BOSTER
Antibody and ELISA Experts

How to BECOME AN ELISA EXPERT in 4 days

ELISA 101

Your all-in-one guide for your ELISA experiments from start to finish

- Master the ELISA from principle to experiment
- Next steps if you experience non-specific binding
- Best practices in sample preparation
- Avoid pitfalls of poor standard curve
- All-inclusive troubleshooting guide

Contents

Introduction

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

ELISAs are typically performed in 96-well (or 384-well) polystyrene plates, which will passively bind antibodies and proteins. It is this binding and immobilization of reagents that makes ELISAs so easy to design and perform. Having the reactants of the ELISA immobilized to the microplate surface makes it easy to separate bound from non-bound material during the assay. This ability to wash away nonspecifically bound materials makes the ELISA a powerful tool for measuring specific analytes within a crude preparation.

General ELISA Procedure

Unless you are using a kit with a plate that is pre-coated with antibody, an ELISA begins with a **coating** step, in which the first layer, consisting of a target antigen or antibody, is adsorbed onto a 96-well polystyrene plate. This is followed by a **blocking** step in which all unbound sites are coated with a blocking agent. Following a series of washes, the plate is **incubated with enzyme-conjugated antibody**. Another series of washes removes all unbound antibody. A **substrate** is then added, producing a calorimetric signal. Finally, the plate is **read**.

Because the assay uses surface binding for separation, several washes are repeated in each ELISA step to remove unbound material. During this process, it is essential that excess liquid is removed in order to prevent the dilution of the solutions added in the next assay step. To ensure uniformity, specialized plate washers are often used.

ELISAs can be quite complex and include multiple intervening steps, especially when measuring protein concentration in heterogeneous samples such as blood. The most complex and varying step in the overall process is detection, where multiple layers of antibodies can be used to amplify signal.

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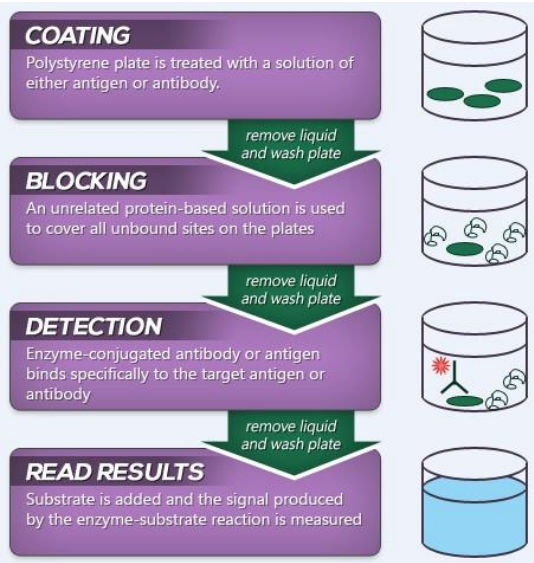
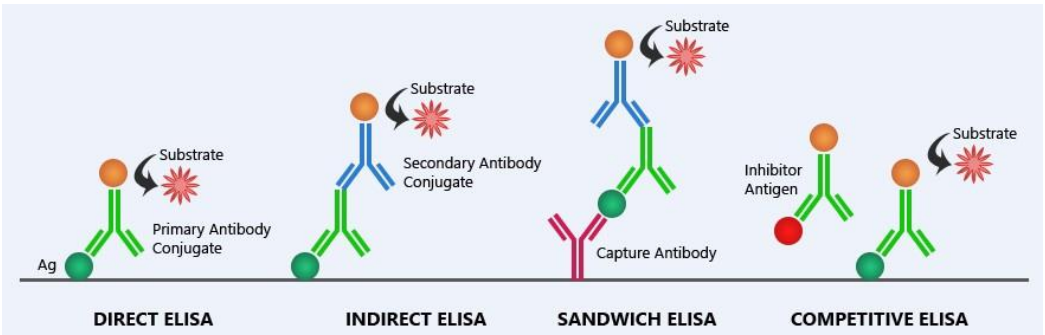


ELISA Types

ELISAs can be performed with a number of modifications to the basic procedure: **direct, indirect, sandwich** or **competitive**. The key step, immobilization of the antigen of interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is then detected either directly (enzyme-labeled primary antibody) or indirectly (enzyme-labeled secondary antibody). The detection antibodies are usually labeled with alkaline phosphatase (AP) or horseradish peroxidase (HRP). A large selection of substrates is available for performing the ELISA with an HRP or AP conjugate. The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal-detection (spectrophotometer, fluorometer or luminometer).

Among the standard assay formats discussed and illustrated below, where differences in both capture and detection were the concern, it is important to differentiate between the particular strategies that exist specifically for the *detection* step. However an antigen is captured to the plate (by direct adsorption to the surface or through a pre-coated "capture" antibody, as in a sandwich ELISA), it is the detection step (as either direct or indirect detection) that largely determines the sensitivity of an ELISA.



1. Direct ELISA

For direct detection, an antigen coated to a multi-well plate is detected by an antibody that has been directly conjugated to an enzyme. This detection method is a good option if there is no commercially available ELISA kits for your target protein.

Advantages

- Quick because only one antibody and fewer steps are used.
- Cross-reactivity of secondary antibody is eliminated.

Disadvantages

- Immunoreactivity of the primary antibody might be adversely affected by labeling with enzymes or tags.
- Labeling primary antibodies for each specific ELISA system is time-consuming and expensive.
- No flexibility in choice of primary antibody label from one experiment to another.
- Minimal signal amplification.

2. Indirect ELISA

For indirect detection, the antigen coated to a multi-well plate is detected in two stages or layers. First an unlabeled primary antibody, which is specific for the antigen, is applied. Next, an enzyme-labeled secondary antibody is bound to the first antibody. The secondary antibody is usually an anti-species antibody and is often polyclonal. The indirect assay, the most popular format for ELISA, has the advantages and disadvantages:

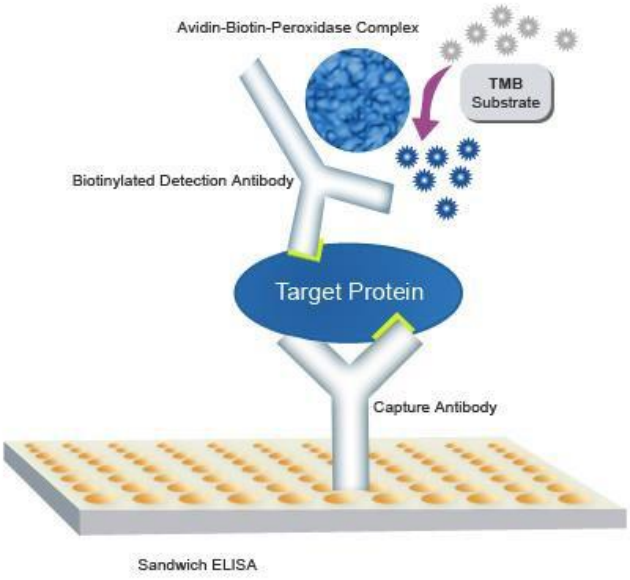
Advantages

- A wide variety of labeled secondary antibodies are available commercially.
- Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection.
- Maximum immunoreactivity of the primary antibody is retained because it is not labeled.
- Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification.

Disadvantages

- Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal.
- An extra incubation step is required in the procedure.

3. Sandwich ELISA

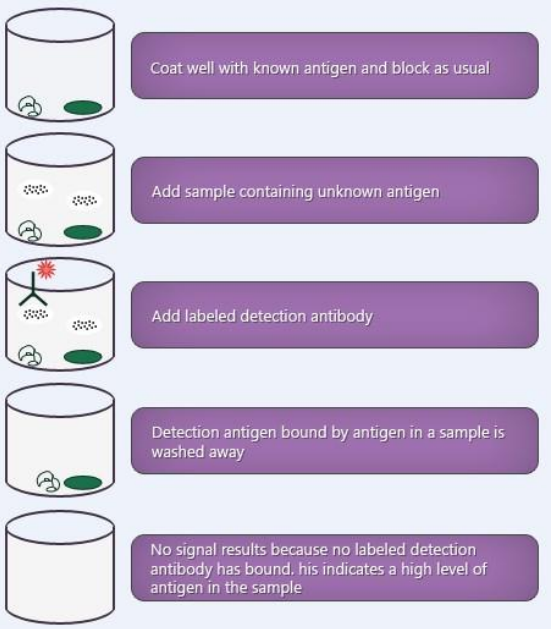


Sandwich ELISAs typically require the use of matched antibody pairs, where each antibody is specific for a different, non-overlapping part (epitope) of the antigen molecule. A first antibody (known as capture antibody) is coated to the wells. The sample solution is then added to the well. A second antibody (known as detection antibody) follows this step in order to measure the concentration of the sample. The diagram above shows the schematics for Boster's ELISA assay which is based on the sandwich format. This type of ELISA has the following advantages:

- High specificity: the antigen/analyte is specifically captured and detected
- Suitable for complex (or crude/impure) samples: the antigen does not require purification prior to measurement
- Flexibility and sensitivity: both direct or indirect detection methods can be used

4. Competitive ELISA

The key event of competitive ELISA (also known as inhibition ELISA) is the process of competitive reaction between the sample antigen and antigen bound to the wells of a microtiter plate with the primary antibody. First, the primary antibody is incubated with the sample antigen and the resulting antibody–antigen complexes are added to wells that have been coated with the same antigen. After an incubation period, any unbound antibody is washed off. The more antigen in the sample, the more primary antibody will be bound to the sample antigen. Therefore, there will be a smaller amount of primary antibody available to bind to the antigen coated on the well, resulting in a signal reduction. The main advantage of this type of ELISA arises from its high sensitivity to compositional differences in complex antigen mixtures, even when the specific detecting antibody is present in relatively small amounts.



Summary of Key Steps in Different ELISA Types

	Indirect	Direct	Sandwich	Competitive
Capture Ab Coating	X	X	√	X
Antigen Coating	√	√	X	√
Blocking	√	√	√	√
Sample (Antigen) Incubation	X	X	√	√
Primary Ab Incubation	√	√	√	√
Secondary Ab Incubation	√	X	√	√
Substrate Prep	√	√	√	√
Signal Detection	√	√	√	√
Data Analysis	√	√	√	√

ELISA Data Interpretation

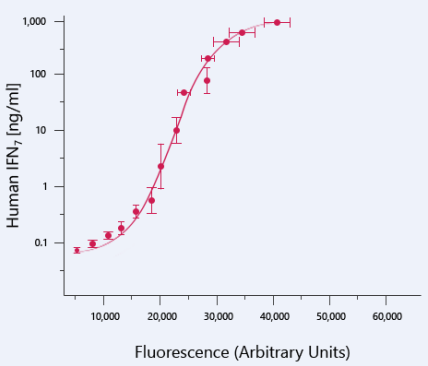
The ELISA assay yields three different types of data output:

1) **Quantitative**: ELISA data can be interpreted in comparison to a standard curve (a serial dilution of a known, purified antigen) in order to precisely calculate the concentrations of antigen in various samples.

2) **Qualitative**: ELISAs can also be used to achieve a yes or no answer indicating whether a particular antigen is present in a sample, as compared to a blank well containing no antigen or an unrelated control antigen.

3) **Semi-Quantitative**: ELISAs can be used to compare the relative levels of antigen in assay samples, since the intensity of signal will vary directly with antigen concentration.

ELISA data is typically graphed with optical density vs log concentration to produce a sigmoidal curve as shown below. Known concentrations of antigen are used to produce a standard curve and then this data is used to measure the concentration of unknown samples by comparison to the linear portion of the standard curve. In fact, it is the relatively long linear region of the curve that makes the ELISA results accurate and reproducible. The unknown concentration can be determined directly on the graph or with curve fitting software which is typically found on ELISA plate readers.



Sample Preparation

The procedure below provides a general guidance for the preparation of commonly tested samples for use in ELISA assays. *At Boster, we are working on our detailed sample preparation protocols that cover more than 20 sample types and expecting to update this handbook in the near future.* Please check with the literature for experiments similar to yours for your new assay development. Generally:

- Protein extract concentration is at least 1-2 mg/mL.
- Cell and tissue extracts are diluted by 50% with binding buffer.
- Samples are centrifuged at 10,000 rpm for 5 min at 4°C to remove any precipitate before use.

1. Cell Culture Supernatants

Centrifuge cell culture media at 1,500 rpm for 10 min at 4°C. Assay immediately. Aliquot supernatant immediately and hold at -80°C, avoiding freeze/thaw cycles.

2. Cell Extracts

Place tissue culture plates on ice. Remove the media and gently wash cells once with ice-cold PBS. Remove the PBS and add 0.5 ml extraction buffer per 100 mm plate. Tilt the plate and scrape the cells into a pre-chilled tube. Vortex briefly and incubate on ice for 15-30 min. Centrifuge at 13,000 rpm for 10 min at 4°C

(this creates a pellet from the insoluble content). Aliquot the supernatant into clean, chilled tubes (on ice) and store samples at -80°C, avoiding freeze/thaw cycles.

3. Conditioned Media

Plate the cells in complete growth media (with serum) until the desired level of confluence is achieved. Remove the growth media and gently wash cells using 2- 3 mL of warm PBS. Repeat the wash step. Remove the PBS and gently add serum-free growth media. Incubate for 1-2 days. Remove the media into a centrifuge tube. Centrifuge at 1,500 rpm for 10 min at 4°C. Aliquot the supernatant and keep samples at -80°C, avoiding freeze/thaw cycles.

4. Tissue Extract

Mince tissue on ice in ice-cold buffer, preferably in the presence of protease inhibitors. Place the tissue in micro-centrifuge tubes and dip into liquid nitrogen to snap freeze. Keep samples at -80°C for later use or keep on ice for immediate homogenization.

For every 5 mg of tissue, add 300 µL of extraction buffer to the tube and homogenize:

- 100 mM Tris, pH 7.4
- 150 mM NaCl
- 1 mM EGTA
- 1 mM EDTA
- 1% Triton X-100 0.5%
- 0.5% sodium deoxycholate

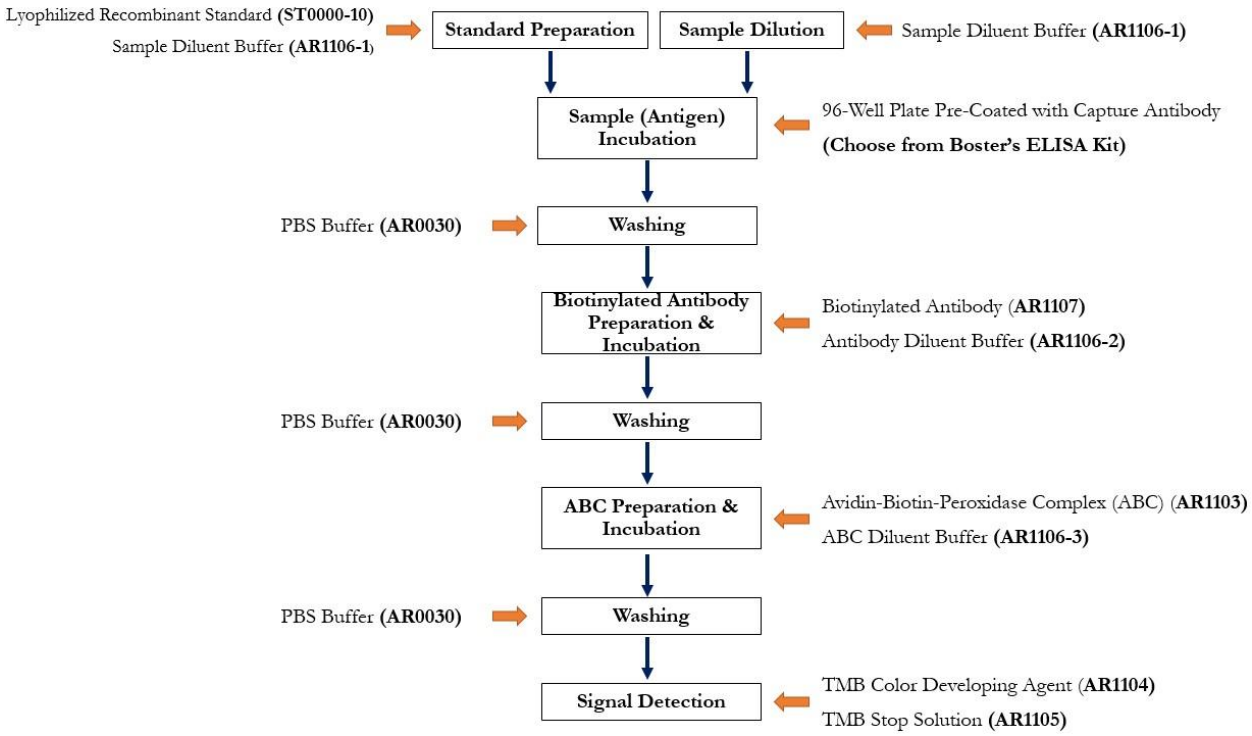
(This portion of the buffer can be prepared ahead of time and stored at 4°C. Immediately before use, the buffer must be supplemented with phosphatase inhibitor cocktail [as directed by manufacturer], protease inhibitor cocktail [as directed by manufacturer] and PMSF to 1 mM to generate a complete extraction buffer solution.)

Rinse the blade of the homogenizer twice with 300 µL extraction buffer. Place the sample on a shaker at 4°C for 2 hours.

Centrifuge the sample for 20 min at 13,000 rpm at 4°C. Aliquot the supernatant into pre-chilled tubes sitting in ice. Keep the samples at -80°C, avoiding freeze/thaw cycles.

Note: Lysis buffer volume must be determined according to the amount of tissue present. Typical concentration of final protein extract is at least 1 mg/mL.

Recommended Protocols



Reagent Preparation

1. Standard Solutions (Best used within 2 hours)
   - 10,000 pg/mL: Add 1 mL of sample diluent buffer into one tube of standard (10 ng per tube) and mix thoroughly. Note: Store this solution at 4°C for up to 12 hours (or -20°C for 48 hours) and avoid freeze-thaw cycles.
   - 5,000 pg/mL: Mix 0.3 mL of 10,000 pg/mL with 0.3 mL of sample diluent buffer and mix thoroughly.
   - 2,500 pg/mL: Mix 0.3 mL of 5,000 pg/mL with 0.3 mL of sample diluent buffer and mix thoroughly.
   - Perform similar dilutions until the standard solutions with these concentrations (pg/mL) are made: 1,250, 625, 312, 156 and 78.
   - Add 100 µL of each of the diluted standard solutions to the appropriate empty wells. Repeat in duplicate or triplicate for accuracy.

2. Biotinylated Antibody
   - Calculate the total volume needed for the assay by multiplying 0.1 mL/well and the number of wells required. Add 2-3 extra wells to the calculated number of wells to account for possible pipetting errors.
   - Generate the required volume of diluted antibody by performing a 1:100 dilution (For each 1 µL concentrated antibody, add 99 µL antibody dilution buffer) and mixing thoroughly.

3. Avidin-Biotin-Peroxidase (ABC) [Diluted ABC solution shouldn't be prepared > 1 hr before experiment]
   - Calculate the total volume needed for the assay by multiplying 0.1 mL/well and the number of wells required. Add 2-3 extra wells to the calculated number of wells to account for possible pipetting errors.
   - Generate the required volume of diluted ABC solution by performing a 1:100 dilution (For each 1 µL concentrated ABC solution, add 99 µL ABC dilution buffer) and mixing thoroughly.

Sandwich ELISA

All of the ELISA kits from Boster use the **sandwich format** and **avidin-biotin chemistry**. Our ELISA assays require the dilutions of standard solutions, biotinylated antibody (detection antibody) and avidin-biotin-peroxidase.

1. Capture Antibody Coating

   (These steps are not required if the pre-adsorbed Picokine ELISA kits from Boster are used)
   - Dilute the capture antibody to a final concentration of 1-10 μg/mL in bicarbonate/carbonate antigen-coating buffer (100 mM NaHCO3 in deionized water; pH adjusted to 9.6).
   - Pipette 100 μL of diluted antibody to each well of a microtiter plate.
   - Cover the plate with adhesive plastic and incubate at 4°C overnight (or 37°C for 30 min).
   - Remove the coating solution and wash the plate 3X with 200 μL PBS (Phosphate Buffered Saline) buffer (10 mM Na2HPO4 and 1.8 mM NaH2PO4 in deionized water with 0.2% Tween 20; pH Adjusted to 7.4) with for 5 minutes each time. The coating/washing solutions can be removed by flicking the plate over a sink. The remaining drops can be removed by patting the plate on a paper towel or by aspiration. Do not allow the wells to dry out at any time.

2. Blocking

   (These steps are not required if the pre-adsorbed Picokine ELISA kits from Boster are used)
   - Pipette 200 μL blocking buffer (5% w/v non-fat dry milk in PBS buffer) per well to block residual protein-binding sites. Alternatively, BSA or BlockACE can be used to replace non-fat dry milk.
   - Cover the plate with adhesive plastic and incubate for 1-2 hour(s) at 37°C (or at 4°C overnight).
   - Remove the blocking solution and wash the plate 2X with 200 μL PBS for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

3. Reagent Preparation

   - Prepare for the diluted standard solutions, biotinylated antibody and ABC solutions as shown on p.9.

4. Sample (Antigen) Incubation

   - Serially dilute the sample with blocking buffer immediately before use. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.
   - Pipette 100 μL of each of the diluted sample solutions and control to each empty well. Repeat in duplicate or triplicate for accuracy. The negative control should be species- and isotype-matched as well as non-specific immunoglobulin diluted in PBS buffer.
   - Cover the plate with adhesive plastic and incubate for 2 hours at room temperature.
   - Remove the content in the wells and wash them 3X with 200 μL PBS buffer for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

5. Biotinylated Antibody Incubation

   - Pipette 100 μL of diluted antibody to the wells with control, standard solutions and diluted samples.

- Cover the plate with adhesive plastic and incubate for 1 hour at 37°C (or 2 hours at room temperature). These incubation times should be sufficient to receive a strong signal. However, if a weak signal is observed, perform incubation overnight at 4°C for a stronger signal.
- Remove the content in the wells and wash them 3X with 200 µL PBS for 5 min each time. Flick the plate and pat the plate as described in the coating step.

6. ABC Incubation

- Pipette 100 µL of diluted ABC solution to the wells with control, standard solutions and diluted samples.
- Cover the plate with adhesive plastic and incubate for 0.5 hour at 37°C.
- Remove the content in the wells and wash them 3X with 200 µL PBS buffer for 5 min each time. Flick the plate and pat the plate as described in the coating step.

7. Substrate Preparation

Prepare the substrate solution immediately before use or bring the pre-made substrate to room temperature. The two widely used enzymes for signal detection are horse radish peroxidase (HRP) and alkaline phosphatase (AP), and their corresponding substrates, stopping solutions, detection absorbance wavelengths and color developed are as follows:

Enzyme	Substrate*	Stop Solution	Absorbance (nm)	Color Developed
HRP	TMB	2M H_2SO_4	450	Yellow
AP	pNPP	0.75M NaOH	405	Yellow

* TMB: 3,3',5,5'-tetramethylbenzidine; pNPP: p-nitrophenyl-phosphate

Note:

- The TMB substrate must be kept at 37°C for 30 min before use.
- Hydrogen peroxide can also act as a substrate for HRP.
- Sodium azide is an inhibitor of HRP. Do not include the azide in buffers or wash solutions if HRP-labeled conjugate is used for detection.

8. Signal Detection

- Pipette 90 µL of substrate solution to the wells with the control, standard solutions and diluted samples.
- Incubate the plate at 37°C in the dark. If TMB is used, shades of blue will be observed in the wells with the most concentrated solutions. Other wells may show no obvious color.
- Color should be developed in positive wells after 15 min. After sufficient color development, pipette 100 µL of stop solution to the appropriate wells (if necessary).
- Read the absorbance (OD: Optical Density) of each well with a plate reader.

9. Data Analysis

- Prepare a standard curve using the data produced from the diluted standard solutions. Use absorbance on the Y-axis (linear) and concentration on the X-axis (log scale).
- Interpret the sample concentration from the standard curve.

Indirect ELISA

This is a general protocol in which antigen coating and blocking may not be required if the wells from the manufacturer have been pre-adsorbed with the antigen.

1. Antigen Coating

- Dilute purified antigens to a final concentration of 1-10 μg/mL in bicarbonate/carbonate antigen-coating buffer (100 mM NaHCO3 in deionized water; pH adjusted to 9.6).
- Pipette 100 μL of diluted antigen to each well of a microtiter plate.
- Cover the plate with adhesive plastic and incubate at 4°C overnight (or 37°C for 30 min).
- Remove the coating solution and wash the plate 3X with 200 μL PBS (Phosphate Buffered Saline) buffer (10 mM Na2HPO4 and 1.8 mM NaH2PO4 in deionized water with 0.2% Tween 20; pH Adjusted to 7.4) with for 5 minutes each time. The coating/washing solutions can be removed by flicking the plate over a sink. The remaining drops can be removed by patting the plate on a paper towel or by aspiration. Do not allow the wells to dry out at any time.

2. Blocking

- Pipette 200 μL blocking buffer (5% w/v non-fat dry milk in PBS buffer) per well to block residual protein-binding sites. Alternatively, BSA or BlockACE can be used to replace non-fat dry milk.
- Cover the plate with adhesive plastic and incubate for 1-2 hour(s) at 37°C (or at 4°C overnight).
- Remove the blocking solution and wash the plate 2X with 200 μL PBS for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

3. Reagent Preparation

- Prepare for the diluted standard solutions as shown on p.9.

4. Primary Antibody Incubation

- Serially dilute the primary antibody of choice with blocking buffer. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.
- Pipette 100 μL of each diluted antibody per well. Repeat in duplicate or triplicate for accuracy. The negative control should be species- and isotype-matched as well as non-specific immunoglobulin diluted in PBS buffer.
- Cover the plate with adhesive plastic and incubate for 1 hour at 37°C (or 2 hours at room temperature). These incubation times should be sufficient to receive a strong signal. However, if a weak signal is observed, perform incubation overnight at 4°C for a stronger signal.
- Remove the diluted antibody solution and wash the wells 3X with 200 μL PBS for 5 min each time. Flick the plate and pat the plate as described in the coating step.

- Serially dilute the primary antibody of choice with blocking buffer. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.
- Pipette 100 μL of each diluted antibody per well. Repeat in duplicate or triplicate for accuracy. The negative control should be species- and isotype-matched as well as non-specific immunoglobulin diluted in PBS buffer.
- Cover the plate with adhesive plastic and incubate for 1 hour at 37°C (or 2 hours at room temperature). These incubation times should be sufficient to receive a strong signal. However, if a weak signal is observed, perform incubation overnight at 4°C for a stronger signal.
- Remove the diluted antibody solution and wash the wells 3X with 200 μL PBS for 5 min each time. Flick the

plate and pat the plate as described in the coating step.

5. Secondary Antibody Incubation

- Serially dilute the conjugated secondary antibody with blocking buffer immediately before use. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.
- Pipette 100 μL of diluted secondary antibody solution to each well.
- Cover the plate with adhesive plastic and incubate for 2 hours at room temperature.
- Remove the content in the wells and wash them 3X with 200 μL PBS buffer for 5 min each time. Flick the plate and pat the plate as described in the coating step.

6. Substrate Preparation

Prepare the substrate solution immediately before use or bring the pre-made substrate to room temperature. The two widely used enzymes for signal detection are horse radish peroxidase (HRP) and alkaline phosphatase (AP), and their corresponding substrates, stopping solutions, detection absorbance wavelengths and color developed are as follows:

Enzyme	**Substrate***	**Stop Solution**	**Absorbance (nm)**	**Color Developed**
HRP	TMB	2M H_2SO_4	450	Yellow
AP	pNPP	0.75M NaOH	405	Yellow

* TMB: 3,3',5,5'-tetramethylbenzidine; pNPP: p-nitrophenyl-phosphate

Note:

- The TMB substrate must be kept at 37°C for 30 min before use.
- Hydrogen peroxide can also act as a substrate for HRP.
- Sodium azide is an inhibitor of HRP. Do not include the azide in buffers or wash solutions if HRP-labeled conjugate is used for detection.

7. Signal Detection

- Pipette 90 μL of substrate solution to the wells with the control and standard solutions.
- Incubate the plate at 37°C in the dark. If TMB is used, shades of blue will be observed in the wells with the most concentrated solutions. Other wells may show no obvious color.
- Color should be developed in positive wells after 15 min. After sufficient color development, pipette 100 μL of stop solution to the wells (if necessary).
- Read the absorbance (OD: Optical Density) of each well with a plate reader.

8. Data Analysis

- Prepare a standard curve using the data produced from the diluted standard solutions. Use absorbance on the Y-axis (linear) and concentration on the X-axis (log scale).
- Interpret the sample concentration from the standard curve.

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Direct ELISA

This is a general protocol in which antigen coating and blocking may not be required if the wells from the manufacturer have been pre-adsorbed with the antigen.

1. Antigen Coating

- Dilute purified antigens to a final concentration of 1-10 μg/ml in bicarbonate/carbonate antigen-coating buffer (100 mM NaHCO3 in deionized water; pH adjusted to 9.6).
- Pipette 100 μL of diluted antigen to each well of a microtiter plate.
- Cover the plate with adhesive plastic and incubate at 4°C overnight (or 37C for 30 min).
- Remove the coating solution and wash the plate 3X with 200 μL PBS (Phosphate Buffered Saline) buffer (10 mM Na2HPO4 and 1.8 mM NaH2PO4 in deionized water with 0.2% Tween 20; pH Adjusted to 7.4) with for 5 minutes each time. The coating/washing solutions can be removed by flicking the plate over a sink. The remaining drops can be removed by patting the plate on a paper towel or by aspiration. Do not allow the wells to dry out at any time.

2. Blocking

- Pipette 200 μL blocking buffer (5% w/v non-fat dry milk in PBS buffer) per well to block residual protein-binding sites. Alternatively, BSA or BlockACE can be used to replace non-fat dry milk.
- Cover the plate with adhesive plastic and incubate for 1-2 hour(s) at 37°C (or at 4°C overnight).
- Remove the blocking solution and wash the plate 2X with 200 μL PBS for 5 min each time. Flick the plate and pat the plate as described in the coating step.

3. Reagent Preparation

- Prepare for the diluted standard solutions as shown on p.9.

2. Primary Antibody Incubation

- Serially dilute the conjugated primary antibody with blocking buffer immediately before use. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.
- Pipette 100 μL of diluted secondary antibody solution to each well.
- Cover the plate with adhesive plastic and incubate for 2 hours at room temperature.
- Remove the content in the wells and wash them 3X with 200 μL PBS buffer for 5 min each time. Flick the plate and pat the plate as described in the coating step.

3. Substrate Preparation

Prepare the substrate solution immediately before use or bring the pre-made substrate to room temperature. The two widely used enzymes for signal detection are horse radish peroxidase (HRP) and alkaline phosphatase (AP), and their corresponding substrates, stopping solutions, detection absorbance wavelengths and color developed are as follows:

Enzyme	Substrate*	Stop Solution	Absorbance (nm)	Color Developed
HRP	TMB	2M H_2SO_4	450	Yellow
AP	pNPP	0.75M NaOH	405	Yellow

* TMB: 3,3',5,5'-tetramethylbenzidine; pNPP: p-nitrophenyl-phosphate

Note:

- The TMB substrate must be kept at 37°C for 30 min before use.
- Hydrogen peroxide can also act as a substrate for HRP.
- Sodium azide is an inhibitor of HRP. Do not include the azide in buffers or wash solutions if HRP-labeled conjugate is used for detection.

4. Signal Detection

- Pipette 90 μL of substrate solution to the wells with the control and standard solutions.
- Incubate the plate at 37°C in the dark. If TMB is used, shades of blue will be observed in the wells with the most concentrated solutions. Other wells may show no obvious color.
- Color should be developed in positive wells after 15 min. After sufficient color development, pipette 100 μL of stopping solution to the wells (if necessary).
- Read the absorbance (OD: Optical Density) of each well with a plate reader.

5. Data Analysis

- Prepare a standard curve using the data produced from the diluted standard solutions. Use absorbance on the Y-axis (linear) and concentration on the X-axis (log scale).
- Interpret the sample concentration from the standard curve.

To be continued on next page

Competitive ELISA

This is a general protocol in which antigen coating and blocking may not be required if the wells from the manufacturer have been pre-adsorbed with the antigen.

1. Antigen Coating

- Dilute purified antigens to a final concentration of 20 μg/ml in bicarbonate/carbonate antigen-coating buffer (100 mM NaHCO3 in deionized water; pH adjusted to 9.6).
- Pipette 100 μL of diluted antigen to each well of a microtiter plate.
- Cover the plate with adhesive plastic and incubate at 4°C overnight (or 37C for 30 min).
- Remove the coating solution and wash the plate 3X with 200 μL PBS (Phosphate Buffered Saline) buffer (10 mM Na2HPO4 and 1.8 mM NaH2PO4 in deionized water with 0.2% Tween 20; pH Adjusted to 7.4) with for 5 minutes each time. The coating/washing solutions can be removed by flicking the plate over a sink. The remaining drops can be removed by patting the plate on a paper towel or by aspiration. Do not allow the wells to dry out at any time.

2. Blocking

- Pipette 200 μL blocking buffer (5% w/v non-fat dry milk in PBS buffer) per well to block residual protein-binding sites. Alternatively, BSA or BlockACE can be used to replace non-fat dry milk.
- Cover the plate with adhesive plastic and incubate for 1-2 hour(s) at 37°C (or at 4°C overnight).
- Remove the blocking solution and wash the plate 2X with 200 μL PBS for 5 min each time. Flick the plate and pat the plate as described in the coating step.

3. Reagent Preparation

   a. Prepare for the diluted standard solutions as shown on p.9.

4. Sample (Antigen) Incubation
   a. Serially dilute the sample with blocking buffer immediately before use. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.
   b. Pipette 100 μL of diluted sample to each well.
   c. Cover the plate with adhesive plastic and incubate for 2 hours at room temperature.
   d. Remove the content in the wells and wash them 3X with 200 μL PBS buffer for 5 minutes each time. Flick the plate and pat the plate as described in the coating step.

5. Primary Antibody Incubation
   a. Serially dilute the primary antibody of choice with blocking buffer. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.
   b. Pipette 100 μL of each diluted antibody per well. Repeat in duplicate or triplicate for accuracy. The negative control should be species- and isotype-matched as well as non-specific immunoglobulin diluted in PBS buffer.
   c. Cover the plate with adhesive plastic and incubate for 1 hour at 37°C (or 2 hours at room temperature). These incubation times should be sufficient to receive a strong signal. However, if a weak signal is observed, perform incubation overnight at 4°C for a stronger signal.
   d. Remove the diluted antibody solution and wash the wells 3X with 200 μL PBS for 5 min each time. Flick the plate and pat the plate as described in the coating step.

6. Secondary Antibody Incubation

a. Serially dilute the conjugated secondary antibody with blocking buffer immediately before use. The optimal dilution should be determined by a titration assay according to the antibody manufacturer.
b. Pipette 100 µL of diluted secondary antibody solution to each well.
c. Cover the plate with adhesive plastic and incubate for 2 hours at room temperature.
d. Remove the content in the wells and wash them 3X with 200 µL PBS buffer for 5 min each time. Flick the plate and pat the plate as described in the coating step.

7. Substrate Preparation

Prepare the substrate solution immediately before use or bring the pre-made substrate to room temperature. The two widely used enzymes for signal detection are horse radish peroxidase (HRP) and alkaline phosphatase (AP), and their corresponding substrates, stopping solutions, detection absorbance wavelengths and color developed are as follows:

Enzyme	Substrate*	Stop Solution	Absorbance (nm)	Color Developed
HRP	TMB	2M H_2SO_4	450	Yellow
AP	pNPP	0.75M NaOH	405	Yellow

* TMB: 3,3',5,5'-tetramethylbenzidine; pNPP: p-nitrophenyl-phosphate

Note:

a. The TMB substrate must be kept at 37°C for 30 min before use.
b. Hydrogen peroxide can also act as a substrate for HRP.
c. Sodium azide is an inhibitor of HRP. Do not include the azide in buffers or wash solutions if HRP-labeled conjugate is used for detection.

8. Signal Detection

a. Pipette 90 µL of substrate solution to the wells with the control, standard solutions and diluted samples.
b. Incubate the plate at 37C in the dark. If TMB is used, shades of blue will be observed in the wells with the most concentrated solutions. Other wells may show no obvious color.
c. Color should be developed in positive wells after 15 minutes. After sufficient color development, pipette 100 µL of stopping solution to the wells (if necessary).
d. Read the absorbance (OD: Optical Density) of each well with a plate reader.

9. Data Analysis

a. Prepare a standard curve using the data produced from the diluted standard solutions. Use absorbance on the Y-axis (linear) and concentration on the X-axis (log scale).
b. Competitive ELISA yields an inverse curve: Higher values of antigen in the samples yield a smaller amount of color change.
c. Interpret the sample concentration from the standard curve.

To be continued on next page

Troubleshooting Guide

The following guide serves as a checklist for the possible causes and solutions with respect to some of the most commonly encountered problems from the ELISA assays.

1. Weak or No Signal

	Possible Cause	Solution
1	Blocking protein in coating solution	Eliminate blocking protein from coating solution
2	Capture antibody (or antigen) does not bind to plate	Use ELISA plate, not tissue culture plate Try longer coating time Increase concentration of coating components
3	Problem with the standard	Use new sample Check that the standard is appropriately handled
4	Incubation time too short	Follow the manufacturer guideline (If the problem persists, try incubating samples at 4°C overnight)
5	Incubation temperature too low	Ensure incubations are done at correct temperature Before proceeding, all reagents, including plate, should be at room temperature or as recommended by the manufacturer
6	Incompatible sample type	Use sample that the assay is known to detect a positive control (Include such control in your experiment)
7	Incompatible assay buffer	Ensure assay buffer is compatible with the target of interest
8	Target present below detection limit	Decrease dilution factor or concentrate samples
10	Incorrect/Insufficient/No substrate	Check the substrate identity Increase concentration or amount of substrate Follow manufacturer guidelines
11	Incorrect/Insufficient/No antibody	Check the antibody identity Repeat the assay with higher antibody concentrations to find the optimal one for your experiment
12	Antibody stored at 4°C for several weeks or subjected to repeated freeze-thaw cycles	Use fresh aliquot of antibody that has been stored at -20°C or below

13	Incorrect reagents added/ prepared; Missing reagents	Check protocol, ensure correct reagents are added in proper order and prepared to correct concentrations (e.g. TMB for HRP-labeled antibodies)
14	Expired/Contaminated reagents	Make and use fresh/uncontaminated reagents
15	Enzyme inhibitor present	Avoid sodium azide in HRP reactions Avoid phosphate in AP reactions
16	Incorrect storage of components	Double check storage conditions on kit level (Most kits need to be stored at 4°C)
17	Ultra vigorous plate washing	Gently pipette wash buffer (manual method) Ensure correct pressure (automatic wash system)
18	Wells dry out	Cover the plate using sealing film or tape for all incubations
19	Wells scratched with pipette or pipette tips	Carefully dispense/aspirate solutions into and out of wells
20	Plate read at incorrect detection wavelength	Use recommended wavelength/filter Ensure plate reader is set correctly for type of substrate used
21	Slow color development	Prepare substrate immediately before use Allow longer incubation Ensure stock solution is unexpired and uncontaminated
22	Epitope recognition impeded by adsorption to plate	Conjugate peptide to large carrier protein before coating onto plate

2. Saturated Signal

	Possible Cause	Solution
1	High sample concentration	Use higher sample dilutions (Determine the optimal dilutions by titration assay)
2	Excessive substrate	Decrease concentration or amount of substrate: Follow manufacturer guidelines (The substrate provided with the ELISA kit might require further dilution)
3	Substrate color changed before use	Make substrate immediately before use
4	Non-specific antibody binding	Try different formulations in coating solutions

		Ensure wells are pre-processed to prevent non-specific binding Use affinity-purified antibody and preferably one that is pre-adsorbed. Use serum (5-10%) from same species as secondary antibody (bovine serum is also recommended)
5	Incubation time too long	Follow the manufacturer guidelines (If the problem persists, try incubating samples at 4°C overnight)
6	Excess antibody	Repeat the assay with lower antibody concentrations to find the optimal one for your experiment
7	Contaminated buffers with metals or HRP	Make and use fresh buffers
9	Insufficient washing	Follow the manufacturer guidelines At the end of each washing step, flick the plate over a sink and pat the plate on a paper towel
10	Plate sealers not used or re-used	During incubations, cover plates with plate sealers. Use a fresh sealer every time the used sealer is removed from the plate
11	Plate read at incorrect detection wavelength	Use recommended wavelength/filter Ensure plate reader is set correctly for type of substrate used
12	Excess time before plate reading	Read your plate within 30 minutes after adding the substrate (If the reading is not performed within this time frame, add a stopping solution after sufficient color is developed in the plate)

3. High Background

	Possible Cause	Solution
1	Insufficient washing	Follow the manufacturer guidelines At the end of each washing step, flick the plate over a sink and pat the plate on a paper towel
2	Ineffective/Contaminated blocking buffer	Try higher blocking protein concentration Increase blocking time

		Use fresh buffer
3	Excess antibody	Repeat the assay with lower antibody concentrations to find the optimal one for your experiment
4	Excess substrate	Decrease concentration or amount of substrate Follow manufacturer guidelines (Note: The substrate provided with the ELISA kit might require further dilution)
5	Cross reactivity (Detection antibody reacts with coating antibody)	Run appropriate controls
6	Non-specific antibody binding	Try different formulations in coating solutions Ensure wells are pre-processed to prevent non-specific binding Use affinity-purified antibody and preferably one that is pre-adsorbed Use serum (5-10%) from same species as secondary antibody (bovine serum is also recommended)
7	Insufficient Tween in buffers	Use PBS containing 0.05% Tween
8	Suboptimal salt concentration in washing buffer	Optimize salt concentration as high concentration can reduce non-specific interactions
9	Incubation temperature too high	Optimize incubation temperature for your assay (antibodies bind optimally at very specific temperature)
10	Reagents were not mixed properly	Thoroughly mix all reagents and samples before pipetting solutions into wells
11	Blanks contaminated with samples	Change pipette tips when switching between blanks and samples Put a lid on pates to avoid any spilling between wells
12	Sample contaminated with enzymes	Test samples with substrate alone to check for contaminating enzymes
13	Contaminated TMB substrate	Use a clean container to check that the substrate in not contaminated (TMB substrate should be clear and colorless before adding to wells)

14	Substrate incubation in light	Carry out substrate incubation in dark or follow recommendation from manufacturer
15	Uneven evaporation of solution from wells during incubation	Always incubate with a lid on the plate
16	Precipitate created in wells upon substrate addition	Increase dilution factor of sample or decrease concentration of substrate
17	Incubation time too long	Follow the manufacturer guidelines (If the problem persists, try incubating samples at 4°C overnight)
18	Incorrect standard curve dilutions	Check pipetting techniques Double check calculations
19	Plates stacked during incubations, leading to uneven temperature distribution	Avoid stacking plates
20	Dirty or defective plates	Clean the plate bottom
21	Unstopped color development	Use Stopping solution to prevent over-development
22	Excess time before plate reading	Read your plate within 30 minutes after adding the substrate (If the reading is not performed within this time frame, add a stopping solution after sufficient color is developed in the plate) Note: Color continues to develop even after adding the stopping solution (although at a slower rate)
23	Incorrect plate reading setting	Use recommended wavelength/filter Ensure plate reader is set correctly for type of substrate used

4. Low Sensitivity

	Possible Cause	Solution
1	Assay format not sensitive enough	Switch to a more sensitive detection system (e.g. colorimetric to chemiluminescence) Switch to a more sensitive assay type (e.g. direct ELISA to sandwich ELISA) Increase incubation time and/or temperature
2	Improper storage of ELISA kit	Store all reagents as recommended

		Note: All reagents may not have identical storage requirements
3	Insufficient target	Reduce sample dilution or concentrate sample
4	Inactive substrate	Ensure reporter enzyme has the expected activity
5	Poor target adsorption to wells	Covalently link target to wells
6	Insufficient substrate	Increase concentration or amount of substrate
7	Incompatible sample type	Use a sample that the assay is known to detect a positive control Include positive control in your experiment
8	Interfering ingredients in buffers and sample	Check reagents for any interfering chemicals, e.g. sodium azide in antibodies inhibit HRP enzyme; EDTA used as anti-coagulant for plasma collection inhibits enzymatic reactions
9	Mixing or substituting reagents from different kits	Avoid mixing components from different kits
10	Incorrect plate reading setting	Use recommended wavelength/filter Ensure plate reader is set correctly for type of substrate used

5. Poor Standard Curve

	Possible Cause	Solution
1	Improper standard solution	Confirm dilutions are done correctly Make new standard curve as appropriate
2	Standard improperly reconstituted	Briefly spin vial before opening Inspect for undissolved material after reconstituting
3	Standard degraded	Store and handle standard as recommended Prepare standards no more than two hours before use
4	Improper curve fitting	Try plotting using different scales, e.g. log-log, 5-parameter logistic curve fit
5	Pipetting error	Use calibrated pipettes and proper pipetting technique
6	Insufficient washing	Follow the manufacturer guidelines

		At the end of each washing step, flick the plate over a sink and pat the plate on a paper towel
7	Poorly mixed reagents	Thoroughly mix reagents
8	Poor/variable adsorption of reagents to plate	Extend incubation time Check coating buffer Use a different plate as appropriate Check homogeneity of samples
9	Plates stacked during incubation	Keep plates separated if not using rotating plates
10	Dirty or defective plates	Clean the plate bottom

6. Poor Replicate Data

	Possible Cause	Solution
1	Bubble in wells	Ensure no bubbles are present prior to reading plate
2	Insufficient washing of wells	Carefully wash wells Follow recommended protocols Check that all ports of the plate washer are unobstructed
3	Incomplete reagent mixing	Ensure all reagents are mixed thoroughly
4	Inconsistent pipetting	Use calibrated pipettes and proper pipetting techniques If a multi-channel pipette is used, ensure that all channels deliver the same volume
5	Inconsistent sample prep or storage	Ensure consistent sample prep and optimal sample storage conditions (e.g. minimize freeze/thaw cycles)
6	Particulates in samples	Remove the particulates by centrifugation
7	Plate sealers not used or re-used	During incubations, cover plates with plate sealers Use a fresh sealer every time the used sealer is removed from the plate
8	Cross-well contamination	Ensure plate sealers and pipette tips are not contaminated with reagents

9	Edge effect (higher or lower OD in peripheral wells than in central wells)	Ensure plates and reagents are kept at room temperature before pipetting into wells unless otherwise instructed During incubation, seal the plate completely with a plate sealer and avoid stacking plates

7. Inconsistent Assay-to-Assay Results

	Possible Cause	Solution
1	Insufficient washing of wells	Carefully wash wells Follow recommended protocols Check that all ports of the plate washer are unobstructed
2	Variation in incubation temperature	Adhere to recommended incubation temperature Avoid incubating plates in area where environmental conditions vary
3	Variation in protocol	Adhere to the same protocol from run to run
4	Plate sealers not used or re-used	During incubations, cover plates with plate sealers Use a fresh sealer every time the used sealer is removed from the plate
5	Incorrect dilutions	Confirm dilutions are done correctly for standard solutions, etc Make new standard curve as appropriate
6	Contaminated buffers	Make and use fresh buffers
7	Plates stacked during incubation	Keep plates separated if not using rotating plates

8. Slow Color Development

	Possible Cause	Solution
1	Substrates too old, contaminated or used at incorrect pH	Make and use fresh substrates at correct pH: they should be prepared immediately before use
2	Expired/Contaminated solutions	Make and use fresh reagents
3	Incorrect incubation temperature	Ensure plates and reagents are kept at room temperature before pipetting into wells unless otherwise instructed

		During incubation, seal the plate completely with a plate sealer and avoid stacking plates
4	Low antibody concentration	Repeat the assay with higher antibody concentrations to find the optimal one for your experiment
5	Low substrate concentration	Add more substrate to the wells Make substrate no more than one hour before use Note: Typical ELISA sensitivity is ~0.1 pg/mL with exact value depends on antibody used.

9. Plate Imaging Problem

	Possible Cause	Solution
1	Oversaturated image after acquisition	Use full resolution image to analyze results (Do not use jpeg or other compressed formats)
2	Blurry spots in images	Re-focus your camera before taking a new image
3	Repeated pixel values or rectangular spots	Use lower bin size, higher image resolution and/or lossless file type
4	Flat standard in images	Reduce acquisition time

FAQs

1. **The ELISA protocols do not recommend shaking during incubations. Have you tested shaking and decided against it or is it unnecessary?**

   We tested our protocols with and without shaking during incubations and determined that there is no difference between the two approaches. Therefore, we believe that shaking is not necessary.

2. **Is your ELISA kit suitable for use with tissue lysates? If so, what are the protocols?**

   Theoretically, our ELISA kit can work with tissue lysates. Our general sample preparation protocol for tissue lysates is as follows:

   - Rinse the tissue with PBS to remove excess blood.
   - Chop the tissue into 1-2 mm pieces.
   - Using a tissue homogenizer, homogenize the samples in PBS or lysate solution such as the Mammal Tissue Protein Extraction Reagent (Boster Bio Catalog Number AR0101) at a ratio of 10 mL lysate solution to 1 g of tissue.
   - Centrifuge the homogenates at approximately 5000 x g for 5 min.
   - Assay immediately or store the homogenates at -20°C (avoid repeated freeze-thaw cycles).

3. **My samples contain very low cytokine concentration. What is the minimum concentration that can be measured with confidence using your ELISA kits?**

   Low cytokine concentrations are typical for many biological samples. When determining the minimum concentration that can be reliably measured by ELISA, consider the following:

   - The standard curve: As the concentration range of ELISA is typically 0 - 1000 pg/mL, the data points on the standard curve for this range correspond to 0, 15.6, 31.25, 62.5, 125, 250, 500, and 1000 pg/mL.
   - The assay sensitivity: Boster's Picokine ELISA kits typically have a reported sensitivity of 10 pg/mL. The concentration detected in many biological samples will fall between the 0 and 15.6 pg/mL data points of the standard curve. As long as the value detected is above the statistical sensitivity of the ELISA, (e.g., 5 pg/mL or greater), the value is statistically significant. Results below this detection limit are of questionable validity.

4. **Is your ELISA kit suitable for use with tissue homogenates?**

   For most cases, yes. If there is enough target protein present in the tissue of interest, the ELISA kit will work. Also, if there are a known alternative processing of the protein in a specific tissue that results in protein reactivity change to the kit, we will note it in our product datasheet.

5. **Is your ELISA kit suitable for use with any non-validated sample types?**

   In order to use an ELISA kit with a non-validated sample type, it is necessary to perform a spike and recovery study to determine if a non-validated sample type will work with a particular kit. To do this:

   - Divide the sample into two aliquots.
   - In one of the aliquots, you should "spike in" a known amount of the kit standard.
   - Perform a dilution series to compare the spiked to the unspiked sample.

   Generally, samples with expected recovery and linearity between 80-120% are acceptable. This method can be used to validate any sample type that has not been previously evaluated by Boster.

6. **Can I extend the standard curve?**

   No one can guarantee the assay accuracy once the concentrations outside the specified range within the curve are used. A specific range is generated to provide the statistical confidence for the assay accuracy.

7. **What causes high variability between sample duplicates?**

   The two main reasons for high sample variability in an assay are inconsistent pipetting and washing. Thus, it is important to perfect these techniques. However, some of this variability is unavoidable — this is the rationale for calculating the average results from sample duplicates. Another possible culprit for high variability is the "edge effect" in which the outermost wells of the plate are more vulnerable to drying out due to evaporation. Plate stacking will also cause variability because temperature will be unevenly distributed across the plates.

8. **What are the differences between the sandwich ELISA and competitive ELISA?**

   Sandwich ELISAs typically require the use of matched antibody pairs, where each antibody is specific for a different, non-overlapping part (epitope) of the antigen molecule. A first antibody (known as capture antibody) is coated to the wells. The sample solution is then added to the well. A second antibody (known as detection antibody) follows this step in order to measure the concentration of the sample. Higher signal output reflects higher concentration of the target antigen in the sample.

   The key event of competitive ELISA is the process of competitive reaction between the sample antigen and antigen bound to the wells of a microtiter plate with the primary antibody. First, the primary antibody is incubated with the sample antigen and the resulting antibody–antigen complexes are added to wells that have been coated with the same antigen. After an incubation period, any unbound antibody is washed off. The more antigen in the sample, the more primary antibody will be bound to the sample antigen. Therefore, there will be a smaller amount of primary antibody available to bind to the antigen coated on the well, resulting in a signal reduction.

9. **Why do my wells turn green after I add the stop solution?**

   The green color is a result of incomplete mixing between the substrate and stop solution. After adding the stop solution, gently tap the plate or place it on a shaker until the mixture in the wells turns yellow.

10. **Why does a brown or orange-brown precipitate appear in my wells after adding the stop solution? How can I resolve this issue?**

    The precipitate is a result from insufficient washing after incubation with the HRP- labeled detection antibody. To resolve this issue, perform a 30-second soak during each wash step followed by a complete removal of all liquid in the wells.

11. **If I don't use all the wells from a microtiter plate for my current ELISA assay, how can I preserve the unused wells for future use?**

    The microtiter plate typically has removable strips of wells. Unused wells may be removed from the plate, returned to the foil pouch containing the desiccant pack and stored at 2-8°C for up to one month.

Ordering Information

With more than 20 years of experience and trust from 10,000+ scientists, Boster is proud of offering more than **700 PicoKine™ ELISA kits** that help accelerate scientific discovery in research areas including **immunology**, **neuroscience** and **cancer**. Each of our ELISA kits has sufficient reagents for 96 tests per kit. The table below shows some of the most commonly used ELISA kits. Boster supplies kits that detect a variety of antigens, proteins and peptides. See the **Target Protein Index** for a complete list of target proteins our ELISA kits were designed to detect.

		Sample Types*									
Target	**Species**	**CCS**	**Se**	**P(h)**	**P(e)**	**P(c)**	**U**	**M**	**CL**	**T**	**Cat. No.**
Adiponectin	Human	√	√	√	√		√			√	EK0595
Angiopoietin-2	Mouse	√	√	√	√						EK0938
BDNF	Human	√	√	√	√	√					EK0307
CRP	Rat	√	√	√	√						EK0978
CTLA4	Mouse	√	√	√							EK0717
EGFR	Human	√	√	√	√			√			EK0327
FGF21	Human	√	√	√	√						EK0994
IFN Gamma	Human	√	√								EK0373
IL-6	Human	√	√	√	√	√					EK0410
IL-8	Human	√	√	√	√	√					EK0413
IL-10	Human	√	√	√	√	√					EK0416
Leptin	Mouse	√	√	√	√						EK0438
MCP-1	Rat	√	√								EK0902
NGF Beta	Rat	√	√								EK0471
P53	Human								√		EK0895
PD-1	Human	√									EK0959
TGF Beta 1	Human	√	√		√		√				EK0513
TNF Alpha	Mouse	√	√	√	√						EK0527

* Cell Culture Supernates [CCS], Serum [Se], Plasma: Heparin [P(h)], Plasma: EDTA [P(e)], Plasma: Citrate [P(c)], Urine [U], Milk [M], Cell Lysate (CL), Tissue (T)

We also offer a variety of ELISA components that can be purchased separately from the kits:

- Lyophilized recombinant standard (1 ng to 100 ng) [Catalog # e.g. ST0000-100 for 100 ng]
- 96-well plate (No antibody pre-coated) [Catalog # AR1100]
- Avidin-Biotin-Peroxidase Complex (ABC) [Catalog # AR1103]
- Buffers (Sample diluent, antibody diluent, ABC diluent) [Catalog # AR1106-1, AR1106-2, AR1106-3]
- Buffers (PBS, TBS) [Catalog # AR0030, AR0031]
- TMB color developing agent and stop solution [Catalog # AR1104 and AR1105]
- Biotinylated antibody [Catalog # AR1107]

Target Protein Index (By Species)

Human

A2M	CA9	CD40L	Cystatin C	Fractalkine	IGFBP6	JAM-A
ACE	Cadherin-2	CD44	DAN	FSTL1	IGFBP7	Kallikrein 14
ACE2	Caspase 3	CD47	DCR3	Furin	IL-1 alpha	Kallikrein 3
Activin A	Caspase 8	CD48	Decorin	Galectin-1	IL-1 beta	Kallikrein-6
ADA	Cathepsin B	CD5	Diablo	Galectin-2	IL-10	Kallistatin
ADAM12	Cathepsin D	CD56	DKK-1	Galectin-3	IL-11	KIM1
ADAM15	Cathepsin E	CD5L	DKK-3	Galectin-3BP	IL-12(p40)	Kininogen-1
ADAM8	Cathepsin L	CD6	DLL1	Galectin-4	IL-12(p70)	KIT
ADAM9	Cathepsin S	CD73	DTK	Galectin-7	IL-13	KLK1
ADAMTS1	CCL1	CD93	E-Cadherin	Galectin-9	IL-15	KLK11
ADAMTS13	CCL13	CD97	EDIL3	G-CSF	IL-16	KLK12
ADAMTS4	CCL14	CEA	EGF	GDF-15	IL-17	KLK13
Adiponectin	CCL15	CEACAM1	EGFR	GDNF	IL-17C	KLK5
AFP	CCL16	CFD	EG-VEGF	GH	IL-17E	KLK8
Aggrecan	CCL17	CFH	Elafin	GHR	IL-17RA	Laminin
AGP1	CCL18	Chemerin	Emmprin	GM-CSF	IL-17RB	LAP(TGF-beta1)
AGRP	CCL19	Chitinase 3-like 1	Endostatin	Gp130	IL-17RC	LBP
ALCAM	CCL21	Clusterin	Endothelin	GPNMB	IL-18	LDLR
ALK-1	CCL23	C-MET	Eotaxin	Granulysin	IL-18BP	Leptin
Amphiregulin(AR)	CCL24	COMP	EPCAM	Granzyme A	IL-18R1	LIFR
ANG	CCL27	complement C5a	EPCR	Granzyme B	IL1R1	Livin
Angiopoietin-1	CCL28	COMT	Epiregulin	GREM2	IL1R2	LOX-1
Angiopoietin-2	CCL4	Corin	ErbB-2	HAI-2	IL-1RA	LOXL2
Angiostatin K1-3	CCL7	CRISP3	ERBB3	HBEGF	IL1RL1	LRIG3
ANGPTL3	CCL8	CRLF2	ESM1	HE4	IL-2	Lumican
ANGPTL4	CD10	CRP	FABP4	Hemopexin	IL-20	LYVE-1
APOA1	CD105	CSF1R	FASL	Hepcidin	IL-22	Marapsin
APOE	CD13	CSF3R	Fetuin A	HGF	IL-27	MBP-C
APP	CD163	CT-1	Fetuin B	HO-1	IL-28A	MCP-1
AXL	CD169	CXCL1	FGF19	HSP27	IL-29	M-CSF
Azurocidin	CD200	CXCL10	FGF21	ICAM-1	IL-3	MDC
B7-1	CD21	CXCL11	FGF4	ICAM-2	IL-31	Mer
BAFF	CD23	CXCL13	FGF7	ICAM-3	IL-33	Mesothelin
BCAM	CD244	CXCL14	FGF9	ICAM5	IL-34	MFGE8
BDNF	CD25	CXCL16	Fibronectin	IDS	IL-37	MIA
Betacellulin	CD26	CXCL4	Fibulin-3	IFN gamma	IL-4	MICA

Biglycan	CD28	CXCL5	Ficolin-1	IGF1R	IL-5	MICB
BMP-2	CD30	CXCL6	Ficolin-2	IGF2R	IL-6	Midkine
BMP-4	CD32	CXCL7	Ficolin-3	IGFBP-1	IL-6R alpha	MIF
BMP-5	CD320	CXCL9	FLRG	IGFBP2	IL-7	MIP-1 alpha
BMP-7	CD36	CYR61	Flt-3ligand	IGFBP-3	IL7R	MIP-3 alpha
CA125	CD40	Cystatin B	FOLR1	IGFBP4	IL-8	MMP-1

Target Protein Index (By Species)
Human - continued

MMP-12	NT-4	Prostasin	Seprase	TAFI	TIMP-3	TNFSF4
MMP13	Oncomodulin	P-Selectin	Serpin C1	Tenascin-C	TIMP-4	TNFsR I
MMP-14	OPG	PSP94	sE-Selectin	Tetranectin	Tissue factor	TRAIL
MMP-2	OPN	PTX3	sFAS	TFF1	TLR1	TREML1
MMP-3	OSM	Rage	SFRP5	TFF2	TLR2	TrkA
MMP-7	P53	RANK	SHBG	TFF3	TLR3	Trypsin
MMP-8	PAI-1	Rantes	Siglec-7	TFPI	TNF alpha	Tryptase
MMP-9	P-Cadherin	RBP4	SLAM	TFPI2	TNF beta	TSLP
MPO	PCSK9	REG-4	sL-Selectin	TGF alpha	TNFRSF13B	TSP2
MSP	PD-1	Relaxin 1	SPARC	TGF beta 1	TNFRSF13C	uPA
MyD88	PDGF-AB	Relaxin 2	SPARCL1	TGF-beta 2	TNFRSF14	uPAR
Nectin-4	PD-L1	Relaxin 3	SP-D	TGF-beta 3	TNFRSF16	VCAM-1
Nesfatin-1	PECAM-1	Renin	SPHK1	TGFBI	TNFRSF17	VE-Cadherin
Neuregulin-1	PEDF	Resistin	SPINK1	TGFBR2	TNFRSF18	VEGF
Neuropilin-1	Periostin	R-Spondin-1	SPINT1	TGFBR3	TNFRSF4	VEGF-C
Neurotrophin-3	Persephin	R-Spondin-3	STC1	THBS1	TNFRSF7	VEGFR2
NGAL	PLAT	R-Spondin-4	sTNFsRII	Thioredoxin	TNFRSF9	WIF1
NGF	PLGF	S100A12	Survivin	Thrombomodulin	TNFSF11	WISP1
Nidogen-1	PON1	SAA	sVEGFR1	TIE2	TNFSF12	XCL1
NOV	Progranulin	SCF	Syndecan-1	TIMP-1	TNFSF13	
NRCAM	Prolactin	SCGB1A1	Syndecan-4	TIMP-2	TNFSF14	

Target Protein Index (By Species)
Rat

Activin A	CD44	EGF	ICAM-1	IL-3	Neuropilin-1	sL-Selectin
ADAM12	CD80	Endothelin	IFN gamma	IL-4	Neuropilin-2	TGF beta 1
Adiponectin	CD86	Eotaxin	IGF-1	IL-6	Neurotrophin-3	TGF-beta 2
Angiopoietin-1	CNTF	EPO	IGF-2	KIM1	NGAL	TGF-beta 3
BDNF	CRP	FAS-L	IGFBP5	Laminin	NGF	TIMP-1
BMP-2	CXCL1	FGF1	IL-1 alpha	Lumican	PDGF-AB	TIMP-2

BMP-7	CXCL2	Fibronectin	IL-1 beta	MCP-1	RAGE	TNF alpha
BTC	CXCL3	Fractalkine	IL-10	MIP-1 alpha	RANK	TNFSF12
Cadherin-2	CXCL5	GDF5	IL-13	MIP-3 alpha	Rantes	TrkA
CCL4	Cystatin C	GDNF	IL-17F	MMP-2	SAP	VEGF
CCL7	DKK1	GM-CSF	IL-18	MMP-8	SCF	VEGFR3
CD36	E-Cadherin	HGF	IL-2	MMP-9	sE-Selectin	

Target Protein Index (By Species)

Mouse

ACE	CD13	CXCL4	GM-CSF	IL-34
ACE2	CD14	CXCL5/ENA-78	Gp130	IL-4
Activin A	CD163	CXCL9	GREM2	IL-5
ADAM12	CD169	Cystatin C	HAI-2	IL-6
Adiponectin	CD200	DAN	Hemojuvelin	IL-7
ALCAM	CD22	Decorin	HGF	IL7R
ALK-1	CD23	DKK1	ICAM-1	JAM-A
Amphiregulin	CD25	DLL4	IDS	KIM1
Angiopoietin-1	CD26	DMP-1	IFN gamma	Kremen-1
Angiopoietin-2	CD30	E-Cadherin	IGF-1	Laminin
ANGPTL3	CD30L	EGF	IGF-2	LBP
AXL	CD32	EGFR	IGFBP-1	LDLR
B7-1	CD36	Elastase	IGFBP2	Leptin receptor
BAFF	CD40	Endostatin	IGFBP-3	LOX-1
BCAM	CD40L	Endothelin	IGFBP5	L-Selectin
BDNF	CD44	Eotaxin	IL-1 alpha	Lumican
beta IG-H3	CD48	Epiregulin	IL-1 beta	MADCAM-1
BMP-2	CD56	EPO	IL-10	Marapsin
BMP-4	CD5L	ESM1	IL-11	MBL2
BMP-7	CD6	FABP4	IL-12(p40)	MCP-1
BTC	CD70	FAS	IL-12(p70)	M-CSF
Cadherin-2	CD86	FASL	IL-13	MDC
Cathepsin B	CFH	FCRN	IL15RA	MER
Cathepsin D	Chemerin	FGF1	IL-17	MFGE8
CCL1	Chitinase 3-like 1	FGF21	IL-17C	MIP-2
CCL12	Chordin	FGF23	IL-17D	MMP-12
CCL17	Clusterin	Fibronectin	IL-17F	MMP-2
CCL19	C-MET	Flt-3ligand	IL-17RC	MMP-3

CCL20	Complement C5a	FOLR1	IL-1RA	MMP-9
CCL21	Cripto	Fractalkine	IL1RL1	MPO
CCL24	CRP	Galectin-1	IL-2	MSP
CCL28	CSF1R	Galectin-3	IL-20	Neuropilin-1
CCL3	CTLA4	Galectin-7	IL-21	Neuropilin-2
CCL6	CXCL1	GAS6	IL-23	Neurotrophin-3
CCL7	CXCL10	G-CSF	IL-27 p28	NGAL
CCL8	CXCL13	GDF-15	IL-3	NGF
CCL9	CXCL16	GDF5	IL-31	NOPE
CD10	CXCL3	GDNF	IL-33	Oncostatin M

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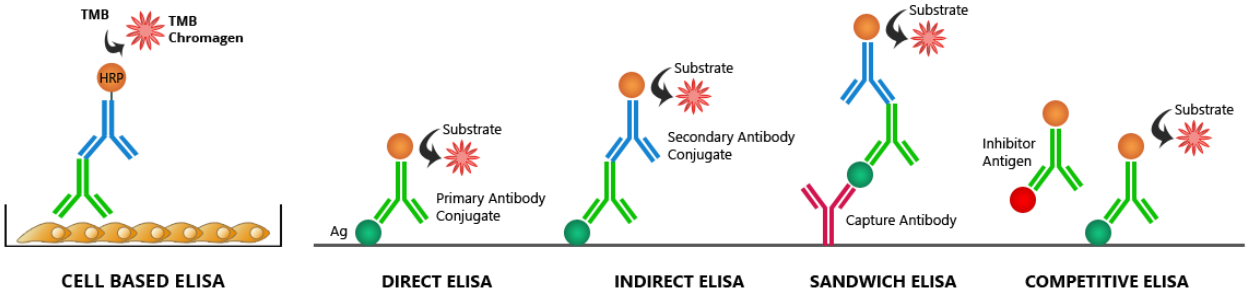
Ver. 100117

Cell-based ELISA: Principles and Protocols

ELISA Types

ELISAs can be performed with a number of modifications to the basic procedure: direct, indirect, sandwich, competitive, or cell-based. The key step, immobilization of the antigen of interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. In the case of cell-based ELISA, whole cells are seeded and fixed to the bottom of the wells. The antigen is then detected either directly (enzyme-labeled primary antibody) or indirectly (enzyme-labeled secondary antibody). The detection antibodies are usually labeled with alkaline phosphatase (AP) or horseradish peroxidase (HRP). A large selection of substrates is available for performing the ELISA with an HRP or AP conjugate. The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal-detection (spectrophotometer, fluorometer or luminometer).

Among the standard assay formats discussed and illustrated below, where differences in both capture and detection were the concern, it is important to differentiate between the particular strategies that exist specifically for the detection step. However an antigen is captured to the plate (by direct adsorption to the surface, through a pre-coated "capture" antibody, as in a sandwich ELISA, or by whole cells fixation, as in cell-based ELISA), it is the detection step (as either direct or indirect detection) that largely determines the sensitivity of an ELISA.



Cell-based ELISA Key Principles

Cell-Based ELISA, also referred to as In-Cell ELISA (ICE), In-Cell Western (ICW), cytoblot, fixed-cell ELISA (FICE), or FACE (Fast Activated Cell-based ELISA) is a plate-based immunoassay which utilizes an indirect ELISA format to allow for rapid high-throughput assessment of the relative amounts of a target protein, determination of the degree of post-translational modifications, or screening on the effects (including dose-dependent) of various drugs, metabolites, and treatment conditions on protein expression in whole fixed cells of various types or different cell lines. In this assay the target antigen is being detected directly in the cells without sample preparation common to the other ELISA types. Cells are either grown/cultured directly in the microplate wells pre-coated with extracellular matrix (adherent cells), or seeded afterwards (non-adherent/suspension cells) directly from a cell culture into the assay microplate and allowed to attach for several hours or overnight.

Each well can be treated with inhibitors (i.e. siRNA or chemicals) or activators to stimulate a cellular response. After treatment the cells are fixed, permeabilized and blocked in the wells, effectively being "freezed" in the particular context with no further sample prep perturbations.

Primary antibodies specific for target antigen are added and allowed to bind to their respective targets within the particular subcellular compartment. HRP- or AP-conjugated secondary antibodies specific for the primary antibody are added and allowed to bind to their respective epitopes. Then an HRP- or AP- substrate is added and the signal is detected. Depending on the selected substrate (chromogenic or fluorogenic), the detection format of the assay can be

colorimetric or fluorimetric, and the signal is read with a standard plate reader or microplate fluorometer, respectively. In some assay formats, e.g. fluorescent and infrared cell-based ELISAs, secondary antibodies can be directly conjugated to a fluorophore or to an IR or NIR dye, instead of an enzyme, thus omitting the step with substrate addition. Fluorescence or infrared imaging systems are used for signal detection. Fluorimetric and IR detection have the advantage of multiplexing, i.e. the possibility to detect more than one target in the same well, thus minimizing well to well variability.

Typical to the cell-based ELISA format is the use of normalization methods correcting for well-to-well cell number variations and allowing target protein levels to be accurately assessed and compared across multiple samples.

Normalization is applied at several levels:

1. In addition to the antibody against the protein of interest, a second normalization antibody against a resident/housekeeping protein which is known to be expressed constitutively and at high levels with low variability between cell lines and experimental conditions (e.g. GAPDH) is used to serve as an internal positive control in normalizing the target absorbance values when protein concentration differences are due to experimental treatment. The quantity of GAPDH in a sample is directly proportional to the number of cells in the sample. Typically, GAPDH expression is used to normalize expression signal from the gene-of-interest with respect to cell number, thus facilitating a direct comparison of target protein levels from one well to another in a comparative quantitative fashion. This has been particularly useful for RNAi studies or other drug treatment studies.

   In the case of fluorimetric detection, normalization and target protein detection can be performed in the same well using different host species primary antibodies for the two proteins, species-specific HRP- or AP-conjugated secondary antibodies in combination with spectrally distinct fluorogenic substrates for each one of the enzymes.

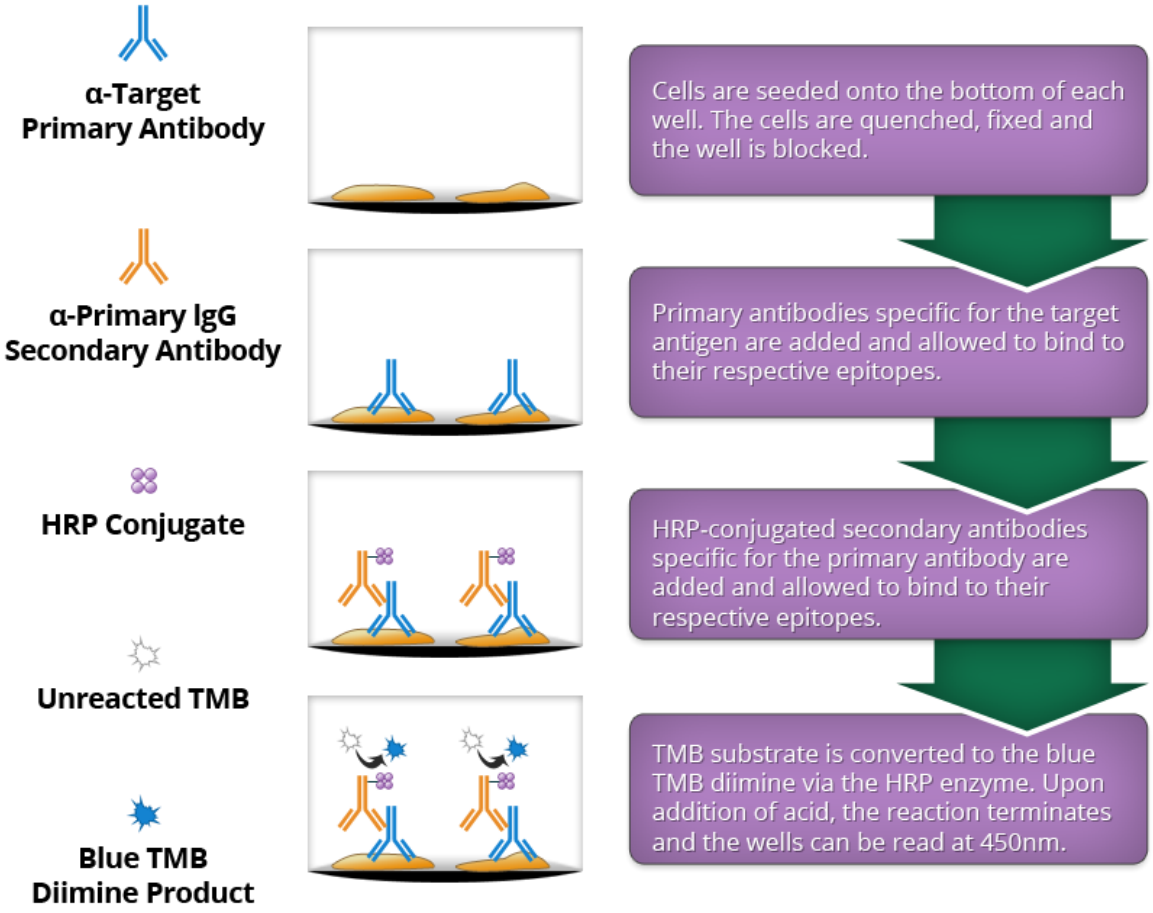
   In the case of colorimetric detection, target and normalization assessment must be performed in separate wells.

2. Following the colorimetric measurement of HRP activity via substrate addition, the Crystal Violet whole-cell staining method can be used to determine cell density. After staining, the absorbance values are normalized to cell amounts, by which the plating difference is adjusted.

3. Janus Green whole cell staining intensity (a mitochondrial dye) can also be used to determine cell number in each well, and the absorbance (HRP or AP activity) values from each well can be normalized to cell number to account for any differences in seeding density between wells.

4. If a phosphorylated target is about to be detected (using Cell-Based Phospho ELISA kits), a phospho-specific antibody and a normalization antibody that recognizes the total protein regardless of its phosphorylation status will be provided for normalization purposes. The absorbance values of the phosphorylated protein are to be normalized to that of the total protein in each well to correct for well-to-well variations.

The following flowchart shows the general protocol steps of a typical cell-based ELISA experiment:



ADVANTAGES of cell-based ELISA:

- **Fast and simple** - the assays are performed directly on cells which have been fixed in the microplate wells, eliminating the need for time-consuming cell lysis, lysate transfer, protein extraction or special sample preparation. Cell fixation to data acquisition requires approximately 30 minutes of hands on time.
- **Flexible** - detection of protein markers is possible in either adherent or suspension cells
- **Relevant biological context** - data are more meaningful because proteins are detected in their cellular context, and cells are "frozen" at a desired time point, in their biologically relevant state eliminating further modifications from sample processing, e.g. measure specific protein level or post-translational modifications (e.g. phosphorylation or cleavage events).
- **Quantification accuracy** - maximized by normalization adjustments accounting for differences in cell number or in total protein relative to phosphorylated protein from well to well.
- **High throughput** - 96- and 384-well formats make it easy in one experiment to either test a few targets of interest across a range of culture conditions, drug treatments, or cell lines in parallel, or test multiple targets across a few conditions.
- **Reproducible** - because of its simplicity, cell-based ELISA provides highly reproducible results.
- **Cost-effective** - no capture antibody means less reagent cost than sandwich ELISAs.

- **Instrumentation compatibility** - signal detection is compatible with standard ELISA plate readers.

Required Materials and Equipment:

Materials and reagents

- **TBS**: 50 mM Tris-HCL, 150 mM NaCl, pH adjusted to 7.6
- **Quenching Buffer**: 1% H2O2, 0.055% NaN3
- **Blocking Buffer**: 2% BSA, 0.5% Triton X-100, 0.05% NaN3
- **Wash Buffer**: 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH adjusted to 7.6
- **Ani-target Primary Antibody** (rabbit polyclonal)
- **Anti-GAPDH Antibody** (mouse monoclonal)
- **Primary Antibody Diluent**: 1% BSA, 0.5% Triton X-100, 0.05% NaN3
- **Enzyme*-Conjugated Anti-Rabbit IgG Secondary Antibody** (*HRP or AP) - for the anti-target rabbit primary antibody
- **Enzyme*-Conjugated Anti-Mouse IgG Secondary Antibody** (*HRP or AP) - for anti GAPDH mouse primary antibody
- **Enzyme substrate** (see Table below for possibilities)
- **Stop Solution**: 1M H2SO4 for HRP, 0.75M NaOH for AP
- **1 % SDS Solution** - for Crystal Violet solubilization
- **Crystal Violet Solution**: 0.05% Crystal violet, 0.5% Ethanol
- **4%** and/or **8% formaldehyde** (or higher concentration that could be diluted) for cell fixation
- **Deionized or sterile water**
- **Poly-L-Lysine** (Boster SKU AR0003 for suspension cells)

Equipment

- Cell culture incubator
- 96-Well Cell Culture Clear-Bottom Microplate
- Microplate absorbance reader or microplate fluorometer (fluorescence reader) for measuring the signal (O.D. absorbance or fluorescence of the enzyme reaction product, depending on the selected substrate - see Table for more details)
- Micropipettes with capability of measuring volumes ranging from 1 µl to 1 ml and pipette tips
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer for washing

- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent papers or vacuum aspirator
- Test tubes or microfuge tubes capable of storing ≥1 ml
- Disposable reagent reservoirs / containers for preparing the necessary quantities prior to use
- Orbital shaker

Recommended Protocols: Cell-based ELISA

Cell Seeding

1. Seed 200 µl of 20,000 **adherent cells** in culture medium in each well of a 96-well plate pre-coated with extracellular matrix (*the plates are sterile and pre-treated for cell culture*). For **suspension cells and loosely attached cells**, coat the plates with 100 µl of 10 µg/ml Poly-L-Lysine to each well of a 96-well plate for 30 minutes at 37°C prior to adding cells.

   Adherent cells can be grown in the recommended assay plates or seeded directly into the assay plate and allowed to attach for several hours to overnight.

   The **optimal cell seeding density** (the number of cells plated onto each well) depends on the cell type, cell size (diameter, in case of the adherent cells), the expression level or abundance of the target protein in the cells, treatment conditions and incubation time. The cells used for testing should be around 75-90% confluent. Generally, the cells should form a monolayer onto the well surface. Typically for HeLa cells, seed 30,000 cells per well overnight for treatment the following day.

   Note: Various experimental conditions can cause detachment of adherent cells. These detached cells are frequently lost during the fixation and washing steps of a typical cell-based ELISA protocol, and subsequently they go unaccounted for during data analysis. For example, adherent cells undergoing apoptosis will readily detach from a culture plate. This often leads to their loss and thus underestimation of the proportion of apoptotic cells. For such cases, try using a protocol which eliminates the loss of the apoptotic and detaching cells (recommended for cell-based ELISA on adherent cells undergoing apoptosis/detachment).

2. Incubate the cells for overnight at 37°C, 5% CO2. Depending on the cell line and treatment, the typical incubation time is 0.5-16 hours.

Cell Treatment

3. Treat the cells as desired in total volume of 100 µL of treatment media for 96-well microplate (up to 10% serum is acceptable). Mix solution by gently agitating the plate and incubate according to your treatment protocols or according to your empirical determination.

   When the cells get fully attached, the media can be removed and replaced with media containing treatment of interest. The cells can be treated with inhibitors, activators, stimulators (chemicals, proteins/peptides) or a combination of the substances listed above. The cells can be treated with UV and serum starvation to meet the needs of the end-user.

   Note: When treatment with drug of interest is performed, it is recommended to include wells with untreated cells and cells treated with the drug solvent only.

4. Remove the cell culture medium and rinse with 200 µl of TBS (*50 mM Tris-HCL, 150 mM NaCl, pH adjusted to 7.6*), twice.

   Note: See BosterBio's TBS buffer (AR0031) for our recommended reagent concentrations and molarity.

Cell Fixation

5. Fix the cells by incubating (approx. 15 min) with 100 µl of Fixing Solution (*4% or 8% formaldehyde solution in TBS*) for 20 minutes at room temperature.

Fixing Solution is used to fix and crosslink the cells to the plate after cell culture. It is prepared by adding formaldehyde to 1x TBS with light mixing. 4% formaldehyde solution should be used for adherent cells and 8% formaldehyde - for suspension cells and loosely attached cells. During the incubation, the plates should be sealed with Parafilm.

Warning: Fixing Solution is volatile and formaldehyde is known to be a highly toxic reagent. Wear appropriate personal protection equipment (mask, gloves and glasses) when using this chemical.

6. Remove the Fixing Solution and wash the plate 3 times with 200 µl Wash Buffer (*0.1% Tween-20 in TBS*) for five minutes each time with gentle shaking on the orbital shaker. The plate can be stored at 4°C for a week.

Note: For all wash steps, tap the plate gently on absorbent papers to remove the solution completely.

Quenching

7. Add 100 µl Quenching Buffer *(1% H2O2, 0.055% NaN3*) and incubate for 20 minutes at room temperature.

Quenching Buffer is used to inactivate the endogenous peroxidase activity of the seeded cells.

8. Wash the plate 3 times with Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker. After the last wash, invert the plate and gently tap the plate on absorbent papers to remove residual fluid. Proceed immediately to the next step. Do not allow the plate to air dry between steps.

Blocking

9. Add 200 µl of Blocking Buffer (*2% BSA, 0.5% Triton X-100, 0.05% NaN3*) and incubate for 1 hour at room temperature.

Blocking Buffer is used to block additional binding sites in each well.

10. Wash 3 times with 200 µl Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker. After the last wash, invert the plate and gently tap the plate on absorbent papers to remove residual fluid. Proceed immediately to the next step. Do not allow the plate to air dry between steps.

Primary Antibody Incubation

11. Dilute the primary antibodies stock solutions (target-specific antibody and/or - anti-normalization protein antibody) with Antibody Diluent (*1% BSA, 0.5% Triton X-100, 0.05% NaN3*), blocking buffer, wash buffer, or other appropriate blocker to the needed final concentration depending on the assay, according to the antibody provider instructions, or as determined by user. Adjust the total volume of the antibody solutions based on the number of targets and number of wells being tested.

Note: Optimal concentrations for primary antibodies should be determined by each laboratory for each assay. The 1X solutions should be prepared prior to use. The diluted primary antibodies can be stored at 4°C for up to two weeks.

12. Add 50 µl of 1x primary antibodies (target-specific antibody and/or anti-normalization protein antibody) to the corresponding wells, cover with Parafilm and incubate for 16 hours (overnight) at 4°C. If the target expression is known to be high, incubate for 2 hours at room temperature with gentle shaking on the shaker.

Note: The two primary antibodies must be from different host sources (e.g. rabbit target Ab and mouse normalization antibody), so that the secondary antibodies must discriminate and not cross react between the

hosts of the primary antibody. This may be ignored if your anti-target and anti-normalization protein primary antibodies are applied in different wells.

Note: For accuracy, repeat in duplicate or in triplicate.

Normalization Antibody is used to detect the internal **positive controls** (a housekeeping GAPDH protein is typical in cell based ELISA kits) for normalization of OD values of the target protein.

If a **phosphorylated target** is being detected, an antibody against the phospho-target is being used, and two normalization antibodies: 1) antibody against the total (non-phosphorylated) target protein, and 2) Anti-GAPDH Antibody.

To determine the background signal (which can later be subtracted from all measured data/from all other readings), you may include **negative controls** on this step by omitting primary antibody from at least one well containing cells for each experimental condition and detector antibody used (e.g. add only Primary Antibody Diluent or blocking buffer, or TBS, or wash buffer, etc., or add a non-specific immunoglobulin).

13. Wash 3 times with 200 µl of Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker. After the last wash, invert the plate and gently tap the plate on absorbent papers to remove residual fluid. Proceed immediately to the next step. Do not allow the plate to air dry between steps.

Secondary Antibody Incubation

14. Dilute the enzyme-conjugated secondary antibodies stock solutions if needed with antibody diluent (1% BSA, 0.5% Triton X-100, 0.05% NaN3), blocking buffer, wash buffer, or other appropriate blocker to the required final concentration depending on the assay, according to the antibody manufacturer's instructions, or as determined by the user. Adjust the total volume of the antibodies solutions based on the number of targets and number of wells being tested.

The two most widely used enzymes for signal detection are horse radish peroxidase (HRP) and alkaline phosphatase (AP).

Note: Optimal concentrations for secondary antibodies should be determined by each laboratory for each assay. The 1X solutions should be prepared prior to use. The diluted antibodies can be stored at 4°C for up to two weeks.

15. Pipette 50 µL of the diluted and tempered secondary antibody solutions to corresponding wells and incubate for 1.5 hours at room temperature with gentle shaking on the shaker.

Note: The Secondary Antibody solutions are added into each well including the negative control wells where primary antibodies were omitted.

Highlight: HRP is known to be more robust than AP. HRP-conjugated secondary antibodies are recommended to be used for detecting less abundant proteins, while AP-conjugated secondary antibodies can be used with primary antibodies detecting more abundant proteins.

Substrate Preparation & Incubation

16. During the secondary antibody incubation, prepare the substrate solution. If substrate solution is provided pre-made and ready-to-use, transfer the necessary amount according to the number of assayed wells to another container and bring the solution to room temperature. Otherwise, prepare the substrate solution immediately before use and bring it to room-temperature.

The following table presents the most widely used chromogenic and fluorogenic substrates for HRP and AP commonly applied in ELISAs with their corresponding stopping solutions, detection absorbance or excitation/emission wavelengths, color developed if available, etc.:

Enzyme	Substrate Type	Substrate	Substrate Chemical Name	Stop Solution	Detection Wavelength (nm)	Color Development	Additional Details
HRP	Chromogenic	TMB	3,3’,5,5’-tetramethylbenzidine	1M/2M H2SO4	A450	Yellow	TMB is the most popular chromogenic substrate for detecting HRP activity, notably in ELISA. TMB produces upon reaction with peroxidase a blue color, which is measured at 370 nm or 652 nm, and yellow color (stopped reaction after adding an acid - measured at 450 nm). Sensitivity is greater than classic substrates like OPD and ABTS with very low background.
HRP	Chromogenic	OPD	o-Phenylenediamine		A490	Purple	
HRP	Chromogenic	ABTS	(2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate)		A450	Green	
HRP	Fluorogenic/Chromogenic	ADHP	10-Acetyl-3,7-dihydroxyphenoxazine		Ex/ Em = 531/595 A576±5		ADHP is considered to be the most stable and sensitive fluorogenic substrate for horseradish peroxidase (HRP). It has

							been used to detect as low as femtomolar (10-15 M) concentration of primary antibodies in an ELISA format. Its signal can be easily read by either a fluorescence microplate reader or an absorbance microplate reader at the corresponding wavelegths. ε = 54 000
HRP	Fluorogenic	HPPA	3-(p-Hydroxyphenyl)propionic acid		Ex/Em = 325/404		
AP	Chromogenic	pNPP	p-nitrophenyl-phosphate	0.75M NaOH	A405	Yellow	pNPP is a preferred substrate for high sensitivity detection of alkaline phosphatase in ELISA assays
AP	Fluorogenic	MUP	4-Methylumbelliferyl Phosphate		Ex/Em= 360/440		
AP	Fluorogenic	FDP	fluorescein diphosphate		Ex/Em= 490/514	Green	FDP is the most sensitive fluorogenic alkaline phosphatase substrate; widespread in fluorescence-detected ELISAs; However FDP is not very

							thermally stable, shows a sequential alkaline phosphatase mediated hydrolysis, and requires special precautions for storing. ε ≈ 90,000

17. Before adding the substrate, wash 3 times with 200 µl of Wash Buffer for 5 minutes at a time, with gentle shaking on the shaker. After the last wash, invert the plate and gently tap the plate on absorbent papers to remove residual fluid. Proceed immediately to the next step. Do not allow the plate to air dry between steps.
18. Add 50 µl of Substrate solution to each well and incubate for 30 minutes at room temperature in the dark with gentle shaking on the shaker.

The incubation time may vary (15-45 min) depending on the cell number, antibody affinity and the concentration of the target protein.

Warning: Both chromogenic and fluorogenic substrates are light-sensitive. Keep away from light.

In a typical cell-based ELISA kit, a blue color will be developed upon substrate (TMB) addition that can be directly read at 650 nm, or the reaction can be stopped after the incubation time passes, by adding stop solution and absorbance read at 450 nm, as described in the next step.

Signal Detection

19. Add 50 µl of Stop Solution (depending on which enzyme conjugate you are using) to each well and read the signal at the respective wavelength immediately using the absorbance or fluorescence microplate reader (depending on the type of the selected substrate).

In a typical cell-based ELISA kit, the acidic stop solution turns the blue color of the HRP-TMB reaction product of the previous step into yellow. Make sure that each well develops for the same amount of time. Read the absorbance as soon as possible (within max 30 minutes of stopping the reaction) under a microplate reader at 450nm with optional reference wavelength of 665nm. Before reading, clean the bottom of the plate using a lint-free wipe to remove moisture or fingerprints that could interfere with accurate data collection. The use of a stop solution generating a yellow product produces 2-4 fold increase in sensitivity compared to the soluble blue product generated directly upon substrate addition.

Reaction Stopping Solution provides convenience and control by allowing the signal-generating reaction to be terminated at a user-determined time point. After the addition of the stop reagent, the signal remains stable.

Note: Prior to each use, transfer the amount of Stop Solution required for the assay into a secondary container. After use, discard any remaining Stop Solution in the secondary container.

Warning: Sulfuric Acid (H_2SO_4) and sodium hydroxide (NaOH) are corrosive. Wear personal protection equipment, i.e. lab coat, gloves and eye protection.

Optional: Crystal Violet Whole Cell Staining

The following protocol is for colorimetric measurement of the cell density.

Crystal Violet binds to DNA in cell nuclei and gives absorbance readings proportional to cell counts at 595 nm. If normalization of OD value to cell number variation is desired, follow the protocol listed below.

20. After finishing reading the absorbance at the respective wavelength, wash the plate twice with 200 µl of Wash Buffer and twice with 200 µl of 1x TBS for 5 minutes each. Tap the plates on paper towel to remove the excess liquid. Let plate air dry for 5 minutes at room temperature.
21. Add 50 µl of Crystal Violet Solution (0.05% Crystal violet, 0.5% Ethanol) to each well, incubate for 30 minutes at room temperature on the shaker.

Warning: Crystal Violet is an intense stain. Avoid contact with skin and clothing.

22. Tip off Crystal Violet solution into beaker. Wash plate by dipping into bucket of water in the sink with the water continuing to run. Carefully rinse the wells in distilled water until no more color comes off the wells. Allow the plate to dry for 30 minutes.
23. Add 100 µl of 1% SDS Solution into each well and incubate on the shaker at room temperature for 1 hour. SDS Solution is used in the Crystal Violet procedure to solubilize cells and release the dye for subsequent quantification at 595 nm.
24. Read absorbance at 595 nm with microplate reader. OD595 value is proportional to cell numbers. If absorbance is too high, the solubilized Crystal Violet Solution can be diluted 10 times with H2O on a separate 96-well plate.

Data Analysis

Background Correction

Correct the raw cell-based-ELISA signal for the background signal by subtracting the signal of well(s) incubated in the absence of the primary antibody (secondary antibody alone) for each experimental condition from all other readings of the same experimental type/treatment.

Crystal Violet Staining Normalization

To account for differences in cell numbers in various wells, normalize the target-specific signal (OD450) from each well to the whole cell number signal (the OD595 Crystal Violet staining signal) by dividing the background-corrected OD450 values by the corresponding background-corrected OD595 values: OD450/OD595.

GAPDH Normalization

The OD450 values obtained for the target protein can be normalized using the OD450 values obtained for GAPDH.

If a phosphorylated target is being detected, the OD450 values obtained for both the phosphorylated and non-phosphorylated target protein can be normalized using the OD450 values obtained for GAPDH.

Anti-non-phosphorylated target protein Antibody Normalization

The OD values obtained for the phosphorylated target protein can be normalized using the OD values obtained for the non-phosphorylated target protein via the proportion, OD450 (Anti-phospho-protein Antibody)/OD450 (Anti-non-phospho-protein Antibody).