be transported to the laboratory at ambient temperature (22±3°C, avoid extremes) and put into culture within 30 hours of collection. Under no circumstances should blood be stored in refrigerator.

2. Dispensing Blood
Blood samples must be evenly mixed before aliquoting. Use a roller-rocket or gently invert tubes about 10 times immediately prior to dispensing.

Note: It is important to keep cell damage to an absolute minimum as the test requires viable lymphocytes.

Dispense three 1.5 mL aliquots of heparinized blood from each animal into wells of a 24-well tissue culture tray (see Table 2 for recommended layout). This should be performed under aseptic conditions using either sterile disposable pipettes with automatic pipette filler or sterile transfer pipettes.

3. Addition of Stimulation Antigens
Add 100 µL (24 well culture plate) or 25 µL (96 well culture plate) of either PBS (nil antigen control), avian PPD or bovine PPD using aseptic technique to the appropriate 3 wells containing the blood previously dispensed in Step 1 of the procedure above. Antigens are best dispensed using a repetitive delivery pipette such as the Eppendorf “Combitip” system, fitted with sterile 5 mL tips and set on 1. The antigens must be mixed thoroughly into the aliquoted blood. Preferably use a microplate shaker set for 1 minute on high. If a suitable machine is not available, swirl each 24-well or 96 well culture tray ten times both clockwise and counter clockwise on a flat smooth surface. Hold the lid or to get blood on the lid. Avoid frothing of blood.

Optimal performance of this test is dependent on the stimulation antigens being completely mixed in with the blood.

Table 2 Recommended layout for dispensing blood and antigens into 24-well culture trays

<table>
<thead>
<tr>
<th>Animal</th>
<th>NIL</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>A5</th>
<th>A6</th>
<th>Animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NIL</td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
<td>A4</td>
<td>A5</td>
<td>A6</td>
<td>Animal</td>
</tr>
<tr>
<td>3</td>
<td>NIL</td>
<td>B1</td>
<td>B2</td>
<td>B3</td>
<td>B4</td>
<td>B5</td>
<td>B6</td>
<td>Animal</td>
</tr>
<tr>
<td>5</td>
<td>NIL</td>
<td>C1</td>
<td>C2</td>
<td>C3</td>
<td>C4</td>
<td>C5</td>
<td>C6</td>
<td>Animal</td>
</tr>
<tr>
<td>7</td>
<td>NIL</td>
<td>D1</td>
<td>D2</td>
<td>D3</td>
<td>D4</td>
<td>D5</td>
<td>D6</td>
<td>Animal</td>
</tr>
</tbody>
</table>

Stage two – Bovine IFN-γ EIA

1. Reconstitute freeze dried components, if required, while equilibrating other reagents according to the guidelines outlined in “Procedural Notes”.

2. Add 50 µL of Green Diluent to the required wells.

3. Add 50 µL of test and control samples to the appropriate wells containing Green Diluent. Control samples should be added last to each plate. Mix thoroughly by vortexing plates for 1 minute on a microplate shaker, or, if not available, by pipetting up and down 5 times.

4. Cover each plate with a lid and shake for 1 minute on a plate shaker at a setting of 460 rpm±50 rpm. Remove plate from shaker and incubate at room temperature (18–24°C) for 150 minutes.

5. Shaker contents and wash wells 6 times at room temperature. Fill wells with wash buffer taking care not to cross contaminate adjacent wells. Shake out wash fluid and repeat operation a further 5 times. After the sixth wash, place plate(s) face down on clean filter paper and allow to drain, and flick several times over absorbent paper to remove as much remaining wash buffer as possible.

Table 3 Recommended plasma storage layout

<table>
<thead>
<tr>
<th>Row</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>5N</td>
<td>5A</td>
<td>5B</td>
<td>6N</td>
<td>6A</td>
<td>6B</td>
<td>7N</td>
<td>7A</td>
<td>7B</td>
<td>8N</td>
<td>8A</td>
<td>8B</td>
</tr>
<tr>
<td>C</td>
<td>9N</td>
<td>9A</td>
<td>9B</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>10A</td>
<td>10B</td>
<td>11A</td>
<td>11B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>12N</td>
<td>12A</td>
<td>13B</td>
<td>13N</td>
<td>14A</td>
<td>14B</td>
<td>15N</td>
<td>15A</td>
<td>15B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>16N</td>
<td>16A</td>
<td>16B</td>
<td>17N</td>
<td>17A</td>
<td>17B</td>
<td>18N</td>
<td>18A</td>
<td>18B</td>
<td>19N</td>
<td>19A</td>
<td>19B</td>
</tr>
<tr>
<td>F</td>
<td>20N</td>
<td>20A</td>
<td>20B</td>
<td>21N</td>
<td>21A</td>
<td>21B</td>
<td>22N</td>
<td>22A</td>
<td>22B</td>
<td>23N</td>
<td>23A</td>
<td>23B</td>
</tr>
<tr>
<td>H</td>
<td>27N</td>
<td>27A</td>
<td>27B</td>
<td>28N</td>
<td>28A</td>
<td>28B</td>
<td>29N</td>
<td>29A</td>
<td>29B</td>
<td>30N</td>
<td>30A</td>
<td>30B</td>
</tr>
</tbody>
</table>

N=Nil Control Antigen (PBS); AvPPD=Avian PPD; BoPPD=Bovine PPD

4. Incubation
Incubate tissue culture trays, containing blood and antigens, for 16–24 hours at 37°C in a humidified atmosphere.

5. Harvesting of Plasma Samples
Plasma collection may be facilitated by centrifuging the 24-well trays at 500 g for approximately 10 minutes at room temperature (22±3°C). After the incubation, carefully remove approximately 500 µL of plasma (100 µL for 96 well culture plate) from above the sedimented red cells using a variable-volume pipette (100–1,000 µL) and transfer to separate storage tubes as outlined in the Recommended Plasma Storage Layout Table (Table 3). It is convenient to use 1 mL microtubes in 96-well format storage racks. Use a new pipette tip for each plasma sample.

Note: It is important to minimize harvest of any cellular material along with the plasma. However, contamination of the plasma with a very small amount of erythrocytes during harvesting has no effect on the IFN-γ EIA. Similarly, slight haemolysis of blood samples has little effect on the IFN-γ EIA.

The nil antigen sample for animal No. 1 is stored in well A1. The avian sample in A2 and the bovine sample in A3, and so on for each other animal. Wells C4, C5, C6, C4, G5 and G6 should be left empty. This storage pattern is convenient as it allows transfer of the assay samples from the storage racks directly into the EIA trays using a 12-channel pipette. The empty wells in the EIA plate, after the samples have been transferred, are used for assaying positive and negative controls supplied with the EIA kit.

Samples should be assayed in duplicate. Plasma in row A of the storage rack should be transferred to rows A and B of the EIA microplate, row B to C and D, row C to E and F and row D to G and H. The samples in one full storage rack will require two EIA microplates to assay.

6. Plasma storage
Plasma may be stored at 35° to 46°F (5–3°C) for up to 7 days if not required for assays on the day of collection. Each microtube must be sealed with an appropriate cap before storage. Label sample racks with all relevant information including date, operator initials, tube contents and animal numbers and herd details. For longer periods, samples may be stored frozen at −20°C for several months.

Note: Samples must be allowed to equilibrate to room temperature prior to testing by EIA. Carefully vortex each tube several times immediately prior to assay for IFN-γ.
Note: Automatic microplate washers may be used providing the number of wash cycles and the delay period between each wash has been optimized to remove background reactions. Ensure that unbound conjugate has been adequately removed from the wells to prevent invalid results.

6. Add 100 µL of freshly prepared conjugate reagent to wells. The reagent is 1x conjugate diluted in working strength Blue Diluent according to the Reagent Preparation Table (Table 1). Mix thoroughly as in Step 3.

7. Cover each plate, shake and incubate as in Step 4 for 60±5 minutes.

8. Wash wells as in Step 5.

9. Add 100 µL of freshly prepared enzyme substrate solution to wells. Mix thoroughly as in Step 3.

10. Cover each plate with a lid, shake and incubate as in Step 4 for 30 minutes. Protect from direct sunlight.

11. Add 50 µL of Enzyme Stopping Solution to each well, being careful not to transfer chromogen from well to well, then mix by gentle agitation. Note: The Enzyme Stopping Solution should be added to wells in the same order and at the same speed as the enzyme substrate solution.

12. Read the absorbance of each well within 5 minutes of terminating the reaction using a 450 nm filter with a 620–650 nm reference filter. The absorbance values will then be used to calculate results.

Quality control (valid assay)
The control results must be examined before the sample results can be interpreted. Determine the mean absorbance of negative and positive controls.

Acceptable range of means
- Negative bovine IFN-γ control < 0.130
- Positive bovine IFN-γ control > 0.700

Note: The positive control replicates must not deviate by more than 30% from their mean absorbance.

If either of the above criteria is not met, the EIA run is invalid and must be repeated.

Interpretation
1. Calculate mean nil antigen, avian and bovine PPD absorbance values for each sample.
2. Compare the mean absorbance values of the nil antigen, avian and bovine PPD samples for each animal.
   - Positive = 0D bovine PPD − nil antigen ≥ 0.1; and
   - Negative = 0D bovine PPD − avian ≥ 0.1
3. Blood plasma collected from cattle having an OD value greater than 0.100 above that of avian PPD and nil (PBS) antigen, indicates the presence of Mycobacterium bovis infection.

CAUTION
Immunosuppression caused by recent dexamethasone treatment or parturition may depress IFN-γ responses to mycobacterial antigens. Animals that have received an injection of dexamethasone within one week, or that have calved within 4 weeks, should be retested to reduce the possibility of a false-negative result.

Hyper reactors
If the OD values for both PPD-B and PPD-A exceed 3.5 OD units, the samples have to be diluted with NIL plasma from the same animal, in a ratio of 1 part PPD-B/PPD-A and 2 parts NIL plasma and retested in the EIA. Should the retested samples have OD values which exceed 3.5 units, then the original undiluted samples (PPD-B and PPD-A) need to be diluted in a ratio of 1 part PPD-B/PPD-A and 4 parts NIL plasma and be retested again in the EIA.

As with any biological test, this test may give a false positive or false negative result due to local conditions. A test should be interpreted in the context of all available clinical, historical and epidemiological information relevant to the animal(s) under test. Further confirmatory testing may be required in certain circumstances.

Responsibility for the test interpretation and consequent animal husbandry decisions rests solely on the user, and any consulting veterinarian and appropriate health advisors or authorities.

Thermo Fisher Scientific accepts no responsibility for any loss or damage, however caused, arising from the interpretation of test results.

Limitation of procedure
False results may occur due to:
- Incorrect technique
- Use of any anticoagulant other than heparin
- Excessive levels of circulating IFN-γ
- Immunosuppression
- Use of contaminated reagents
- Other deviations from the recommended test procedure

Abbreviated test procedure
Stage 1 – Whole blood culture
1. Collect blood.
2. Aliquot heparinised blood.
3. Add antigens.
4. Incubate overnight.
5. Harvest plasmas.
6. Store plasmas (if necessary).

Stage 2 – Bovine IFN-γ EIA
1. Reconstitute freeze dried components.
2. Add Green Diluent to required wells.
3. Add test plasmas (simultaneously for each animal) and controls to wells.
4. Incubate for 60 minutes.
5. Wash wells.
6. Prepare Conjugate Reagent and add to wells.
7. Incubate for 60 minutes.
8. Wash wells.
10. Add Substrate to wells.
11. Incubate for 30 minutes.
12. Stop reaction.
14. Validate and interpret results.

For a full list of references for the use of BOVIGAM™, please contact Technical Support.

Customer and technical support
Technical support: visit thermofisher.com/askaquestion
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- Worldwide contact telephone numbers
- Order and web support
- User guides, manuals, and protocols
- Certificates of Analysis
- Safety Data Sheets (SDSs; also known as MSDSs)
- MSDS: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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