ELISA “Do-it-yourself” Kit

User Manual

Supplemental reagents and plates for developing quantitative ELISA

This product is for research use only and is not intended for diagnostic use.

CYTO DIAGNOSTICS

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Overview

Enzyme-linked immunosorbent assay (ELISA) is used to detect and quantify the target antigen from a sample of complex mixtures. To develop an ELISA, the necessary steps are to select the ELISA format, collect the components required for the assay, and determine an effective assay protocol.

Cyto's ELISA "Do-it-yourself" kit contains a comprehensive collection of supplemental reagents and plates, providing an economical way to develop and perform different formats of colorimetric ELISA with reduced preparation time. The kit contains the necessary reagents and materials for developing all formats of ELISA using an HRP-TMB based signal amplification system. The reagents have been extensively validated for various analytes and ELISA formats. The diluents and buffers are carefully formulated and aseptically filtered through a 0.2-micron filter into clean, pre-sterilized containers to ensure consistent performance and prolonged shelf life. The provided 96-well strip microplates with 12 breakable strips of 8 wells have a high binding surface, suitable for the passive adsorption of antibodies and antigens with a molecular weight greater than 30 kDa.

Different formats of ELISA development

The fundamental factor to consider for ELISA development is to select the assay format suitable for the antigen of interest or the desired sensitivity. Depending on the way of capturing and detecting the target antigen, there are four major types of ELISA- direct, indirect, sandwich, and competitive ELISA.

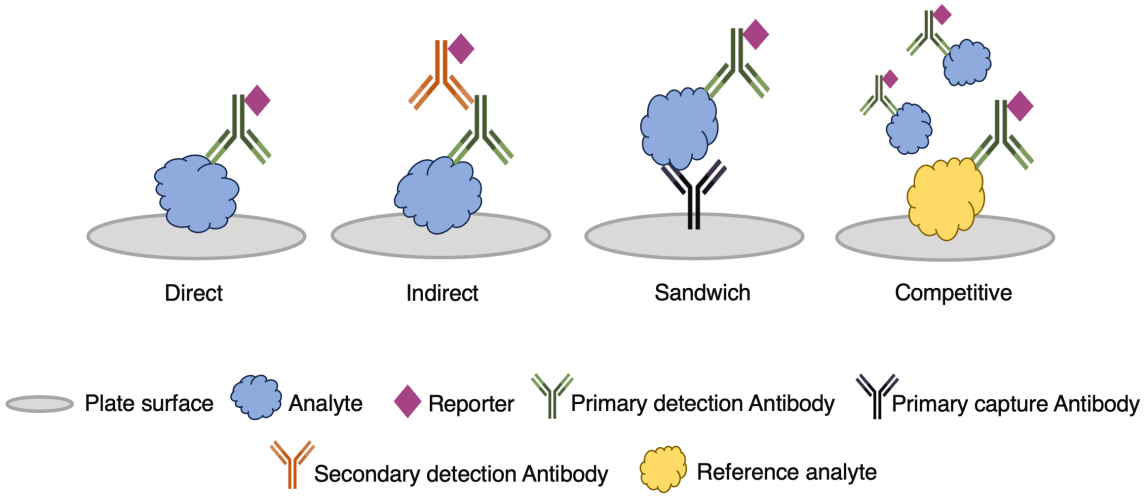

Figure 1: Schematic of different formats of ELISA

Both direct and indirect ELISA techniques involve a target antigen-coated plate surface. This antigen is detected either by a labelled primary antibody (direct ELISA), or a primary antibody bound to a labelled secondary antibody (indirect ELISA).

The term "sandwich" in the following example, suggests the use of two layers of primary antibodies; capture, and detection (can be either direct or indirect or indirect detection). The sandwich ELISA is known as the most robust and sensitive format of the assay.

In a competitive ELISA, also known as an inhibition ELISA, the presence of the target antigen is measured by the signal inhibition or interference resulting from the target antigen competing with a reference antigen to bind to the detection primary antibody. This generates an assay signal that is inversely proportional to the target antigen concentration. Each ELISA type can be implemented and adjusted into a competitive format.

Each format of ELISA offers different level of preference over the others, depending on the objective of detection, reagent availability, time requirement, efficiency, and simplicity of the assay.

Table 1: Brief description of the suitability and challenges of different types of ELISA:

Format	Suitability	Challenges
Direct ELISA	Applicable for simple and fast detection of immunological response to high molecular weight antigens	Labeled primary antibody is required, making it time-consuming, expensive and a rare assay format
Indirect ELISA	Commonly used to quantify total antibody concentration in samples; highly sensitive and more versatile than direct ELISA	With the need of a labeled secondary antibody, possesses risk of cross-reactivity and non-specific binding
Sandwich ELISA	Ability to detect low-to-high molecular weight antigens from complex samples with high sensitivity and specificity	Difficult to optimize a mutually compatible antibody pair for antigen recognition; chance of cross-reactivity between the capture and detection antibodies
Competitive ELISA	Suitable for low concentration and low molecular weight antigens with a limited number of antibody-binding sites, e.g., small molecules	Requirement of a reference antigen contributes to the complexity of protocol development and optimization

Storage and Stability

Store the kit at 4°C immediately upon receipt (unless specified). Kit has a storage time of 6 months from receipt, providing components have not been reconstituted. Observe the storage conditions for individual reconstituted materials in the Reagent Preparation section.

Materials Supplied

Item	Quantity	Storage Condition
Clear 96-well strip microplate [EC-003-05]	5 plates	N/A
1X Coating Buffer [EL-C9-50ML]	150 mL	4°C
1X Stabilizer and Blocking Solution [EL-C1-50ML]	300 mL	4°C
10X Wash Buffer [EL-C2-50ML]	250 mL	4°C
1X Assay Diluent [EL-C3-50ML]	250 mL	4°C
1X Detection Diluent [EL-C4-50ML]	250 mL	4°C
1X One-step TMB Substrate	60 mL	4°C
1X Stop Solution [EL-C8-50ML]	100 mL	4°C
Adhesive Plate Covers	15 units	N/A

Required Materials (not supplied)

The following materials are not included in the kit, but will be required to perform the assay:

- Primary antibodies
- Standard protein
- HRP-conjugate or Secondary antibody-HRP
- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes and pipette tips to deliver 2 µL to 1 mL volumes
- Adjustable 1-25 mL pipettes and pump
- Absorbent paper
- Distilled or deionized water
- Log-log graph paper or computer and software for ELISA data analysis
- Tubes to prepare standard or sample dilutions

Precautions

- Please read the instructions carefully before using the kit
- All kit components have been formulated and quality tested to function successfully as a kit.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not alter reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

Technical Hints

- Kit components are provided in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Ensure plates are properly sealed or covered during incubation steps.
- Keep the standard dilutions on ice while during preparation, but the ELISA procedure should be performed at room temperature.
- Samples generating absorbance values higher than the highest standard should be further diluted in the appropriate diluent.
- Avoid foaming or bubbles when mixing components or adding reagents to the assay well.
- Complete removal of all solutions and buffers during wash steps.
- Do not let the wells dry out between washes or reagent addition steps.

Reagent preparation

- Equilibrate all reagents to room temperature prior to use. The kit contains enough reagents for five 96-well microplates.
- Prepare only as much reagent as is needed on the day of the experiment.
- Prepare 1X Wash Buffer with deionized or distilled water. If the 10X Wash buffer concentrate contains visible crystals, equilibrate to room temperature, and mix gently until dissolved.
- Prepare coating solution of antibody or antigen using the 1X Coating buffer
- If performing Sandwich ELISA, prepare standard protein dilutions in 1X Assay Diluent
- Prepare primary Detection antibody, and Secondary antibody-HRP or HRP conjugate dilutions using 1X Detection Diluent

Standard Preparation

- Prepare serially diluted standards using 1X Coating buffer (if Direct or Indirect ELISA), or 1X Assay Diluent (for Sandwich ELISA) immediately prior to use. Always prepare a fresh set of standards for every use.
- Keep the standard dilutions on ice while during preparation, but the ELISA procedure should be done at room temperature.
- It is recommended to prepare a seven-point standard curve with a minimum of two replicates using 2-fold serial dilutions

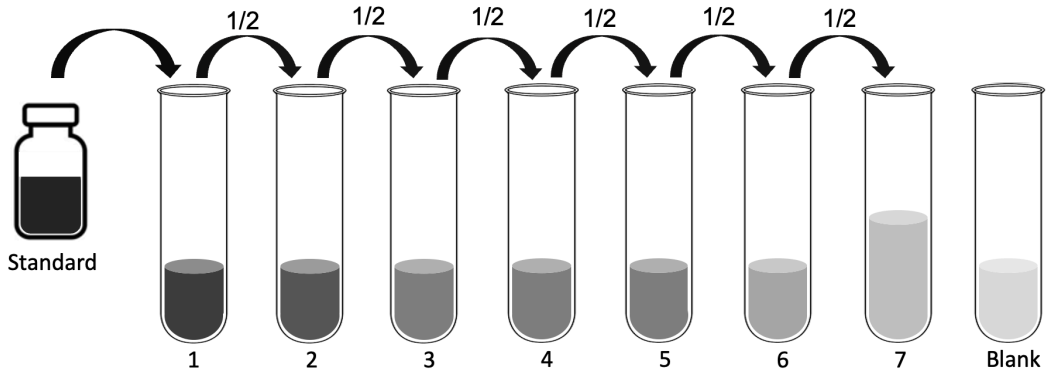

Figure 2: Serial dilution schematic. One tube will contain only diluent without standard protein as the Blank control.

Sample Preparation

- If samples need to be diluted, Assay Diluent should be used for dilution.
- Suggested dilution for normal serum/plasma samples: 10-fold. The concentration measured from the standard curve needs to be multiplied by the dilution factor.
- Samples generating values higher than the highest standard should be further diluted in the appropriate diluents.
- Please note that levels of the target analyte may vary between different specimens. Optimal dilution factors for each sample must be determined by the investigator.

Recommended Assay Protocol

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate. Avoid foaming or bubbles when mixing or applying samples in the wells.

Direct ELISA

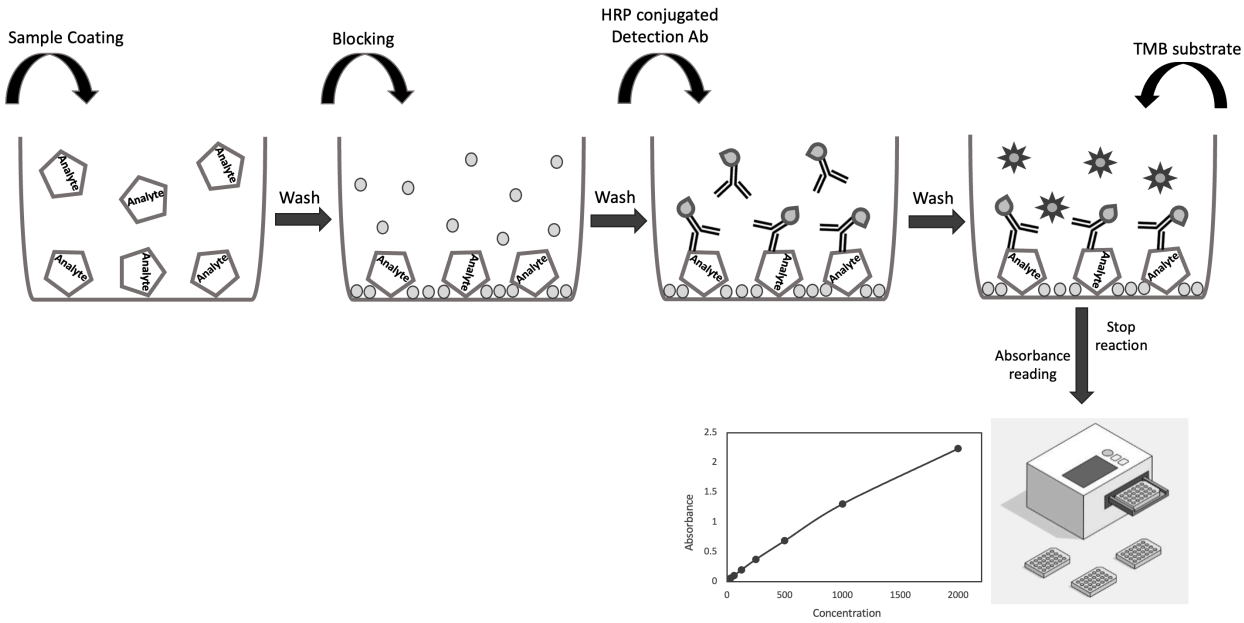

Figure 3: Direct ELISA protocol workflow

The basic protocol of a direct ELISA is as follows:

1. Dilute the target analyte into the 1X Coating Buffer. Optimal coating concentration and standard dilution points to be investigated by the user.
2. Properly mix the solution and add 50-200 µL in each well of the microplate.
3. Incubate the coating solution from 8-24 hours at room temperature protected from light.
4. Minimize evaporation by individually covering the plate with an adhesive sealer.
5. After coating, aspirate the coating solution and wash wells 2-3 times with 300 µL of 1X Wash Buffer to remove the free reagents from the coated analytes.
6. Block the remaining unoccupied binding sites on plate surface with 300 µL of 1X Stabilizer and Blocking Solution for at least 1 hour at room temperature.
7. Aspirate blocking solution and add 100 µL of HRP conjugated anti-analyte primary antibody for detection. Optimal detection antibody concentration to be investigated by the user.
8. Cover the plate and incubate for 1-2 hours at room temperature. After incubation, wash the wells 3-5 times with 300 µL of 1X Wash buffer and aspirate properly.
9. Add 100 µL of TMB substrate to each well and incubate at room temperature in the dark to develop colored product that is proportional to the level of target analyte.
   Note: Stop solution may need to be added sooner if blue color develops rapidly.
10. Add 50-100 µL of Stop Solution to each well, which will turn the blue color to yellow. Measure absorbance at 450 nm and 540 nm immediately.

Indirect ELISA

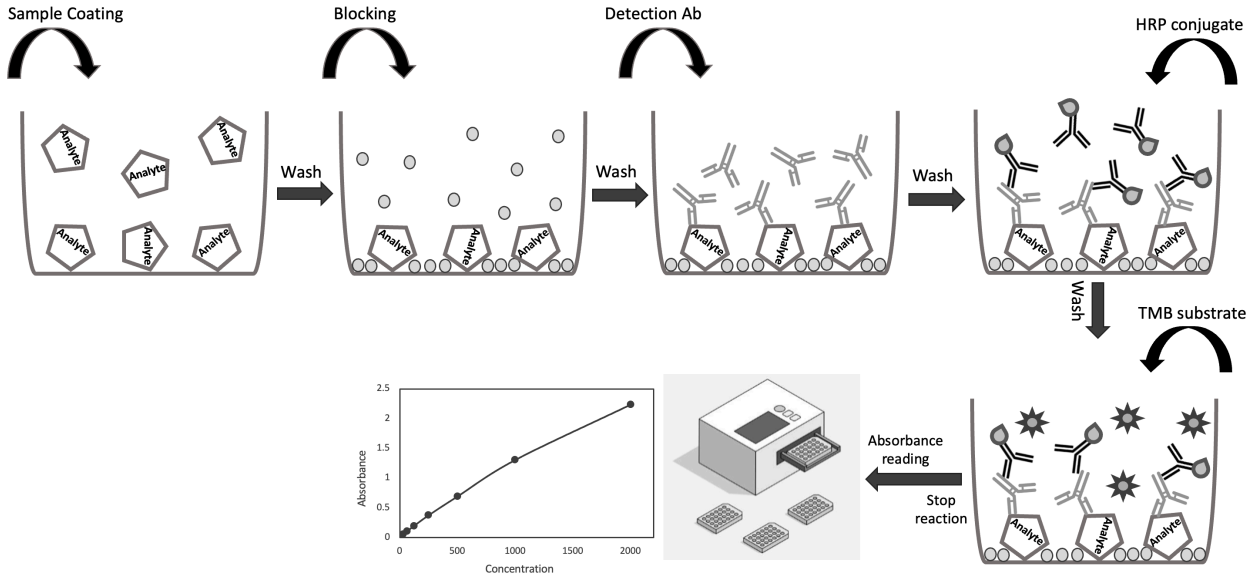

Figure 4: Indirect ELISA protocol workflow

The basic protocol of an indirect ELISA is as follows:

1. Dilute the target analyte into the 1X Coating Buffer. Optimal coating concentration and standard dilution points to be investigated by the user.
2. Properly mix the solution and add 50-200 µL in each well of the microplate.
3. Incubate the coating solution from 8-24 hours at room temperature protected from light.
4. Minimize evaporation by individually covering the plate with an adhesive sealer.
5. After coating, aspirate the coating solution, wash wells 2-3 times with 300 µL of 1X Wash Buffer to remove the free reagents from the coated analytes.
6. Block the remaining unoccupied binding sites on plate surface with 300 µL of 1X Stabilizer and Blocking Solution for at least 1 hour at room temperature.
7. Aspirate blocking solution and add 100 µL of anti-analyte primary antibody for detection. Optimal detection antibody concentration to be investigated by the user.
8. Cover the plate and incubate for 1-2 hours at room temperature. After incubation, wash the wells 3-4 times with 300 µL of 1X Wash buffer and aspirate properly.
9. Add 100 µL of HRP conjugated secondary antibody that can recognise the anti-analyte primary detection antibody. Optimal HRP conjugate concentration to be investigated by the user.
10. Cover the plate and incubate for 1-2 hours at room temperature. After incubation, wash the wells 3-5 times with 300 µL of 1X Wash buffer and aspirate properly.
11. Add 100 µL of TMB substrate to each well and incubate at room temperature in the dark to develop colored product that is proportional to the level of target analyte.
    Note: Stop solution may need to be added sooner if blue color develops rapidly.
12. Add 50-100 µL of Stop Solution to each well, which will turn the blue color to yellow. Measure absorbance at 450 nm and 540 nm immediately.

Sandwich ELISA

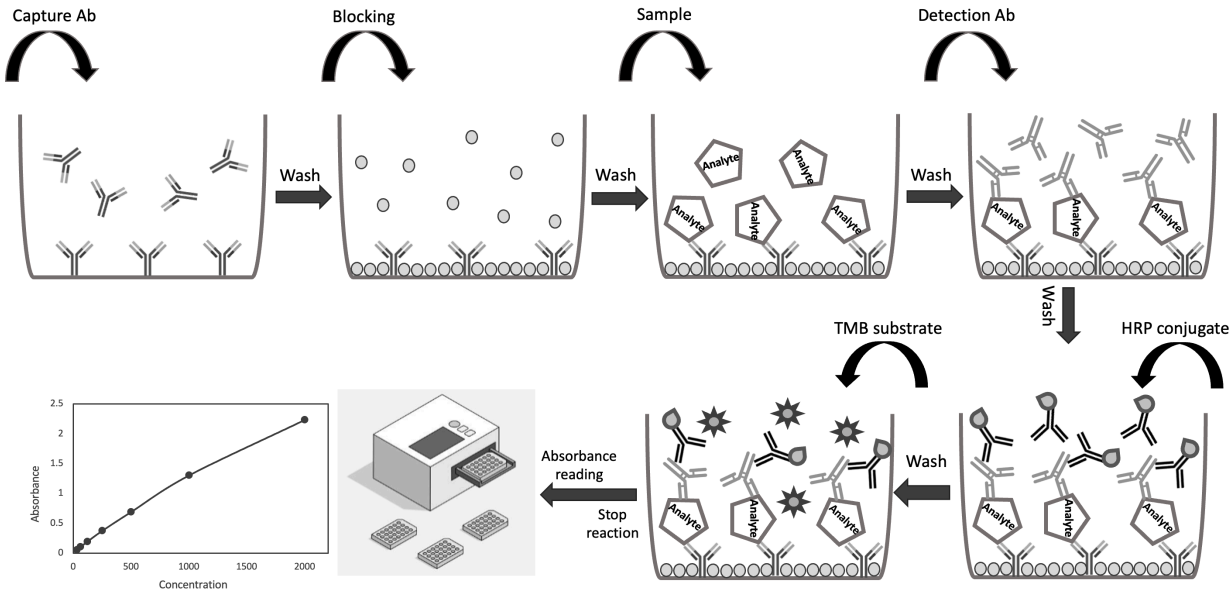

Figure 5: Sandwich ELISA protocol workflow

The basic protocol of a sandwich ELISA is as follows:

1. Dilute the anti-analyte primary capture antibody into the 1X Coating Buffer. Optimal capture primary antibody coating concentration to be investigated by the user.
2. Properly mix the solution and add 50-200 µL in each well of the microplate.
3. Incubate the coating solution from 8-24 hours at room temperature protected from light.
4. Minimize evaporation by individually covering the plate with an adhesive sealer.
5. After coating, aspirate the coating solution, wash wells 2-3 times with 300 µL of 1X Wash Buffer to remove the excess unbound capture primary antibody.
6. Block the remaining unoccupied binding sites on plate surface with 300 µL of 1X Stabilizer and Blocking Solution for at least 1 hour at room temperature.
7. Aspirate blocking solution and add 50-100 µL of analyte (or standard curve dilution points) to the dedicated coated wells. Optimal standard dilution points to be investigated by the user.
13. Cover the plate and incubate for 1-2 hours at room temperature. After incubation, wash the wells 3-4 times with 300 µL of 1X Wash buffer to remove the free reagents from the captured analytes.
8. Add 100 µL of anti-analyte primary detection antibody. Optimal detection antibody concentration to be investigated by the user.
9. Cover the plate and incubate for 1-2 hours at room temperature. After incubation, wash the wells 3-4 times with 300 µL of 1X Wash buffer and aspirate properly.
10. If the primary detection antibody is HRP conjugated, move to step 13.
11. If the primary detection antibody is not HRP conjugated, add 100 µL HRP conjugated secondary antibody (or Streptavidin-HRP if primary detection antibody is biotinylated) that can recognise the anti-analyte primary detection antibody. Optimal HRP conjugate concentration to be investigated by the user.

12. Cover the plate and incubate for 1-2 hours at room temperature. After incubation, wash the wells 3-5 times with 300 µL of 1X Wash buffer and aspirate properly.
13. Add 100 µL of TMB substrate to each well and incubate at room temperature in the dark to develop colored product that is proportional to the level of target analyte.
    Note: Stop solution may need to be added sooner if blue color develops rapidly.
14. Add 50-100 µL of Stop Solution to each well, which will turn the blue color to yellow. Measure absorbance at 450 nm and 540 nm immediately.

Competitive ELISA

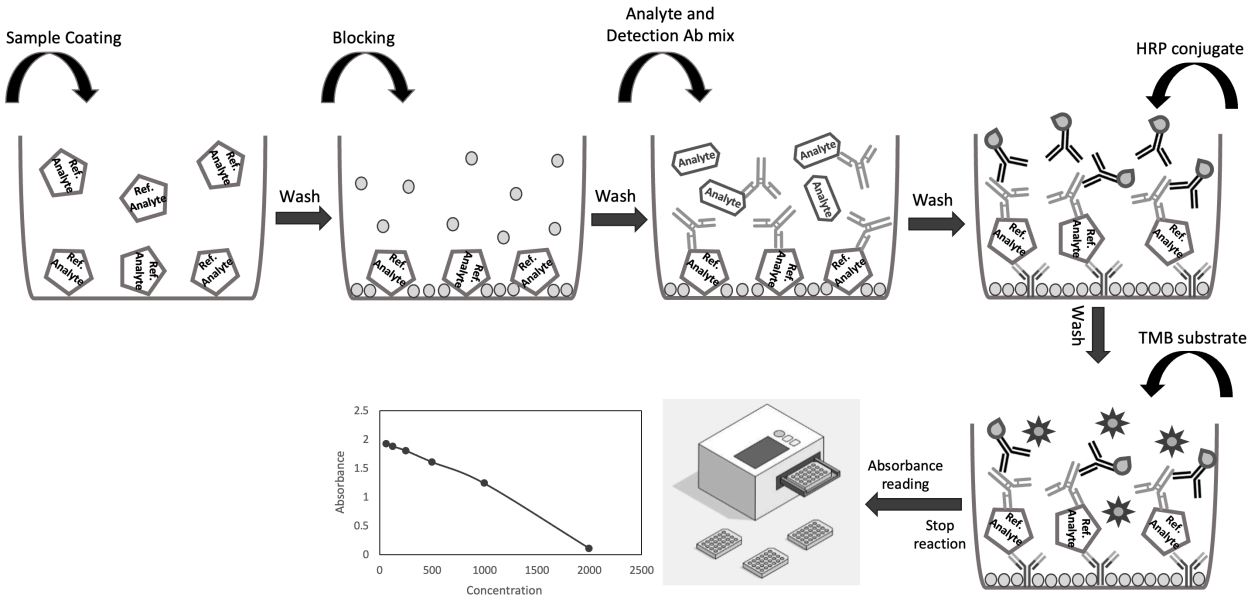

Figure 6: Competitive ELISA protocol workflow

The basic protocol of a competitive ELISA is as follows:

1. Dilute the reference analyte into the 1X Coating Buffer. Optimal coating concentration to be investigated by the user.
2. Properly mix the solution and add 50-200 µL in each well of the microplate.
3. Incubate the coating solution from 8-24 hours at room temperature protected from light.
4. Minimize evaporation by individually covering the plate with an adhesive sealer.
5. After coating, aspirate the coating solution, wash wells 2-3 times with 300 µL of 1X Wash Buffer to remove the free reagents from the coated analytes.
6. Block the remaining unoccupied binding sites on plate surface with 300 µL of 1X Stabilizer and Blocking Solution for at least 1 hour at room temperature.
7. In separate microtubes, prepare sample analyte and primary antibody mixture by adding 50 µL of analyte (or standard curve dilution points) to 50 µL of antibody for each well in the assay. Optimal standard dilution points and primary antibody concentration to be investigated by the user. Incubate for 1 hour at room temperature.

8. Aspirate blocking solution from the microplate wells and add 100 µL of the pre-incubated mixture to the dedicated wells. Cover the plate and incubate for 1 hour at room temperature.
9. After incubation, wash the wells 3-4 times with 300 µL of 1X Wash buffer to remove the free reagents from the bound primary antibody.
10. If the primary detection antibody is HRP conjugated, move to step 13.
15. If the primary detection antibody is not HRP conjugated, add 100 µL HRP conjugated secondary antibody (or Streptavidin-HRP if primary detection antibody is biotinylated) that can recognise the anti-analyte primary detection antibody. Optimal HRP conjugate concentration to be investigated by the user.
11. Cover the plate and incubate for 1-2 hours at room temperature. After incubation, wash the wells 3-5 times with 300 µL of 1X Wash buffer and aspirate properly.
12. Add 100 µL of TMB substrate to each well and incubate at room temperature in the dark to develop colored product that is proportional to the level of target analyte.
    Note: Stop solution may need to be added sooner if blue color develops rapidly.
13. Add 50-100 µL of Stop Solution to each well, which will turn the blue color to yellow. Measure absorbance at 450 nm and 540 nm immediately.

Data Analysis

Analyze the data as described below.

- Subtract readings at 540 nm from the readings at 450 nm.
- Calculate the mean absorbance for each set of duplicate standards, controls, and samples, and subtract the average Blank absorbance value.
- Plot the standard curve on log-log graph paper, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit trend line through the standard points.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Troubleshooting

Problem	Reason	Solution
Poor standard curve	– Inaccurate pipetting – Improper standards dilution	– Check pipettes – Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	– Incubation times too brief – Inadequate reagent volumes or improper dilution	– Ensure sufficient incubation times; change to overnight standard/sample incubation – Check pipettes and ensure correct preparation
Large CV%	– Inaccurate pipetting	– Check pipettes
High background	– Plate is insufficiently washed – Contaminated wash buffer	– Review manual for proper wash technique. If using a plate washer, check all ports for obstructions – Prepare fresh wash buffer
Low sensitivity	– Improper storage of the ELISA kit	– Store the reconstituted protein at -80°C, all other assay components 4°C. Keep substrate solution protected from light.

Please contact our Technical Support team for more information.