Kingfisher Biotech

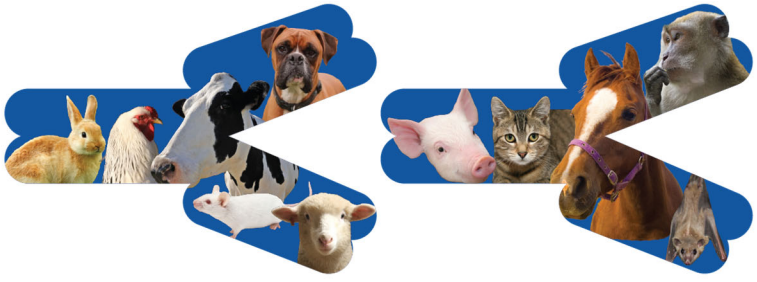
ELISpot

Technical Guide

Reagents for Animal Model and Animal Health Research

1000 Westgate Drive, St. Paul, MN 55114
Customer.Service@KingfisherBiotech.com
KingfisherBiotech.com
651.646.0089

ELISpot Technical Guide



Reagents for Animal Model and Animal Health Research

1000 Westgate Drive, St. Paul, MN 55114
Customer.Service@KingfisherBiotech.com
KingfisherBiotech.com
651.646.0089

ELISpot Technical Guide

Introduction

ELISpot assays are enzyme-linked immunosorbent "spot" assays for the determination of the number of single cells producing a specific protein, such as a cytokine. While an ELISA measures the quantity of specific protein in a sample type, an ELISpot enumerates the quantity of individual cells secreting a specific protein. ELISpot assays utilize antibodies which capture and detect the same molecule in the vicinity of the cell releasing it. Spots are generated using a substrate reacting with an enzyme linked to the detection antibody to produce a colorimetric precipitate. The number of spots is a direct measurement of the protein producing cells.

ELISpot Procedure Overview

1. Prepare a sterile antibody coated ELISpot plate.
2. Add cell cultures and stimulant to plate.
3. Incubate cells at 37˚C with 5% CO_2. Do not disturb the plate!
4. Wash plate. Note: Sterile conditions are no longer required.
5. Add detection antibody and incubate.
6. Wash plate.
7. Add streptavidin-AP and incubate.
8. Wash plate.
9. Add BCIP/NBT substrate and incubate.
10. Stop reaction and dry plate.
11. Analyze plate to determine the number of spot forming cells (SFC).

Reagents for Animal Model and Animal Health Research
1000 Westgate Drive, St. Paul, MN 55114
Customer.Service@KingfisherBiotech.com
KingfisherBiotech.com
651.646.0089

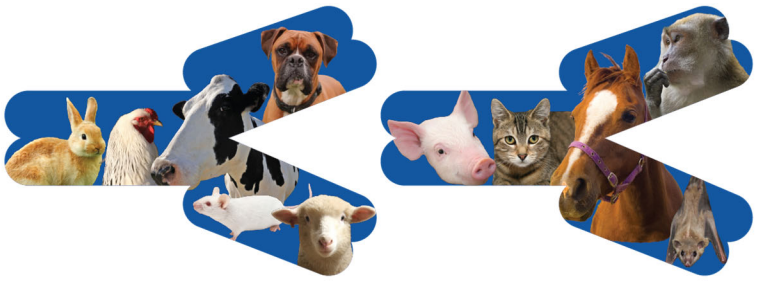
Reagents Required

- Capture Antibody (unconjugated antibody)
- Standard Control (recombinant protein)
- Detection Antibody (biotinylated antibody)
- Sterile DPBS
- Complete Medium
- Wash Buffer
- Reagent Diluent
- Streptavidin-Alkaline Phosphatase
    - Streptavidin-AP; Catalog # AR2308-001
- 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium
    - BCIP/NBT; Catalog # AR2309-025

Additional Materials Required

- 96-well, sterile, filter membrane ELISpot plate
    - ELISpot Plate; Catalog # AR2307-001
    - Millipore MultiScreen$_{HTS}$-IP Filter Plates (MSIP S4W 10)
- Ultrapure water
- Precision pipettors, with disposable plastic tips
- Polypropylene or polyethylene tubes to prepare standard, samples, and working solutions.

  Note: Do not use polystyrene, polycarbonate or glass tubes. They bind protein and antibodies.
- Containers to prepare buffers
- A wash bottle or an automated 96-well plate washer
- Disposable reagent reservoirs



Additional Equipment Required

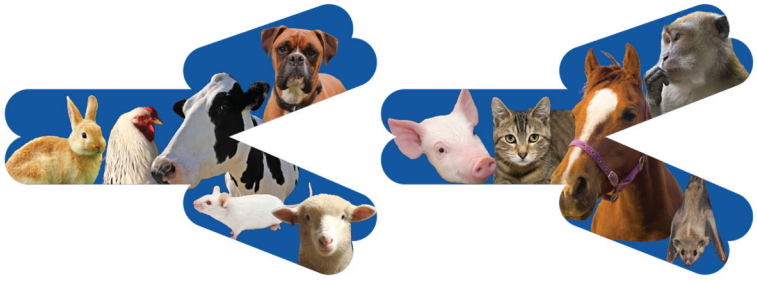
- Sterile environment to prepare cell cultures, such as a laminar flow hood
- 37˚C incubator with 5% CO_2

Time Requirements

- 1 overnight incubation for coated plate preparation
- 1-2 overnight incubation for cell stimulation
- 2.25+ hours for assay development

General ELISpot Suggestions

- Store all reagents at the recommended temperature.
- Do not use reagents if they become visibly contaminated during storage.
- Reagents should be at room temperature before use.
- Prepare reagents immediately prior to use. Buffers may be prepared in advance.
- Vigorous plate washing is essential; however, care should be taken to avoid puncturing the filter membrane ELISpot plate during wash steps. If using an automatic plate washer, confirm and adjust the height of the manifold dispenser prior to use.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Take care not to contaminate the BCIP/NBT Solution. Do NOT return leftover BCIP/NBT Substrate to bottle. If the solution is blue before use, DO NOT USE IT!
- Individual components may contain preservatives. Wear gloves while performing the assay. Please follow proper disposal procedures.
- Do not use sodium azide as a preservative for buffers. Sodium azide interferes with substrate reactions.



- Avoid getting liquid on the bottom of the plate membrane. It may cause sample leakage. Do not remove lower gasket on plate until assay is complete.

Reagent and Sampling Handling and Preparation

Sterile DPBS Preparation

- Recommended buffer: 0.008M sodium phosphate, 0.002M potassium phosphate, 0.14M sodium chloride, 0.01M potassium chloride, pH 7.4

To prepare 1L of DPBS:

1. Fill a suitable container with ~750 mL ultrapure water.
2. Dissolve solid reagents in ultrapure water. Mix well.
    - 1.14 g Sodium Phosphate, Dibasic (FW: 142.0)
    - 0.27 g Potassium Phosphate, Monobasic (FW: 136.0)
    - 8.18 g Sodium Chloride (FW: 58.44)
    - 0.75 g Potassium Chloride (FW: 74.55)
3. Fill to 1L volume.
4. Verify pH.
5. Filter the solution into a sterile container, in sterile environment, using a 0.2 µm membrane.

Sterile Blocking Buffer Preparation: Complete Medium

- Recommend medium containing 10% heat-inactivated Fetal Bovine Serum (FBS) or Equine Serum (ES) in desired cell culture medium, such as RPMI.
- If required, serum-free medium for cell culture can also be used.

Reagent Diluent Preparation

- Recommended buffer: 4% Bovine Serum Albumin (BSA) in DPBS, 0.2 µm filtered
- Alternative buffers may be used as desired. For example:
    - PBS or TBS solutions may be used to replace DPBS.
    - BSA may be replaced with alternative blocking reagents.

        Note: Do not use fetal bovine serum as a blocking reagent. Any free biotin present interferes with the assay
    - BSA concentration may be altered as desired.
- Do not use sodium azide as a preservative. Sodium azide interferes with assay.

To prepare 1L of Reagent Diluent:

1. Fill a suitable container with ~750 mL ultrapure water.
2. Dissolve solid reagents in ultrapure water. Mix well.
    - 1.14 g Sodium Phosphate, Dibasic (FW: 142.0)
    - 0.27 g Potassium Phosphate, Monobasic (FW: 136.0)
    - 8.18 g Sodium Chloride (FW: 58.44)
    - 0.75 g Potassium Chloride (FW: 74.55)
    - 40 g BSA (4% solution)
3. Fill to 1L volume.
4. Verify pH.
5. If desired, filter the solution into a sterile container using a 0.2 µm membrane.

10X Wash Buffer Preparation

- Recommended buffer: 10X DPBS plus 0.5% Tween®-20
- Alternative buffers may be used as desired. For example, PBS or TBS solutions may be used to replace DPBS.
- Do not use sodium azide as a preservative. Sodium azide interferes with assay.

To Prepare 1L of 10X Wash Buffer:

1. Fill a suitable container with ~750 mL ultrapure water.
2. Dissolve reagents in ultrapure water. Mix well.
    - 11.4 g Sodium Phosphate, Dibasic (FW: 142.0)
    - 2.7 g Potassium Phosphate, Monobasic (FW: 136.0)
    - 81.8 g Sodium Chloride (FW: 58.44)
    - 7.5 g Potassium Chloride (FW: 74.55)
    - 5 mL Tween 20® (0.5% solution)
3. Fill to 1L volume.
4. Verify pH.
5. If desired, filter the solution into a sterile container using a 0.2 µm membrane.

1X Wash Buffer Preparation

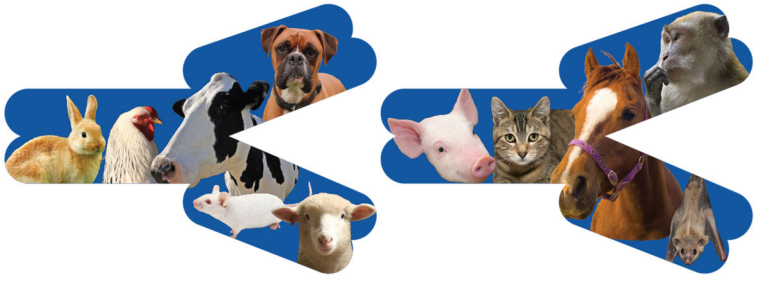
- Recommended buffer: 1X DPBS plus 0.05% Tween®-20
- Alternative buffers may be used as desired. For example, PBS or TBS solutions may be used to replace DPBS.
- Do not use sodium azide as a preservative. Sodium azide interferes with assay.

To prepare 500 mL of 1X Wash Buffer:

1. Dilute 50 mL of 10X Wash Buffer into 450 mL of ultrapure water.
2. Mix well.

    Note: A typical wash bottle will hold 500 mL of solution.

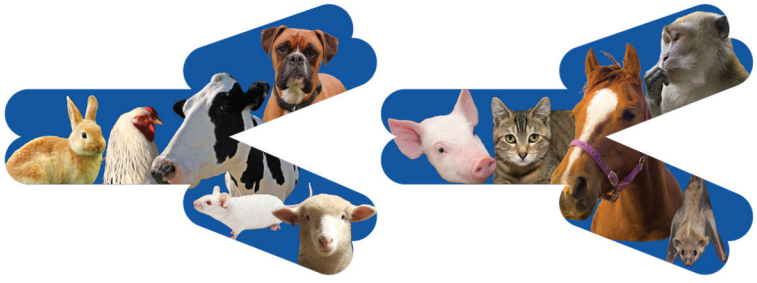
    .



1000 Westgate Drive, St. Paul, MN 55114
Customer.Service@KingfisherBiotech.com
KingfisherBiotech.com
651.646.0089

Sample Handling and Preparation

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.
- Typically, fresh peripheral blood mononuclear cells (PBMCs) obtained by ficoll-hypaque are utilized in the ELISpot assay, however the assay can be optimized for various cell types.
- Cryopreserved cells can be utilized in the assay. Rest cells for one hour at 37°C, 5% CO_2 prior to counting and use.
    - If desired, add >50 U/mL of a recombinant nuclease (for example, Benzonase) to Complete Medium wash during initial cell thawing to help eliminate clumping of cell cultures in assay. Resuspend cells with fresh Complete Medium.
      Smith, J. *et.al.*, Clinical and Diagnostic Laboratory Immunology, Sept. 2001, p 871-879
- Prior to addition of stimulant, cells should be washed thoroughly to remove any interfering substances such as natural cytokines that have been produced by the cells.
- 100 µL of total sample volume per well is recommended.
    - During initial testing, use a wide range of cell concentrations (for example, 2 x 10^3 cells/mL –2 x 10^6 cells/mL) to optimize cell number that results in distinct spot formation.
    - Prepare cells and stimuli at a 2X concentration (double) in Complete Medium. Add 50 µL stimuli and 50 µl cells to appropriate wells to create the final desired stimuli and cell concentration (1X).
    - Alternatively, mix stimuli and cells at the desired concentration separately before adding 100 µL total sample volume per well.
- Samples should be assayed in triplicate each time the assay is performed.
- Once stimulated cells are added to the plate, plates should not be agitated during incubation.
- To avoid edge effects, do not stack plates during incubation.
- Prepare one or more dilutions of cell culture and stimuli if it is suspected that the quantity of spots obtained will exceed optimal assay quantitation.
- If the integrity of the sample is of concern, make a note on the Plate Template and interpret results with caution.



Capture Antibody Working Solution Preparation

- Alternative capture antibody concentrations can be prepared as desired. Please see **Optimal Antibody Concentrations** for more details.

To prepare a 4 µg/mL capture antibody working solution:

1. Dilute 44 µL of 1 mg/mL capture antibody stock in 11 mL DPBS.
2. Mix well.
3. If desired, sterile filter the solution into a sterile container using a 0.2 µm membrane.

Detection Antibody Working Solution Preparation

- Alternative detection antibody concentrations can be prepared as desired. Please see **Optimal Antibody Concentrations** for more details.

To prepare a 4 µg/mL detection antibody working solution:

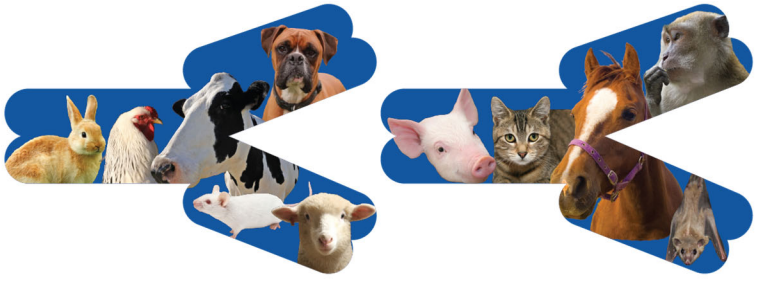
1. Dilute 44 µL of 1 mg/mL detection antibody stock in 11 mL Reagent Diluent.
2. Mix well.

Streptavidin-Alkaline Phosphatase (AP) Working Solution Preparation

- Alternative Streptavidin-AP concentrations can be prepared as desired or based on substrate utilized in the assay.

To prepare streptavidin-AP working solution:

1. Dilute 0.5 mL Streptavidin-AP stock (Streptavidin-AP; Catalog # AR2308-001) in 11.5 mL Reagent Diluent.
2. Mix well.



Control Preparation

Standard Control Preparation

- Use a 50 ng/mL solution of recombinant protein as the standard control:

1. Reconstitute 5 µg recombinant protein vial with 100 µL of Complete Medium to achieve a concentration of 50 µg/mL. Aliquot protein in 10 µL aliquots and store at -20º to minimize freeze-thaw cycles.
2. To prepare a 50 ng/mL (0.05 µg/mL) standard control, dilute 2 µL of 50 µg/mL stock in 2 mL Complete Medium. Mix well.

Blank Control Preparation

- Use Complete Medium only as the blank control.

Positive Control Preparation

- Use cells incubated with a polyclonal activator as the positive control to verify assay functionality.
- Cell culture stimuli can be altered based on functional response desired from target protein.
- Examples of positive control stimuli include:
    - Lectin from *Phaseolus vulgaris* phytohemagglutinin (PHA-P)
    - Phorbol 12-myristate 13-acetate (PMA)/Inomycin calcium salt
    - Concanavalin A (ConA)

Negative Control Preparation

- Use cells in Complete Medium alone as the negative control.

Detailed Assay Procedure

Step 1: Prepare Antibody Coated Plate (Sterile Conditions)

1. Prepare Capture Antibody in sterile DPBS at desired working concentration.
2. Add 100 µL of Capture Antibody Working Solution to appropriate wells. Cover plate and incubate at room temperature (20-25°C) for 12-24 hours.

   Note: Incubation time and temperature may be altered as desired for optimal antibody binding.
3. Empty Capture Antibody Working Solution from plate.
4. Add 250 µL of Blocking Buffer (Complete Medium) to appropriate wells.
5. Cover plate and incubate at room temperature for 1-3 hours.

   Note: Incubation time and temperature may be altered as desired for optimal blocking.

   Tip: Prepare controls and samples for ELISpot during Blocking Buffer incubation.
6. Empty Blocking Buffer from plate.
7. Antibody coated plate is now ready for use.

Step 2: Add Controls and Samples and Incubate (Sterile Conditions)

- Use a Plate Template to record the locations of the standards and unknown samples within the wells.

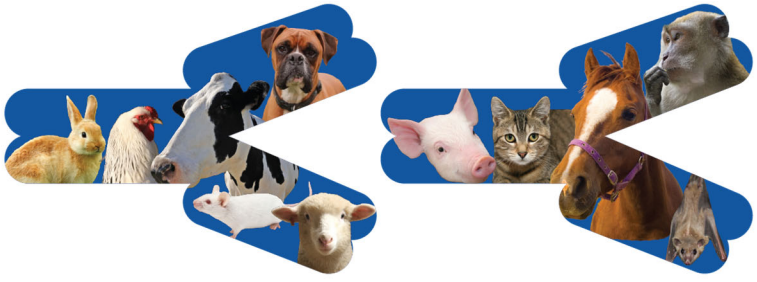
1. Add 100 µL of appropriately prepared controls or samples to each well. Run each control or sample in triplicate.
2. Cover plate and incubate as desired (typically 12-48 hours) at 37°C, 5% CO_2.

   Note: Do NOT agitate plate during incubation!

Step 3: Wash Plate

1. Remove plate cover and empty contents.

   Note: Follow universal precautions when handling and disposing of infectious agents.



1000 Westgate Drive, St. Paul, MN 55114
Customer.Service@KingfisherBiotech.com
KingfisherBiotech.com
651.646.0089

2. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure five additional times for a total of SIX washes. Blot plate onto paper towels or other absorbent material.

   Note: Vigorous plate washing is essential; however, care should be taken to avoid puncturing the filter membrane ELISpot plate during wash steps. If using an automatic plate washer, confirm and adjust the height of the manifold dispenser prior to use. Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Step 4: Add Detection Antibody and Incubate

1. Add 100 µL of Detection Antibody Working Solution to each well. Mix well by gently tapping the plate several times.
2. Cover plate and incubate plate for one (1) hour at room temperature.

   Note: Incubation time and temperature can be altered as desired.

   For example:

   - Detection Antibody Working Solution can be incubated overnight at 2-8ºC.
   - Incubation time can be increased to 2 hours.
   - Incubation temperature can be increased to 37ºC.
   - Plate can be incubated with shaking using an orbital shaker.

Step 5: Wash Plate

1. Remove plate cover and empty contents.

   Note: Follow universal precautions when handling and disposing of infectious agents.

2. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure five additional times for a total of SIX washes. Blot plate onto paper towels or other absorbent material.

   Note: Vigorous plate washing is essential; however, care should be taken to avoid puncturing the filter membrane ELISpot plate during wash steps. If using an automatic plate washer, confirm and adjust the height of the manifold dispenser prior to use. Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Step 6: Add Streptavidin-AP and Incubate

1. Add 100 µL of Streptavidin-AP Working Solution to each well.
2. Cover plate and incubate plate for one (1) hour at room temperature.

Step 7: Wash Plate

1. Remove plate cover and empty contents.

   Note: Follow universal precautions when handling and disposing of infectious agents.

2. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure five additional times for a total of SIX washes. Blot plate onto paper towels or other absorbent material.

   Note: Vigorous plate washing is essential; however, care should be taken to avoid puncturing the filter membrane ELISpot plate during wash steps. If using an automatic plate washer, confirm and adjust the height of the manifold dispenser prior to use. Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Step 8: Add BCIP/NBT Substrate and Incubate

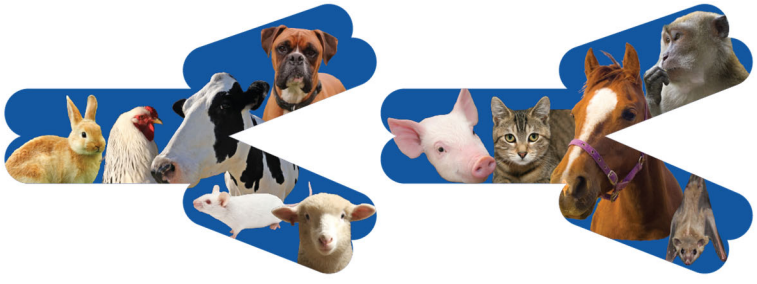
- Take care not to contaminate the BCIP/NBT Solution. Do NOT return leftover BCIP/NBT Substrate to bottle. If the solution is blue before use, DO NOT USE IT!

1. Add 100 µL of BCIP/NBT Substrate Solution into each well.
2. Allow the enzymatic color reaction to develop at room temperature, for up to 15 minutes. The substrate reaction yields a blue precipitate.

   Note: Incubation time can be adjusted based on spot development.

Step 9: Stop Reaction

1. After desired precipitate formation, stop the reaction by flooding the plate with ultrapure water multiple times to ensure removal of all substrate.
2. Remove lower gasket from plate, and flood underside with ultrapure water.



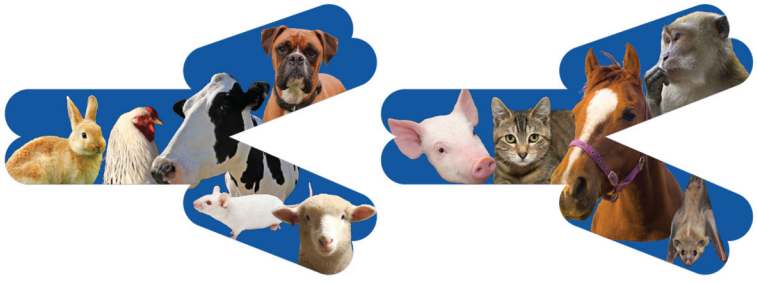
3. Carefully blot plate onto paper towels or other absorbent material.
4. Allow the plate to dry 60 minutes to overnight until completely dry.

Step 10: Measure Spot Forming Cells (SFC)

1. Analyzed spot formation in an ELISpot reader.

   Note: Plate can be manually evaluated with a dissection microscope, if desired.

   Note: Store plate protected from light in sealed container for later re-analysis.



1000 Westgate Drive, St. Paul, MN 55114
Customer.Service@KingfisherBiotech.com
KingfisherBiotech.com
651.646.0089

Performance Improvements

The following information is a general guide only. A variety of options may be utilized to obtain optimal results. Assay verification is required.

If the assay is not sensitive enough, it will not produce optimal spot formation. Suggested options for improving the assay include:

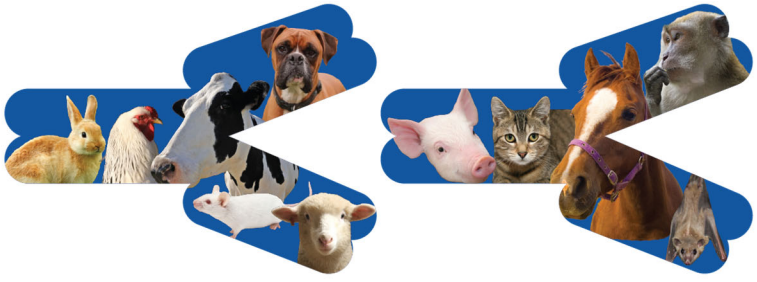
- Increasing Capture Antibody concentration
- Increasing Detection Antibody concentration
- Increasing assay incubation times
- Increasing assay incubation temperatures
- Shaking the plate during Detection Antibody and/or Streptavidin-AP incubation. Do NOT shake the plate during sample incubation!

Maximizing Assay Sensitivity: Titer Assay

To maximize assay sensitivity, the optimal Capture Antibody and Detection Antibody concentrations are required. To verify the optimal concentrations, a titer assay should be performed. For ideal results, the staining intensity of the Positive Standard Control should produce intense, consistent precipitate color while the Blank Control should produce minimal background or color.

Representative ELISpot Titer Assay Procedure

1. Prepare Capture Antibody in DPBS at desired working concentrations.
2. Add 100 µL of Capture Antibody Working Solution to appropriate wells, according to plate schematic.
3. Cover plate and incubate at room temperature (20-25°C) for 12-24 hours.
4. Empty Capture Antibody Working Solution from plate.
5. Add 250 µL of Blocking Buffer (Complete Medium) to wells.
6. Cover plate and incubate at room temperature for 1-3 hours.
7. Empty Blocking Buffer from plate.
8. Prepare 50 ng/mL Positive Standard Control and 0 ng/mL Blank Control in Complete Medium.



9. Add 100 µL of Standard or Blank to appropriate wells, according to plate schematic.

   Note: Run each Standard or Blank in triplicate.

10. Cover plate and incubate at room temperature for 1 hour.

11. Wash plate SIX times with Wash Buffer.

    Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure five additional times for a total of SIX washes. Blot plate onto paper towels or other absorbent material.

    Note: Vigorous plate washing is essential; however, care should be taken to avoid puncturing the filter membrane ELISpot plate during wash steps. If using an automatic plate washer, confirm and adjust the height of the manifold dispenser prior to use. Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

12. Prepare Detection Antibody in Reagent Diluent at desired working concentrations.

13. Add 100 µL of Detection Antibody Working Solution to each well, according to schematic.

14. Cover plate and incubate at room temperature for 1 hour.

15. Wash plate SIX times with Wash Buffer as described in step 11.

16. Prepare Streptavidin-AP in Reagent Diluent at desired working concentration.

17. Add 100 µL of Streptavidin-AP Working Solution to each well.

18. Cover plate and incubate at room temperature for 1 hour.

19. Wash plate SIX times with Wash Buffer as described in step 11.

20. Add 100 µL of BCIP/NBT Substrate Solution to each well.

21. Develop the plate at room temperature for up to 15 minutes or as desired.

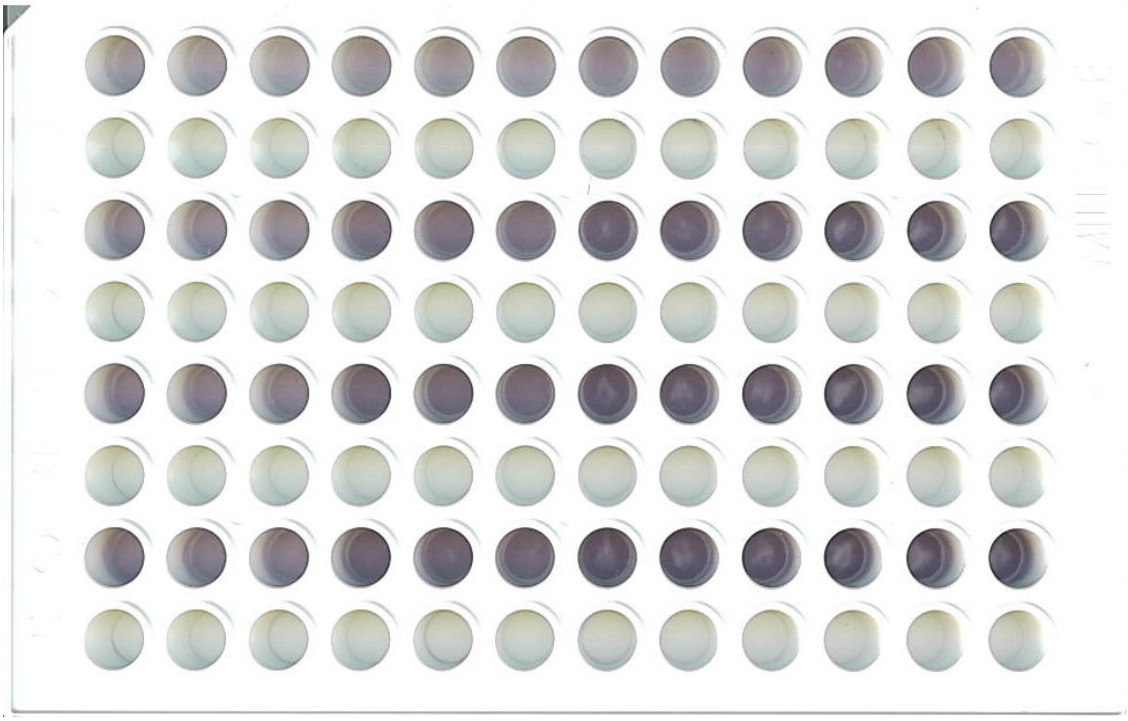
22. Stop reaction by flooding the plate with ultrapure water. Remove under tray and flood bottom of tray with ultrapure water.

23. Dry plate and evaluate results.

Representative ELISpot Titer Plate Schematic

	1	2	3	4	5	6	7	8	9	10	11	12	↓Capture Antibody Concentration
A	50 ng/mL Standard			50 ng/mL Standard			50 ng/mL Standard			50 ng/mL Standard			1 µg/mL
B	0 ng/mL Standard			0 ng/mL Standard			0 ng/mL Standard			0 ng/mL Standard			
C	50 ng/mL Standard			50 ng/mL Standard			50 ng/mL Standard			50 ng/mL Standard			2.5 µg/mL
D	0 ng/mL Standard			0 ng/mL Standard			0 ng/mL Standard			0 ng/mL Standard			
E	50 ng/mL Standard			50 ng/mL Standard			50 ng/mL Standard			50 ng/mL Standard			5 µg/m:
F	0 ng/mL Standard			0 ng/mL Standard			0 ng/mL Standard			0 ng/mL Standard			
G	50 ng/mL Standard			50 ng/mL Standard			50 ng/mL Standard			50 ng/mL Standard			7.5 µg/mL
H	0 ng/mL Standard			0 ng/mL Standard			0 ng/mL Standard			0 ng/mL Standard			
	1 µg/mL			2.5 µg/mL			5 µg/mL			7.5 µg/mL			←Detect Antibody Concentration

Representative ELISpot Titer Data

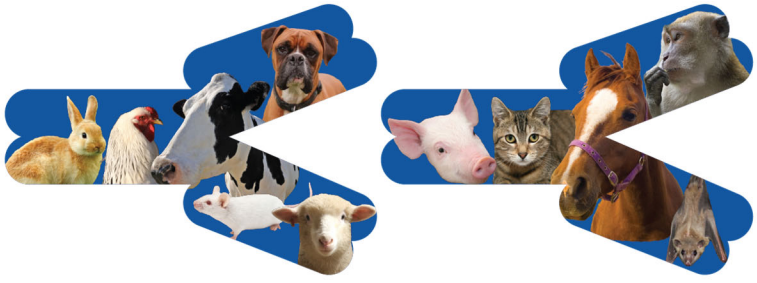


Reagents for Animal Model and Animal Health Research
1000 Westgate Drive, St. Paul, MN 55114
Customer.Service@KingfisherBiotech.com
KingfisherBiotech.com
651.646.0089

Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Notes



Reagents for Animal Model and Animal Health Research
1000 Westgate Drive, St. Paul, MN 55114
Customer.Service@KingfisherBiotech.com
KingfisherBiotech.com
651.646.0089