proteintech®

ELISA
GUIDE

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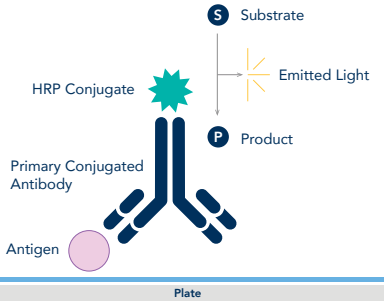
ELISA

INTRODUCTION

ELISAs, also known as Enzyme-linked immunosorbent assays, are the gold standard for the quantitative analysis of biological samples. Their quick and simple protocols make them easy to automate for high-throughput analysis. Initially developed in the 1970s, ELISAs are now widely used in both research and clinical settings.

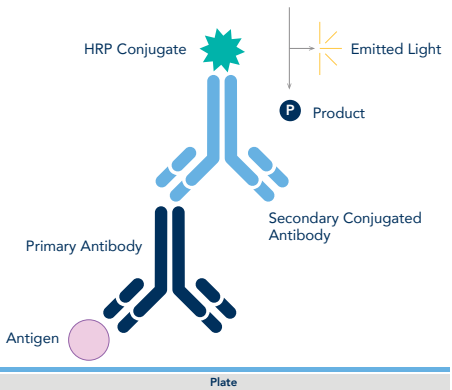
Direct ELISA

Direct ELISAs are the easiest to set up and feature a protocol with fewer steps and reagents compared to other ELISAs. In this assay, plates are simply coated with antigen and then detected with antigen-specific antibodies that are either conjugated to HRP or some other detection molecule.



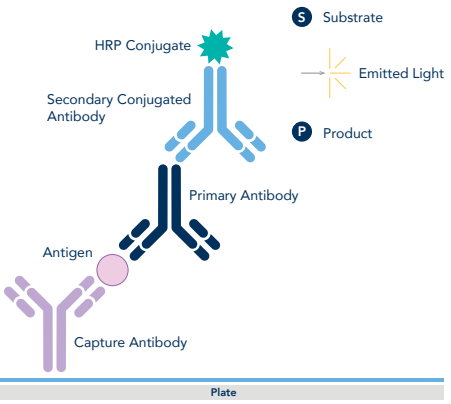
Indirect ELISA

Like a Direct ELISA, an Indirect ELISA also starts with coating a plate with antigens. However, detection is a two-step process that involves a primary antibody followed by an enzyme-conjugated secondary antibody. This method offers increased sensitivity and is most useful for determining total antibody concentration within a sample.



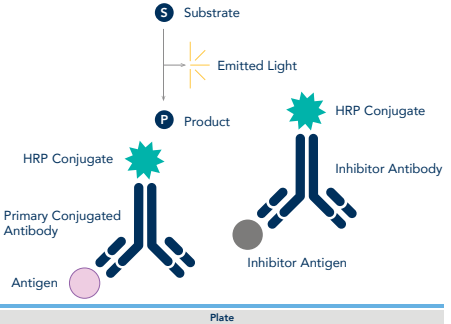
Sandwich ELISA

The sandwich ELISA is the most used ELISA. It relies upon two antibodies, commonly referred to as a matched antibody pair, to recognize the antigen at two different sites. This results in a highly sensitive assay that is best for complex sample types as the antigen of interest does not need to be purified from sample prior to the assay.



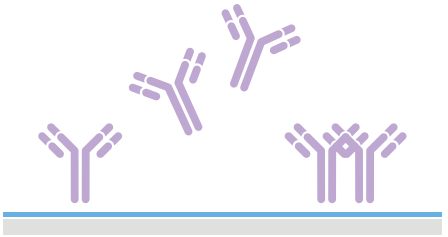
Competitive ELISA

The competitive or competition ELISA is useful for detection of either low abundant antigens or antigens that cannot be bound by antibodies at two different sites. In this assay, plates are coated with a reference/inhibitor antigen, followed by addition of the sample and enzyme conjugated antibody. The sample and reference antigens compete for binding with the antibody, meaning that signal strength will be inversely proportional to amount of antigen present in a sample.



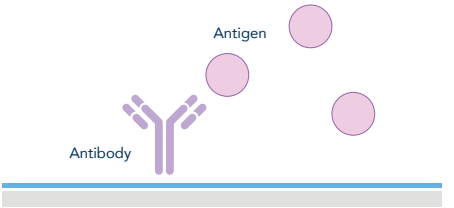
All Proteintech ELISA products are sandwich ELISAs. Learn more and discover our full offering here: https://www.ptglab.com/products/elisa-kits/

Sandwich ELISA Process



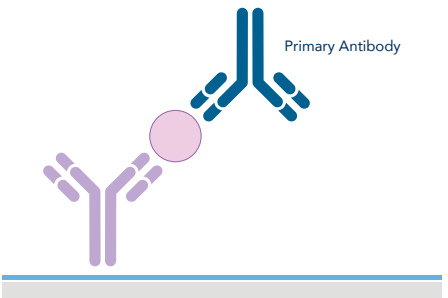
Step 1

Coat Plate with Capture Antibodies and Blocking Reagent



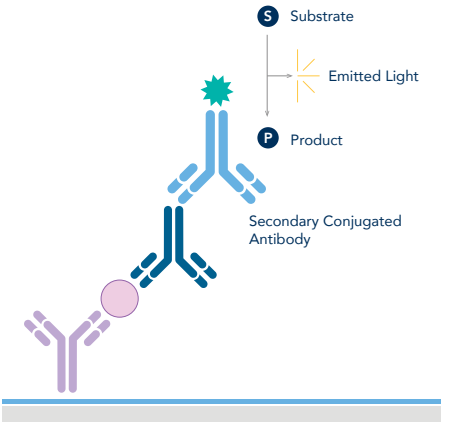
Step 2

Add Samples, Controls, and Standards



Step 3

Add Detection Antibody



Step 4

Add Conjugated-Secondary Antibody Followed by Substrate for Signal Detection

ELISA
BEST PRACTICES

Remember that ELISAs are simply a chemical reaction. Assay performance can be reproducible and reliable if the reaction is allowed to perform in the correct conditions. These conditions can be achieved through pre-experiment planning and proper technique execution during the experiment itself.

Experimental Planning
(Always Plan Ahead)

Verify Reagent Availability
Ensure you have enough of each reagent to complete the planned experiment by calculating sample and reagent volume ahead of time. Make sure all reagents are from the same lot. Mixing lots has the potential to increase variability which could reduce the reliability of the assay.

Map Out Your Plate
Use a template to layout the planned locations of your standards, blanks, controls, and samples. For best statistical analysis, it is recommended to run samples in triplicates while having minimum duplicates of standards and controls.

Sample, Standard, and Reagent Preparation
For consistent assay performance and optimal results, it is important that all assay components are at ideal conditions needed for chemical reactions to occur.

As such, it is important to bring all reagents to room temperature before starting. Some reagents may contain temperature-sensitive components that can come out of the solution when cold. Using reagents at room temperature ensures a more homogenous mixture that will facilitate consistent intra assay performance.

To streamline assay operation, calculate sample dilutions ahead of time.

Experimental Execution

(Pipetting, Washing, and Incubation)

Pipetting

Poor pipette technique is one of the main reasons for variability both between and within assays. Always keep the following things in mind while adding various samples, standards, and reagents during an ELISA.

- Pipette at an angle and avoid touching the bottom of the well as this may dislodge bound antibodies and antigens
- Use multichannel pipettes wherever possible to ensure consistent volumes in each of the wells
- Always use new tips for different samples and standards to reduce cross contamination
- Avoid bubbles wherever possible as this can result in incorrect transferred volume and increased data variability
- Reverse pipette when dealing with viscous samples to avoid blowing out the sample, as this can cause air bubbles

Plate Washing

Poor washing is a common cause of high background, reduced sensitivity, and assay variability. To avoid these issues, it is recommended to use an automated plate washer. The addition of a 30 second rest period after wash buffer addition can also improve the removal of unbound antibodies and antigens. Finally, after each wash step, firmly tap the plates to get rid of as much excess wash buffer as possible.

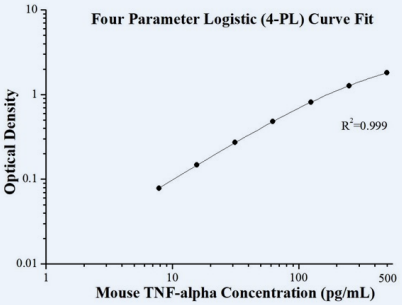
Incubation Time

Remember that ELISAs are simply a series of chemical reactions and sufficient incubation time is needed for each reaction to reach equilibrium. Always follow the suggested protocol recommended by the manufacturer and use all provided/recommended reagents. If incubating multiple plates, do not stack them, as this can cause edge effects due to changes in temperature distribution across a stacked plate.

ELISA
DATA ANALYSIS

The Importance of the Standard Curve

The standard curve allows you to compare your samples to standards of a known concentration. Through using a set of standards ranging from the undetectable to maximum detectable signal of an antibody, a curve can be fit to the standard data which will in turn help you to extrapolate sample concentrations.



Standard Calibration

For best statistical relevance, run all standards in duplicate or triplicate. Understand pipetting precision by calculating the coefficient of variation (CV) between replicates of the same standard. A CV value of greater than 20% could be indicative of pipetting errors, contaminated plate/reagents, uneven temperature distribution, or evaporation during the incubation steps. A lower CV in 10-15% range indicates a good deal of uniformity between standard and sample replicates. To determine background absorbance, add a few blank sample wells. Finally, it is recommended to generate a new standard curve for each plate to correct any differences in pipetting between assay operators and fluctuations in incubation temperatures.

Curve Fitting Strategies

A curve can be fit to a set of standard values through various regression models that utilize a goodness-of-fit measure, known as the R2 value, to explain how well a set of data fits a particular curve. The higher the R2 value (max 1), the higher the percentage of variance that can be explained by the model. The R2 value is calculated by taking the sum of squared residual values, which are the distances between each data point and the mapped curve. Finding a slope that minimizes the distance between each data point and curve will, in theory, give you a higher R2 value.

Linear Regression

This is the simplest form of curve fitting; a line is simply drawn through the points with a slope that minimizes the residual distance between each point to the curve. This goodness-of-fit measure, known as the R2 value, helps to explain how well the dataset fits a particular curve. The higher the R2 value (max 1), the higher the percentage of variance that can be explained by the model. However, this model has a tendency to compress data at lower points on the curve, making it unideal for a wider set of values.

Semi-Log Regression

In this model, the concentration is mapped on a logged scale, which results in a sigmoidal curve. While semi-log regression can better plot a wider range of standard concentrations more evenly, it can sometimes distort the relationship between variables and is not a universal solution for all non-linear relationships.

Log-Log Plots

In a log-log plot, both axes (concentration and optical density) are logged, allowing for visualization of even larger ranges of data. However, this form of regression is not ideal for a smaller set of standard values, as it may obscure smaller details and create a false impression of correlation between standard concentration and optical density.

4 Parameter Logistic (4PL)

The 4PL model most closely matches the non-linear dynamics of biology. It estimates a set of four parameters that help a dataset best fit a "S" shaped curve. With 4PL modeling, you have increased flexibility to work with non-linear datasets and can accurately characterize a full range of responses from baseline to saturation.

Proteintech's ELISA Calculator

Proteintech's ELISA Calculator is a free-to-use, online tool that will take all the hassle out of ELISA data analysis and standard curve modeling. Simply upload your unedited OD data, set the concentration of your standards, and import your plate layout. The tool will create a standard curve for you using the curve fitting method of your choice and automatically extrapolate sample concentrations. Save time on your next experiment and try our ELISA calculator today!

View ELISA Calculator

ELISA

DEVELOPMENT AND VALIDATION STRATEGIES

The are four key areas for consideration when developing ELISAs

1. Antibody Immobilization

Most ELISAs involve some sort of antibody immobilization to a plate. When doing this, it is important to ensure that immobilization does not affect an antibody's ability to bind antigen. Additionally, the choice of plate also matters. Polystyrene plates are recommended, as they have the best capacity of passively absorbing antibodies and proteins. Make sure the color of the plate is compatible with the detection method. Clear plates are best suited for colorimetric detection, while fluorescent and chemiluminescent detection systems are more compatible with black and white plates respectively.

View Matched Antibody Pair Offering

2. Antibody Pair Design and Selection

If designing your own antibody pair, it is critical that the capture and detection antibodies do not have any overlapping epitopes or else the assay won't be very robust. To ensure lot-to-lot consistency, easy scalability, and long-term security of supply, use antibodies that can be easily reproduced, such as recombinant monoclonals. For detection, consider if you would rather use a directly labeled primary or indirectly labeled secondary antibody.

To save time, consider trying out one of Proteintech's Matched Antibody Pairs. These pairs have been created using our in-house proprietary rabbit recombinant and mouse monoclonal technology and have additionally been validated for use in multiplex bead arrays and sandwich ELISAs. Proteintech offers the largest selection of antibody pairs in the industry, with over 3400 combinations in stock.

3. Antibody/Sample Dilution

A checkerboard titration strategy can help to determine optimal ratio of antibody to sample concentration. Spike and recovery can also be used to assess the effect of sample matrix on antibody binding with the analyte. You also want to ensure that the sample matrix does not cause high background and signal to noise ratio, both of which could interfere with the quality of results.

4. Blocking and Wash Buffers

All blocking and washing buffers to be used in the assay should have minimal cross reactivity with any proteins in the sample. They also shouldn't have any enzymes that might interfere with the reaction kinetics. Make sure the salts and detergents in the buffer are of an optimal concentration. A high concentration of detergents and salts can disrupt antigen antibody binding and protein structure. Low detergent concentration can cause high background noise.

Validation Strategies

Spike and Recovery

The spike and recovery strategy assesses the effect of sample matrix on antibody binding to the analyte and in turn determines the accuracy of an assay. In this approach, a known amount of recombinant protein is spiked into the sample matrix. The antibody is then tested for its ability to recover the spiked protein in the sample. For an assay to be deemed reliable and accurate, the recovery of the spiked protein must be within 80-120% of the known or expected concentration. Poor recovery is often a result of background interference and non-specific binding material. It can be corrected by either diluting the sample in diluent that matches the matrix type or diluting the sample matrix itself.

Sample Type		Average% of Expected	Range (%)
Human plasma	1:2	109	103-117
	1:4	94	82-101
Cell culture supernatant	1:16	96	85-106
	1:32	94	88-97

Example Spike and Recovery Results from AuthentiKine Human TNF ELISA Kit (KE00154)

Linearity of Dilution

Linearity of dilution helps to determine the working range of an assay. In this strategy, a sample is serially diluted in a chosen sample diluent and then spiked with a high concentration of recombinant protein. The ability of the assay antibodies to recover the spiked protein is then tested in each of the dilutions. Acceptable linearity (80-120%) over a large range of dilutions means that the assay can be used to identify an analyte at a broad range of concentrations.

		Human Plasma (Sample Diluent PT 1-ec)	Cell Culture Supernatant (Sample Diluent PT 6)
1:2	Average% of Expected	98	98
	Range (%)	90-106	80-112
1:4	Average% of Expected	91	97
	Range (%)	85-96	86-107
1:8	Average% of Expected	93	100
	Range (%)	92-94	95-103
1:16	Average% of Expected	97	99
	Range (%)	93-101	82-109

Example Linearity of Dilution Data from AuthentiKine Human TNF-alpha ELISA Kit (KE00154)

ELISA

COMMERCIAL OFFERINGS FROM PROTEINTECH

Using a commercially available, ready-to-use ELISA kit offers several advantages compared with building a kit on your own. Pre-coated ELISA kits typically include matched and validated antibody pairs along with a set of standards to create a standard curve. Every step in commercial ELISA development is rigorously quality controlled to ensure the highest levels of consistency, accuracy, and reliability between product lots. Using a commercially available kit also removes a lot of user variation that may occur in plate coating and antibody dilution.

Proteintech offers over 1200 ELISA kits against a broad range of human, mouse, and rat targets. Each of our ELISA kits has been developed and extensively validated in house with relevant physiological samples. Sample dilutions have also been pre-determined, and the kits come in a strip-plate format, meaning that an entire plate does not need to be used if you are working with a smaller number of samples.

AuthentiKine™ ELISA Kits

In addition to our traditional ELISA kits, Proteintech also offers AuthentiKine™ ELISA kits which are made using our HumanKine® growth factors and cytokines as immunogens and kit standards. HumanKine proteins are some of the highest quality immunogens in the industry, featuring native glycosylation and folding. As such, these kits are 10-1000x more sensitive than offerings from leading competitors and can measure even the most minute quantities of cytokine in a sample. As such, they are ideal for quantifying of low abundance analytes.

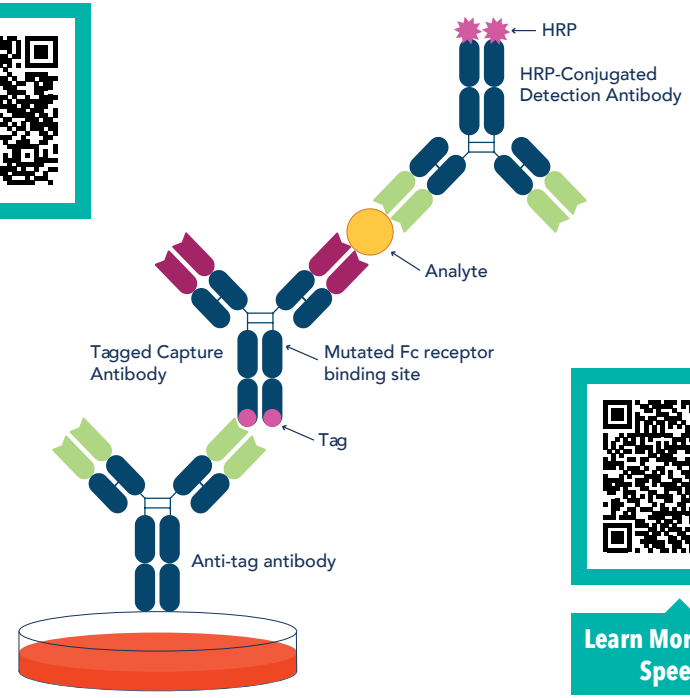
Learn More About Authentikine

Speedy One-Step ELISA Kits

The Speedy Kit is Proteintech's one-step ELISA solution for rapid analyte detection. These kits are made with antibody pairs containing FcZero-backbone capture antibodies, which help to eliminate non-specific binding with Fc receptors that may be present in some serum and plasma samples.

Speedy Kit Advantages:

- Results in 90 minutes or less
- Reduced assay complexity with single wash-step protocols
- Unparalleled precision and sensitivity
- Unmatched cost savings



Learn More About Speedy

ELISA
TROUBLESHOOTING TIPS

High Signal

Cause	Solution
Plate left out too long before reading	Read plate immediately after adding stop solution to prevent signal over development.
Sample readouts are greater than the highest standard	To get samples within the range of the standard curve, you may need to dilute them further in sample buffer.
Contaminated glassware	Always use sterile glassware to prevent any cross contamination and background.
Insufficient Washing	Allow wash buffer to sit in wells for at least 30 seconds to facilitate removal of unbound components. Firmly tap plates after each wash step to get rid of as much wash buffer as possible.
Low salt concentration in wash buffer	Increase salt concentration to reduce background.

Out-of-Range Results

Cause	Solution
Analyte is below detectable range of the assay	Assay is not sensitive enough, consider incorporating a more sensitive detection system or a different assay product all together.
Analyte higher than highest standard	Dilute samples further to get within the detectable range.
Incorrect dilutions prepared	Use the sample dilutions recommended in the protocol as the manufacturer has determined the optimal conditions for assay performance.
Substrate mixed too early	ELISAs are simply a chemical reaction. Only add the substrate 15-20 minutes before you are ready to read the plate and do not overdevelop.
Contaminated Buffers	Always use a fresh pipette tip when drawing liquid out of a buffer bottle or pour buffer into a reagent tray if repeated usage is necessary.

High Variation

Cause	Solution
Uneven volume in each well	Use multichannel pipettes to ensure an even volume of reagents are dispensed into each well and calibrate pipettes regularly to ensure they are dispensing the desired volume.
Non-uniform plate washing	Use plate washers for best results and include a rest step (~30 secs) between each wash.
Cross-well contamination	Use new plate sealers for each experiment.
Bubbles in wells	Pop bubbles before reading, do not blow out pipettes while dispensing samples and reagents.
Stacked plates during incubation	Placing plates in a single layer allows for an even temperature distribution across all wells.
Edge effects	Ensure all reagents are at room temperature before starting and use plate sealers. Peripheral wells tend to respond first to changes in temperature or humidity, leading to drying out and evaporation.

High Background

Cause	Solution
Sample matrix interference	Consider diluting the matrix to reduce the effect of non-specific material in the sample.
Antibody cross-reactivity and non-specific binding	Dilute the sample matrix or consider using a standard diluent that matches the matrix.
Cross well contamination	Use new plate sealers to prevent condensates from falling into the wells.
Insufficient washing	Use a plate washer for the most consistent results.
High or low room temperature	An ambient room temperature of 18-25°C is recommended for chemical reactions to proceed optimally. Take note of the room temperature if you start noticing variations in results across several days.

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