BIONOTE

TB-Feron ELISA Plus

BOVINE
INTERFERON-GAMMA
FOR MYCOBACTERIUM BOVIS ELISA

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Principle of the Test

The BIONOTE TB-Feron ELISA Plus is a sandwich ELISA (enzyme-linked immunosorbent assay) for the qualitative detection of interferon-gamma (IFN-γ). IFN-γ assay is based on the principle that lymphocytes in animal blood are able to immunologically remember the stimulating antigen after stimulation by exogenous or endogenous antigens. When an antigen of *Mycobacterium bovis* is added to the blood of an animal with bovine tuberculosis, antigen-specific effector/memory T cell is rapidly re-stimulated to produce IFN-γ, the cytokine, which is used as a specific marker in cell-mediated immune response (recall response). To measure the IFN-γ in samples, BIONOTE TB-Feron ELISA Plus utilizes a sandwich ELISA method using monoclonal and polyclonal antibodies. It is designed mainly for measurement of bovine IFN-γ and CMI (cell-mediated immunity) after cultivating heparin-treated whole blood with stimulating antigens. It can also be used in the qualitative analysis of IFN-γ with supernatant liquid of cell culture medium or other samples containing lymph.

Materials provided

BIONOTE TB-Feron ELISA Plus contains following items to perform the assay.

1) PPD B (1)
2) PPD A (2)
3) PBS (3)
4) Antibody coated Microplate (4)
5) Negative Control (5)
6) Positive Control (6)
7) 20X Washing Solution (7)
8) Enzyme Conjugate (8)
9) TMB Substrate (9)
10) Stop Solution (10)
11) Adhesive Plate Sealer (11)
12) Instructions for use (12)

Precautions for Use

In order to obtain reproducible results, the following rules must be observed.

1) Blood sample must be treated with heparin and stored at room temperature.
2) Use fresh sample. Hemolyzed or contaminated sample might cause false results.
3) Remove the blood corpuscle in samples before use. They may cause non-specific reaction.
4) Use disposable gloves while handling potentially infectious material and performing the assay. After assay, wash hands with sanitizers.
5) Store all reagents at 2~8 ℃ (35~46 ℉) in the dark. Bring to room temperature (18~25 ℃) 30 minutes prior to use, and return 2~8 ℃ (35~46 ℉) following use.
6) Unused microplate wells should be stored with silica gel in a sealed plastic bag at 2~8 ℃. It should be used as soon as possible. But do not reuse microwells or pour reagents back into their original bottles once dispensed.
7) Do not intermix components from kits with different batch numbers.
8) TMB Substrate (9) and Stop Solution (10) can cause irritation or burns to the skin and eyes. In case of accident, rinse immediately with fresh cold water.
9) Do not expose TMB Substrate (9) to the direct light or any oxidizing agents. Handle all TMB Substrate (9) with new clean glass or plastic ware.
10) Do not use reagents after the expiry date.
11) Avoid contamination of each reagent with sample or other reagents.
12) Optimal results will be obtained by strict adherence to this protocol. Careful pipetting, timing and washing throughout this procedure are necessary to maintain precision and accuracy.
13) The containers and residues should be discarded as biohazard waste and must be handled in accordance with the local regulations.
14) Please reset the test conditions before using an automated analyzer, as the test results may vary.

Collection and Storage of Sample

1) Collect a minimum volume of 5 ml of blood from each animal into a blood collection tube containing heparin as an anticoagulant,, and gently mix blood by inverting several times to dissolve the heparin.
2) Blood samples should be transported to the laboratory at 18~25 ℃ (avoid extremes).
3) Start culturing within 24 hours after collecting blood. Blood should be stored at room temperature (18~25 ℃).

Preparation of Reagents

1) Negative Control (5): The freeze dried Negative Control (5) must be dissolved with 1.0 ml of distilled/deionized water.
2) Positive Control (6): The freeze dried Positive Control (6) must be dissolved with 1.0 ml of distilled/deionized water.
3) 20X Washing Solution (7): The concentrated 20X Washing Solution (7) must be diluted 1 : 19 with distilled/deionized water before use. (e.g. Add 25 ml of 20X Washing Solution (7) to 475 ml of distilled/deionized water and mix thoroughly.)
4) Stability of prepared reagents

Reagent	Storage	Stability
Dissolved Negative Control	–20 ℃ (–4 ℉)	30 days
	2~8 ℃ (35.6~46.4 ℉)	4 hours
Dissolved Positive Control	–20 ℃ (–4 ℉)	30 days
	2~8 ℃ (35.6~46.4 ℉)	4 hours
Diluted washing solution	18~25 ℃ (64.4~77 ℉)	7 days

Procedure of the Test - Pre-treatment of Sample

[Culture of whole blood using a 24-well cell culture tray]

- Dispense stimulating antigen
  Dispense 100 µl of PPD B (1), PPD A (2), and PBS (3) in each well of a 24-well cell culture tray aseptically.

- Addition of Whole blood
  1) Blood samples must be mixed thoroughly before aliquoting. At this time, use roller-rocker or turn the sample tube up and down about 10 times and gently shake it. (The BIONOTE TB-Feron ELISA Plus test requires live lymphocytes, so mix it to the extent that cell damage does not occur.)
  2) Add 1.5 ml of the prepared whole blood to each of the 3 wells in which the stimulating antigens were dispensed. (Use an aseptic pipette sterilized in an aseptic environment.)
     ※ Hemolyzed whole blood may be judged as a false negative, so be careful not to cause hemolysis when collecting or dispensing the sample.
  3) Using a shaker to mix the whole blood with the dispensed stimulating antigen, or rotate it approximately 10 times clockwise or counterclockwise on a flat surface. (Be careful not to bubble or contaminate other wells.)

CAUTION

If the blood volume is less than 5 ml, dispense the stimulating antigen and add blood as follows:

A. Dispense of the stimulating antigen :
Dispense 50 µl of PPD B (1), PPD A (2), and PBS (3) aseptically to each well of a 48-well cell culture tray.

B. Addition of blood :
Add 0.75 ml of the prepared whole blood to each of the 3 wells in which stimulating antigens were dispensed.

- Culture
  Incubate a 24-well cell culture tray containing stimulating antigen and whole blood at 37 ℃ for 16 to 24 hours in a CO_2 incubator.
  * The supply of CO_2 is not required, but humidity control of the incubator is required to prevent drying.

- Plasma separation
  1) After incubation, centrifuge at 500 xg for 10 minutes (room temperature), and transfer the plasma of the supernatant separated from the red blood cells to a clean storage plate or a microtube using a pipette.
  2) At this step, make sure that the red blood cells do not come in. If it is inevitably accompanied, centrifuge at 500 xg for 10 minutes (room temperature) and collect the supernatant again (A small number of red blood cells or slight hemolysis does not significantly affect the test).

- Plasma storage
  Plasma may be stored at 2~8 ℃ (35.6~46.4 ℉) for up to 7 days if not required for assays on the day of collection. For longer storage, samples may be stored frozen at –20 ℃ (–4 ℉) or below.
  However, bring the sample to room temperature (18~25 ℃) for 30 minutes and use after vortexing before performing the ELISA test.

OPTION 1. [Culture of whole blood using tubes for testing (Vacuum vessel, License No.: 109 -100)]
* Test tubes are not included in the ELISA kit.

- Sample preparation and storage
  1) Bring 3 types of test tubes (PPD B, PPD A, PBS) that were kept in the refrigerator to room temperature at least 30 minutes before use.
  2) Collect 1 ml of the collected blood sample using an injection needle and transfer to each of 3 types of the test tubes. Allow the blood to reach the black line (1 ml) of the tube label by a vacuum of the tube.
  3) Shake gently 10 times so that antigens and anticoagulants included in the test tubes can mix well with blood. At this time, be careful not to destroy blood cells by shaking the tube excessively.
  4) Start culturing after collecting blood. If samples are not used immediately, the blood shall be stored at room temperature (18~25 ℃) and must be cultured within 24 hours (Blood refrigeration and freezing are not allowed). Then, mix the tubes again before culturing.
  ※ If it is difficult to collect blood in the test tubes immediately, treat the blood according to [Collection and Storage of Sample] and move it to the laboratory. Then, transfer 1 ml of whole blood into each of the 3 types of the test tubes using a pipette, and shake 10 times to thoroughly mix the antigen with blood. At this step, be careful not to destroy blood cells by shaking the tube excessively.

- Blood culture and plasma collection
  1) Incubate the test tube at 37 ℃ for 16~24 hours to react with a stimulating antigen. When culturing, put the test tube into the rack and position it vertically.
  2) Centrifuge the test tube for 15 minutes at RCF 2,200~2,300 xg.
  3) After centrifugation, collect the plasma above the gel. When collecting plasma, be careful not to touch the gel with the pipette tip.
  4) The collected plasma can be used immediately for the ELISA test. If not, the plasma transferred to another tube can be used.

- Storage of plasma samples
  1) Plasma may be stored at 2~8 ℃ (35.6~46.4 ℉) for up to 7 days if not required for assays on the day of collection. For longer storage, samples may be stored frozen at –20°C (–4 ℉) or below.

OPTION 2. [Culture of whole blood using tubes for testing (Vacuum cluster vessel, License No.: 109 -121)]
* Test tubes are not included in the ELISA kit.

- Sample preparation and storage
  1) Collect at least 4 ml of a whole bovine blood sample with a blood collection tube containing heparin (anticoagulant: heparin must be used), and gently mix up and down using a wrist snap to dissolve the heparin. Please be cautious when shaking the tube as excessive shaking may break the blood cells.
  2) Whole blood sample must be cultured within 24 hours of collection (refrigeration or freezing of the blood is not allowed). The blood sample should be stored and transported at room temperature (18~25 ℃ (64.4~77 ℉)) before being cultured.

- Blood culture
  1) Prepare the number of B, A, and P tubes as with the number of the required samples.
  2) When preparing an individual stimulating antigen cluster tube, carefully open the cap to prevent cross-contamination with the contents of the other cluster tube.
  * Leave the space for the ELISA control solution (NC, PC) for the ELISA test process.

<Example>

PPD B	PPD A	PBS

	1	2	3	4	5	6	7	8	9	10	11	12
A				Sample ⑦	Sample ⑦	Sample ⑦	Sample ⑮	Sample ⑮	Sample ⑮	Sample ㉓	Sample ㉓	Sample ㉓
B				Sample ⑧	Sample ⑧	Sample ⑧	Sample ⑯	Sample ⑯	Sample ⑯	Sample ㉔	Sample ㉔	Sample ㉔
C	Sample ①	Sample ①	Sample ①	Sample ⑨	Sample ⑨	Sample ⑨	Sample ⑰	Sample ⑰	Sample ⑰	Sample ㉕	Sample ㉕	Sample ㉕
D	Sample ②	Sample ②	Sample ②	Sample ⑩	Sample ⑩	Sample ⑩	Sample ⑱	Sample ⑱	Sample ⑱	Sample ㉖	Sample ㉖	Sample ㉖
E	Sample ③	Sample ③	Sample ③	Sample ⑪	Sample ⑪	Sample ⑪	Sample ⑲	Sample ⑲	Sample ⑲	Sample ㉗	Sample ㉗	Sample ㉗
F	Sample ④	Sample ④	Sample ④	Sample ⑫	Sample ⑫	Sample ⑫	Sample ⑳	Sample ⑳	Sample ⑳	Sample ㉘	Sample ㉘	Sample ㉘
G	Sample ⑤	Sample ⑤	Sample ⑤	Sample ⑬	Sample ⑬	Sample ⑬	Sample ㉑	Sample ㉑	Sample ㉑	Sample ㉙	Sample ㉙	Sample ㉙
H	Sample ⑥	Sample ⑥	Sample ⑥	Sample ⑭	Sample ⑭	Sample ⑭	Sample ㉒	Sample ㉒	Sample ㉒	Sample ㉚	Sample ㉚	Sample ㉚

  3) Gently shake the heparin tube again to mix the blood and heparin. Dispense 0.75 ml of bovine blood into B, A, and P cluster tubes containing stimulating antigens.
  4) Apply the plate sealing tape(sealer) onto the cluster tube in which the sample was injected. Fully cover the rack and incubate at 37 °C for 16 to 24 hours.
  * The supply of CO_2 is not required, but humidity control of the incubator is required to prevent drying.

- Plasma separation
  1) After the incubation, the cluster tube rack should be centrifuged (500 xg, 10 min) to collect the supernatant (plasma).
  2) At this step, make sure not to collect the blood cells. If the blood cells have been collected, then centrifuge at 500 xg for 10 minutes and collect the supernatant again.
  3) The collected plasma can be used immediately for the ELISA test. If not, transferred plasma to another tube can also be used.

- Plasma storage
  1) If the samples are not tested immediately, they can be stored at 2~8 ℃ (35.6~46.4 ℉) for up to 7 days. For longer storage, they can be stored frozen at -20 °C (–4 ℉) or below.

Procedure of the Test - ELISA Test

- Preparation of Antibody coated Microplate (4)
  1) Open the sealed plate pouch, take out the required number of plate strips and secure them to the frame. Leave the unused strips in a pouch with silica gel and keep them sealed.

- Conjugate reaction with samples
  1) Dispense 50 µl of Enzyme Conjugate (8) to each well.
  2) Add 50 µl of the dissolved control (A1~A3, B1~B3) and 50 µl of stimulated plasma to each well, as shown in the table below. Then lightly tap the frame to mix well.
  3) Carefully shake the samples for at least 10 seconds to avoid splashing out of the wells. Then seal the plate with an attached Adhesive Plate Sealer (11).
  4) Allow the sealed plate to react at 37 ℃ for 60 minutes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC	NC	a7-B	a7-A	a7-P	a15-B	a15-A	a15-P	a23-B	a23-A	a23-P
B	PC	PC	PC	a8-B	a8-A	a8-P	a16-B	a16-A	a16-P	a24-B	a24-A	a24-P
C	a1-B	a1-A	a1-P	a9-B	a9-A	a9-P	a17-B	a17-A	a17-P	a25-B	a25-A	a25-P
D	a2-B	a2-A	a2-P	a10-B	a10-A	a10-P	a18-B	a18-A	a18-P	a26-B	a26-A	a26-P
E	a3-B	a3-A	a3-P	a11-B	a11-A	a11-P	a19-B	a19-A	a19-P	a27-B	a27-A	a27-P
F	a4-B	a4-A	a4-P	a12-B	a12-A	a12-P	a20-B	a20-A	a20-P	a28-B	a28-A	a28-P
G	a5-B	a5-A	a5-P	a13-B	a13-A	a13-P	a21-B	a21-A	a21-P	a29-B	a29-A	a29-P
H	a6-B	a6-A	a6-P	a14-B	a14-A	a14-P	a22-B	a22-A	a22-P	a30-B	a30-A	a30-P

※ NC: Negative Control, PC: Positive Control, a1-B: a1 sample stimulated by PPD B, a1-A: a1 sample stimulated by PPD A, a1-P: a1 sample stimulated by PBS

- Washing process
  1) Aspirate all liquid from wells and rinse the wells five times with 350 µl of diluted washing solution.
  2) After removing the last wash, shake off the plate strongly to remove any remaining washing solution residue in the well.

  ※ Use the diluted washing solution prepared in advance in accordance with the [Preparation of Reagents].

- Substrate reaction
  1) Add 100 µl of TMB Substrate (9) to each well.
  2) After blocking the light, incubate the wells for 30 minutes at room temperature (18~25 ℃ (64.4~77 ℉)) IN THE DARK.

CAUTION

* Residues of cleaning fluid on the plate may lead to inaccurate results.
* The sealer used for the reaction between conjugate fluid and sample should not be reused

- Stopping the reaction and measuring the absorbance
  1) Add 100 µl of Stop Solution (10) into each of the wells in the same order and at approximately same speed as the TMB Substrate (9).
  2) Measure the absorbance values of the wells at 450 nm in an ELISA plate reader (with a reference wavelength at 620 nm) right after the end of assay.

Interpretation of the Results

- Test validation
  1) The mean absorbance value of the Positive Control (PCx) must be more than 1.0 (≥ 1.0).
  2) The mean absorbance value of the Negative Control (NCx) must be below 0.2 (< 0.2).
  3) If these specifications are not met, the test has to be repeated.

- Interpretation of the results
  1) Compare the mean absorbance values of PBS stimulated samples, PPD A stimulated samples and PPD B stimulated samples.

  * Positive result: Absorbance value (PPD B- PBS) ≥ 0.1 and Absorbance value (PPD B- PPD A) ≥ 0.1
  * Negative result: Absorbance value (PPD B- PBS) < 0.1 or Absorbance value (PPD B- PPD A) < 0.1

  2) If both values subtracted from the absorbance value of PPD B are more than 0.100 (positive result), it is considered as bovine-tuberculosis infected.

  ※ Immunosuppression could interfere with interferon response to mycobacterium antigens. If cows have been injected with dexamethasone within 1 week, or if a calf is less than 4 weeks old, it should be retested to reduce the possibility of false-negative.

Stability and Storage

All reagents should be stored at 2~8 ℃ (35.6~46.4 ℉). Shelf life is 18 months.

Packaging unit

Reagent / Volume	30 Tests/Kit	60 Tests/Kit	150 Tests/Kit	300 Tests/Kit
PPD B (1)	4.0 ml/vial x 1	4.0 ml/vial x 2	4.0 ml/vial x 5	4.0 ml/vial x 10
PPD A (2)	4.0 ml/vial x 1	4.0 ml/vial x 2	4.0 ml/vial x 5	4.0 ml/vial x 10
PBS (3)	4.0 ml/vial x 1	4.0 ml/vial x 2	4.0 ml/vial x 5	4.0 ml/vial x 10
Antibody coated Microplate (4)	1 ea	2 ea	5 ea	10 ea
Negative Control (5)	1.0 ml/vial x 1	1.0 ml/vial x 2	1.0 ml/vial x 5	1.0 ml/vial x 10
Positive Control (6)	1.0 ml/vial x 1	1.0 ml/vial x 2	1.0 ml/vial x 5	1.0 ml/vial x 10
20X Washing Solution (7)	25 ml/bottle x 1	50 ml/bottle x 1	125 ml/bottle x 1	250 ml/bottle x 1
Enzyme Conjugate (8)	10 ml/bottle x 1	20 ml/bottle x 1	50 ml/bottle x 1	100 ml/bottle x 1
TMB Substrate (9)	15 ml/bottle x 1	30 ml/bottle x 1	80 ml/bottle x 1	80 ml/bottle x 2
Stop Solution (10)	15 ml/bottle x 1	30 ml/bottle x 1	80 ml/bottle x 1	200 ml/bottle x 1
Adhesive Plate Sealer (11)	2 ea	4 ea	10 ea	20 ea
Instructions for use (13)	1 ea	1 ea	1 ea	1 ea

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TB-Feron ELISA Plus Quick guide

Pre-treatment of sample

1 Blood collection	Collect the whole blood in heparin tubes *Store at room temperature (18~25 ℃)
2 Whole blood culture	Incubation with stimulating antigens at 37 ℃ for 16-24 hours *Antigen stimulation within 24 hours after collection
3 Plasma separation (centrifugation)	Plate culture: RCF 500 xg, 10 min. For test tube culture: RCF 2,200~2,300 xg, 15 min. For test cluster tube culture : RCF 500 xg, 10 min.

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4 Enzyme conjugate dispension	Dispense 50 µl of Enzyme Conjugate
5 Dispense of the sample	Dispense 50 µl of stimulated plasma and 50 µl of the dissolved control

Reaction at 37 ℃ for 1 hour
Washing 5 times

6 Substrate reaction	Dispense 100 µl of TMB Substrate

Reaction at room temperature
Blocking light

7 Stopping the reaction	Dispense 100 µl of Stop Solution
8 Absorbance measurement	Measured wavelength 450 nm, Reference wavelength 620 nm *It should be measured right after the end of assay

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