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TB Gamma (IGRA ELISA)

MYCOBACTERIUM BOVIS INTERFERON GAMMA RELEASE ASSAY, ELISA

The MYCOBACTERIUM BOVIS INTERFERON GAMMA RELEASE ASSAY, ELISA is based on a principle that live white blood cells of an animal exposed to *Mycobacterium bovis* will release interferon gamma (IFNy) in the presence of *M. bovis* antigens in-vitro. Comparison of IFNy concentration in samples which are exposed (stimulated) to *M.bovis* specific antigens (BPPD), and those which are exposed to placebo reagent (Nil), and/or non-specific *Mycobacterium avium* antigens (APPD), provides information if the animal was previously in contact with *M. bovis*. The test has been validated for use in bovine blood samples and can be used for screening or confirmatory purposes.

Bovine blood samples, in which Li-Heparin was used as anticoagulant, are stimulated for interferon gamma release using Bovine Tuberculin PPD (PPDB), Avian Tuberculin PPD (PPDA) and placebo (Nil) antigens. After stimulation, a sandwich ELISA assay is performed to determine a relative concentration of gamma interferon in samples. The diagnostic test uses microtiter plates coated with IFNy capturing monoclonal antibodies. IFNy present in the sample will bind to antibodies coated on the plate. After the sample is removed, the Conjugate binds to the IFNy which is immobilized on the plate. Following incubation any unbound Conjugate is removed in a washing step, and any remaining Conjugate is revealed using a TMB substrate, which produces color in presence of a HRP. A microplate reader is used to measure optical density of the color produced. The amount of color generated is proportional to the amount of IFNy present in the sample.

Kit Contents

Reagents	2-Plate Kit	5-Plate Kit
5 x Dried Positive Control	**2 x 1 ml**	**5 x 1 ml**
Lyophilized Recombinant bovine IFNy. Has to be reconstituted with 1 ml of Diluent prior to use. Hazard Code: Not classified according to EU regulations.		
Negative Control	**2 ml**	**4 ml**
Ready-to-use; Bovine serum free from bovine IFNy. Contains 0.095% sodium azide as a preservative. Hazard Code: Not classified according to EU regulations.		
Diluent	**5 ml**	**10 ml**
Ready-to-use; proprietary formula. The Diluent contains 0.1% ProClin 300 as a preservative. Hazard Code: Not classified according to EU regulations.		
100X Conjugate	**0.5 ml**	**1 ml**
A proprietary formulation containing anti bovine IFNy antibodies. Hazard Code: Not classified according to EU regulations.		
Substrate	**30 ml**	**70 ml**
Ready-to-use; TMB-buffered solution. Hazard Code: Not classified according to EU regulations.		
10X Wash Buffer	**100 ml**	**2 x 100 ml**
A proprietary formula. Contains 0.1% ProClin 300 as a preservative. Hazard Code: Not classified according to EU regulations.		
Conjugate Diluent	**30 ml**	**70 ml**
A proprietary formulation containing animal serum albumin. Hazard Code: Not classified according to EU regulations.		
Bovine IFNγ AB Coated Plate	**2 plates**	**5 plates**
IFNy AB coated on microplates. Hazard Code: Not classified according to EU regulations.		
Stop Solution	**30 ml**	**70 ml**
Ready-to-use; low concentration acid solution. Hazard Code: **R35** - Causes severe burns; **S26** - In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; **S36/37/39** - Wear suitable protective clothing, gloves, and eye/face protection; **S45** - In case of an accident or if feeling unwell, seek medical advice immediately (show the label on the vial).		
Avian PPD (APPD)	**2 x 1 ml**	**5 x 1 ml**
Sterile, preservative free, liquid containing purified protein derivates prepared from the filtrate of heat killed *Mycobacterium avium.* Hazard Code: Not classified according to EU regulations.		

Bovine PPD (BPPD)	**2 x 1 ml**	**4 x 1 ml**

Sterile, preservative free, liquid containing purified protein derivates prepared from the filtrate of heat killed *Mycobacterium bovis.* Hazard Code: Not classified according to EU regulations.

Required but Not Provided

- Precision single and multi-dispensing micropipettes and **sterile tips** for volumes between 10 to 1000 µl (*e.g.,* single pipettes 10-100 and 100-1000 µl and multi-channel pipette 5-50 and 20-200 µl)
  Note: Use of sterile tips is mandatory only in blood stimulation phase due to possible nonspecific white blood cell stimulation in case of bacterial contamination
- 24-well sterile microplates for sample stimulation (e.g. Greiner cat no 662102)
- 96-well non-antigen-coated transfer plate(s)
- Plastic or glass bottles with screw caps, laboratory beakers or Erlenmeyer flasks to make ready-to-use Wash Buffer
- Sterile, endotoxin free 0.9% NaCl, usually found in local pharmacies as sterile physiological solution
- Reagent reservoirs to transfer reagents into plates
- ELISA microplate reader equipped with 450 nm filter
- Deionized, distilled, or RO purified water to make up the Wash Buffer
- Manual or automatic microplate washing system
- Incubator capable of maintaining a temperature of +37°C
- Microplate cover lids or adhesive foil to cover plates
- Microplate shaker and vortex mixer
- For blood sampling use 10 ml Li-Heparin blood collection tubes (green top)
- For supplies contact our customer support at support@ellielab.com.

Storage & Stability

The kit must be stored at 2-8˚C.

Do not equilibrate the 100X Conjugate to room temperature for use. Use directly from the fridge to make ready to use components. Return to the fridge after use. All other reagents of the kit must be equilibrated at room temperature (20-25°C) for minimum 90 minutes before use.

Kit is transported in a cooled box at a temperature between 0 and 15°C.

Do not use components after the expiration date. Do not mix reagents from different kit serials. Do not expose TMB solution to strong light or any oxidizing agents. Handle TMB solution with clean glass or plastic ware.

Care should be taken to prevent contamination of kit components.

Warnings

- All reagents are for *in vitro* diagnostic use only.
- Do not pipette by mouth.
- Avoid contact with open skin.
- Sodium azide is a toxic substance and is used in some reagents. In case of contact with eyes and skin, flush immediately with copious amounts of water. Sodium azide may react with lead and copper plumbing to form explosive metal azides. Upon disposal of reagents, flush with a large volume of water to help prevent azide build-up.
- Stop Solution contains a dilute acid solution. Use with care to avoid contact with skin and eyes. Avoid exposure to bases, metals, or other compounds that may react with acids. Spills should be cleaned up immediately.

All materials in this kit should be treated according to the product Safety Data Sheet.

Specimen Requirements

Ellie's TB Gamma test can be performed with bovine blood samples.

Stimulation procedure

Stimulation should be done immediately after taking samples or maximum 30 hours later. Blood must not be frozen, refrigerated or overheated at any time during the incubation or transportation.

Blood collection:

Collect blood aseptically using Li-Heparin blood collection tubes and take a minimum 6 ml of venous blood sample.

Transportation of samples:

In case samples are shipped to the laboratory, samples must not freeze or overheat. Transportation temperature should be between 18 and 26 ˚C.

Preparation of the stimulation reagents

Prepare working dilutions of stimulation reagents by diluting APPD and BPPD with sterile endotoxin free 0.9% NaCl (e.g., dilute 160 ul of APPD and BPPD in 840 ul of 0.9%NaCl). Mix well.

Stimulation

1. Mix blood samples evenly immediately before use.
2. For each stimulation transfer 1.5 ml of blood to the wells of a 24-well microtiter plate. Fill a minimum of two wells for each sample for Nil and BPPD stimulation for testing according to the Screening protocol or three wells for Nil, BPPD and APPD stimulation for testing according to the Confirmatory protocol.

3. Add 100 µl of stimulation reagents to each corresponding well, according to the schemes below.

Screening protocol

	1 (Nil)	2 (BPPD)	3 (Nil)	4 (BPPD)	5 (Nil)	6 (BPPD)
A	**Sample 1** Nil	**Sample 1** BPPD	**Sample 5** Nil	**Sample 5** BPPD	**Sample 9** Nil	**Sample 9** BPPD
B	**Sample 2** Nil	**Sample 2** BPPD	**Sample 6** Nil	**Sample 6** BPPD	**Sample 10** Nil	**Sample 10** BPPD
C	**Sample 3** Nil	**Sample 3** BPPD	**Sample 7** Nil	**Sample 7** BPPD	**Sample 11** Nil	**Sample 11** BPPD
D	**Sample 4** Nil	**Sample 4** BPPD	**Sample 8** Nil	**Sample 8** BPPD	**Sample 12** Nil	**Sample 12** BPPD

Confirmatory protocol

	1	2	3	4	5	6
A	**Sample 1** Nil	**Sample 1** APPD	**Sample 1** BPPD	**Sample 5** Nil	**Sample 5** APPD	**Sample 5** BPPD
B	**Sample 2** Nil	**Sample 2** APPD	**Sample 2** BPPD	**Sample 6** Nil	**Sample 6** APPD	**Sample 6** BPPD
C	**Sample 3** Nil	**Sample 3** APPD	**Sample 3** BPPD	**Sample 7** Nil	**Sample 7** APPD	**Sample 7** BPPD
D	**Sample 4** Nil	**Sample 4** APPD	**Sample 4** BPPD	**Sample 8** Nil	**Sample 8** APPD	**Sample 8** BPPD

4. Close plates with plate covers, mix well and proceed with incubation.

5. Incubate at 37± 2˚C for 18-48 hours. Longer incubation increases sensitivity of the assay. Do not mix plates.

Preliminary Steps

Reagent Preparation

All reagents of the kit must be equilibrated to room temperature (20-25˚C) before use, except **100X Conjugate**.

Take the IFN-y coated plate from the foil pouch. If using partial plates, only remove the number of wells needed to test all samples. Place the remaining wells back into the pouch and return them to 2-8˚C.

Wash Buffer Preparation

Prepare ready-to-use Wash Buffer by mixing one part of 10X Wash Buffer with 9 parts of distilled or deionized water. It is very important to equilibrate the Wash Buffer to room temperature before use. Mix well. The amount of Wash Buffer needed to wash one plate is 300 ml. Store the Wash Buffer at room temperature up to one month.

Conjugate Preparation

Dilute the concentrated 100X Conjugate 1:100 with Conjugate Diluent by combining one part of the 100X Conjugate with 99 parts Conjugate Diluent (*e.g.,* the quantity needed for one plate is prepared by mixing 110 µl of concentrated Conjugate and 10.89 ml of Conjugate Diluent). Return the 100X Conjugate to 2-8˚C after use. Protect the prepared dilution from light. The prepared working dilution of Conjugate must be used the same day it is prepared!

Positive Control Preparation

1. Add 1 ml of Diluent into the Positive Control Vial;
2. Incubate for 15 minutes;
3. Mix well;
4. Store at 2-8°C for up to 2 months.

Preparation of Samples and Controls

1. After stimulation, centrifuge blood samples at 2000 ± 200 g for 5 minutes in a plate centrifuge or transfer the blood into microcentrifuge tubes, mark the tubes and centrifuge them at 2000 ± 200 g for 5 minutes. If the centrifuges are not available a small amount of plasma will separate on the top of each well during the incubation step.
2. Use plasma for further testing. Transfer 130 ul of samples and controls into the appropriate wells of the 96 well transfer plate. Pipette controls in duplicate.

Testing Procedure

1. Transfer 100 µl of plasma samples and controls from the 96 well transfer plate into the corresponding Bovine IFN Gamma Coated Plate test wells.

3. Cover the plate with protective foil and incubate for 60 minutes at 36-38°C.

3. Wash the plate:

    - Remove the foil from the plate;
    - Dump the content of the plate;
    - Wash four times with 300 ± 20 µl of Wash Buffer.
    - Tap the plate firmly on ab-sorbent paper after the last wash step.

4. Dispense 100 µl of Conjugate into each well.

5. Incubate for 60 minutes at room temperature (20-25°C).

6. Wash the plate:

    - Dump the content of the plate;
    - Wash four times with 300 ± 20 µl of Wash Buffer;
    - Tap the plate firmly on ab-sorbent paper after the last wash step.

7. Dispense 100 µl of Substrate into each well.

8. Incubate for 15 ± 3 minutes at room temperature.

9. Dispense 100 µl of Stop Solution into each well.

10. Read the results at 450 nm on a microplate reader.

Test Validation

- The mean O.D. of the Negative Controls must read less than 0.200 OD.
- The mean OD of the Positive Controls must read over 0.800 OD.
- The Nil OD value of the test sample must read less than 0.400.
- If a mitogen (Pokeweed) stimulation is done, the OD value of the test sample stimulated with Pokeweed must read over 0.500

If validation criteria are not met, the test results are in-valid and samples have to be retested.

Results & Interpretation

Screening protocol

For screening of samples, results are interpreted according to the following table:

PRESUMED POSITIVE
Sample BPPD - Sample Nil ≥ 0.100

NEGATIVE
Sample BPPD - Sample Nil < 0.100

Recommendation

In case of tuberculosis positive farm, Presumed Positive samples can be considered Positive and no retests are necessary. In case of unknown status of the farm, it is recommended to retest Presumed Positive animals using Confirmatory Protocol.

Confirmatory protocol

For confirmation of TB, it is recommended to run Nil, APPD and BPPD stimulated samples. In the case of Confirmatory Protocol, interpretation of the results are according to the following table:

POSITIVE
Sample BPPD - Sample Nil ≥ 0.100 and Sample BPPD - Sample APPD ≥ 0.100

NEGATIVE
Sample BPPD - Sample Nil < 0.100 or Sample BPPD - Sample APPD < 0.100

Recommendation

Confirming or excluding tuberculosis on a farm or diagnosing an individual animal, requires a combination of epidemiological, clinical, and other diagnostic findings that should be

taken into account when interpreting the result of the test. It is not recommended to rely only on the result of one test or method, notably because mycobacterial infections of farm animals are not fully described and other unknown factors could create false positive or negative results.

Limitation of the Test

The sensitivity of the test is dependent on the phase of the disease and viability of the white blood cells during the stimulation phase.

False results may occur due to:

1. Incorrect technique;
2. Use of any anticoagulant other than heparin;
3. Excessive levels of circulating IFNy;
4. Use of contaminated reagents;
5. Immunosuppression caused by recent dexamethasone treatment (within 7 days), parturition (within 4 weeks) and coinfection with Fasciola Hepatica (Liver fluke);
6. Co-infection with other *Mycobacterium spp.*
7. Other deviations from the recommended test procedure.

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