Equine IFNg ELISpot Kit

Catalog #EIS25135Eq

Target protein alias: IFN-γ , IFNG, IMMG, IFN-gamma

For the quantitative determination of the frequency of cells releasing Equine IFNg

For Research Use Only.Not For Use In Diagnostic Procedures.

Principle Of The Assay

The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies. This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens. ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. A monoclonal antibody specific for Equine IFNg has been pre-coated onto a PVDF (polyvinylidene difluoride)-backed microplate.Appropriately stimulated cells are pipetted into the wells and the microplate is placed into a humidified 37 °C CO2 incubator for a specified period of time. During this incubation period, the immobilized antibody in the immediate vicinity of the secreting cells binds secreted IFNg. After washing away any cells and unbound substances, a biotinylated monoclonal antibody specific for Equine IFNg is added to the wells. Following a wash to remove any unbound biotinylated antibody, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution (BCIP/NBT) is added. A blue-black colored precipitate forms at the sites of cytokine localization and appear as spots, with each individual spot representing an individual IFNg secreting cell. The spots can be counted with an ELISpot reader system or using a stereomicroscope.

PART	DESCRIPTION
Microplate	96-well PVDF-backed microplate coated with a monoclonalantibody
Detection Antibody Concentrate	120 uL of a 100X concentrated solution of biotinylated monoclonal antibody
Streptavidin-AP Concentrate A	120 µL of a 100X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives
Dilution Buffer 1	12 mL of a buffer for diluting Detection Antibody Concentrate with preservatives
Dilution Buffer 2	12 mL of a buffer for diluting Streptavidin-AP Concentrate A with preservatives
Wash Buffer Concentrate	20 mL of a 25X concentrated solution of a buffered surfactant with preservative
BCIP/NBT Substrate	12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT)
Positive Control	Lyophilized(Reconstitute with 300 µL of cell culture medium)

Store the unopened kit at 2-8 °C. Do not use past kit expiration date. This kit is validated for single use only.Note: Results obtained using previously opened or reconstituted reagents may not be reliable.

Limitations Of The Procedure

1. The kit should not be used beyond the expiration date on the kit label.
2. Do not mix or substitute reagents with those from other lots or sources.
3. Any variation in pipetting and washing techniques, incubation time or temperature, or kit age can cause variation in the density of spots, intensity of specific staining, and background levels.

Materials Required But Not Provided With Kit

1. Washer (adjustable injection volume to ensure that each well receives 350μl without overflow).
2. Clean benches, biological safety cabinets, fume hoods.
3. High-precision single-channel pipette (range 0.5-10μl-20μl, 20-200μl, 200-1000μl).
4. High-precision multi-channel pipette (8 or 12, the range of 50-300μl of).
5. 37℃ CO2 incubator.
6. Low temperature centrifuge.
7. Refrigerators (4℃, -20℃, -86℃).
8. Analytical balance.
9. Scissors, tweezers, pliers, etc.
10. Plate mixer, low-frequency oscillator, etc.

Additional Materials Required

1. Centrifuge tubes (capacity of 1.5ml, 5ml, etc.).
2. Disposable pipette tips (range of 0.5-10μl-20μl, 20-200μl, 200-1000μl).
3. Pure water or distilled water.
4. Absorbent paper.

Technical Hints

1. To minimize edge effect, place the microplate (bottom down) onto a piece of aluminum foil (about 4 x 6 inches). Add cells, cover the microplate with the lid, and shape the foil around the edges of the microplate. The foil may be left on the microplate for the rest of the experimental procedure and removed after the BCIP/NBT has been washed off.
2. Do not remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development. It may damage the PVDF membrane filter. The underdrain cover may be removed only after completing the incubation with BCIP/NBT Substrate.
3. Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membrane.
4. Upon completing the experiment, do not dry the microplate at a temperature above 37 °C. It may cause the PVDF membrane filters to crack.
5. The 96-well microplate provided in this kit is not sterile. Due to the short incubation period and the presence of antibiotics in the culture media, microbial contamination has not been shown to be an issue with this ELISpot procedure.
6. This kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the provided microplate and reagents.

Precautions

BCIP/NBT is toxic if swallowed, in contact with skin, or if inhaled. It is a highly flammable liquid and vapor may cause serious irritation and damage to organs. Do not eat, drink, or smoke when using this product. Do not breathe fumes. Use only in a well-ventilated area. Keep away from heat, sparks, open flames, and hot surfaces. Keep the container tightly closed.Some components of this kit contain sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

Sample Preparation

The types of effector and responder cells used, method of cell separation, mode of stimulation, and length of incubation are to be determined by each investigator.

Note

1. The re-dissolved Positive Control cannot be stored again once prepared, so please do not attempt to re-freeze it once it has been reconstituted.
2. Due to shaking/inversion during transport, centrifuging of the tubes/bottles of the kit might be necessary to consolidate the material contained within. Tubes should be shaken manually or centrifuged for 1 min at 1000rpm to pool all material to the bottom.
3. Concentrated washing buffer might crystallize slightly. Use a water bath to help the dissolution during diluting process. The crystals must be totally dissolved when preparing the washing buffer.
4. Only use reagents/components that came directly with this kit. Do not mix batches/lots from other orders of this kit, or from different kits.
5. Ensure the reagents are well mixed. For the reagents in the microplate, adequate mixing is particularly important for accurate test results. It is recommended to employ a micro-oscillator (at the lowest frequency). If a micro-oscillator is not available, please slightly shake the microplate manually for 1 min, in a circular motion in order to make sure the wells are sufficiently mixed.
6. Please ensure the kit has been brought to room temperature prior to beginning the assay.
7. Place the unused microplate strips into the foil bag at 2-8℃ for storage (if you intend to use the strips within a relatively short timeframe).
8. The chromogen reagent is sensitive to light, therefore please avoid exposing to light.
9. Kits that have passed their expiration date should not be used.
10. We recommend regularly checking the thermostat and calibration in order to confirm the incubation temperature remains at a stable 37℃.

Test preparation

1. Please remove Kit from refrigerator 20 minutes in advance, and begin test once it has been brought to room temperature.
2. Dilute the concentrated washing buffer with double distilled water (1:25). Return unused quantity back to the box.
3. Biotinylated Antibody: Remove the appropriate volume of Biotinylated Antibody solution for the quantity of wells intending to be assayed, and dilute with Antibody Diluent in a proportion of 1:100. This should be prepared 30min in advance, and we would absolutely recommend not re-using for additional assaying.
4. Streptavidin-AP: Remove the appropriate amount of Enzyme Conjugate solution for the quantity of wells

intending to be assayed, and dilute with the Enzyme Diluent in a proportion of 1:100. This should be prepared 30min in advance, and we would absolutely recommend not re-using for additional assaying.

Assay Procedure

1. Fill all wells in the microplate with 200 µL of sterile culture media and incubate for approximately 20 minutes at room temperature.
2. When cells are ready to be plated, aspirate the culture media from the wells. Immediately add 100 µL of the appropriate cells or controls to each well (Add cells resuspended in Positive Control solution to the positive control wells.).
3. Incubate cells in a humidified 37 °C CO2 incubator. Optimal incubation time for each stimulus should be determined by the investigator. Do not disturb the cells during the incubation period.
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (250-300 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or
   decanting. Invert the plate and blot it against clean paper towels.
   Note: Adjust the height of the prongs of the manifold dispenser or autowasher to prevent damage to the membranes.
5. Add 100 µL of diluted Detection Antibody mixture into each well, and incubate overnight at 2-8 °C. Alternatively, incubation with detection antibodies can be done for 2 hours at room temperature on a rocking platform.
6. Repeat the wash procedure described in step 4.
7. Add 100 µL of diluted Streptavidin-AP into each well, and incubate for 2 hours at room temperature.
8. Repeat the wash procedure described in step 4.
9. Add 100 µL of BCIP/NBT Substrate into each well, and incubate for 1 hour at room temperature. Protect from light.
10. Decant the BCIP/NBT Substrate solution from the microplate and rinse the microplate with deionized water. Invert the microplate and tap to remove excess water. Remove the flexible plastic underdrain from the bottom of the microplate, wipe the bottom of the plate
    thoroughly with paper towels and dry completely either at room temperature (60-90 minutes) or 37 °C (15-30 minutes).

Calculation Results

The developed microplate can be analyzed by counting spots using either a dissection microscope or a specialized automated ELISpot reader. Specific spots are round and have a dark center with slightly fuzzy edges. Quantitation of results can be done, for example, by calculating the number of spot forming cells (SFC) per number of cells added to the well.

Reproducibility Data

Cells of the corresponding source (5×10^5 cells/mL) were stimulated overnight in a 37 °C, 5% CO_2 incubator with appropriate stimulating reagents (25 ng/mL PMA, 1 µg/mL LPS, 10 µg/mL PHA, 5 µg/mL ConA, etc.). The samples were assayed in eight replicate wells according to the kit procedure, and spots were counted using a stereomicroscope.

Well	Number of Spots Counted
1	465
2	356
3	337
4	415
5	287
6	414
7	323
8	318

Troubleshooting Guide

Observation	Problem	Corrective Action
Following the incubation with BCIP/NBT chromogen and rinsing the microplate with deionized water, the dark blue background color of the filter membrane attenuates visualization and quantitation of spots.	The membrane is wet.	Microplates cannot be analyzed accurately until the PVDF filter membranes are completely dry. Wait until the membrane becomes dry (typically 15-30 minutes at 37 °C or 60-90 minutes at room temperature).
The number of spots in the wells that contained the cells is high, but their contrast as well as intensity of staining in the Positive Control wells is low.	Underdevelopment; perhaps the result of using Streptavidin-AP and/ or BCIP/NBT solutions that have not been brought to room temperature.	Warm the appropriate reagents to room temperature before adding them to the wells.
The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned blue-black.	Cell stimulation problem. Too few cells were added to the wells.	Ensure that reagents used to stimulate the cytokine release from the cells retained their biological activity. One way to check is to perform immunocytochemistry on fixed cells after stimulation. Increase the number of cells added per well.
Following incubation with BCIP/NBT and drying the microplate, the density of the spots makes them difficult to quantify.	Too many cells were added to the wells.	Make dilutions of cells (1 x 106, 5 x 105, 1 x 105, 5 x 104, 1 x 104 cells per well) to determine the optimal number of cells that will result in formation of distinct spots.