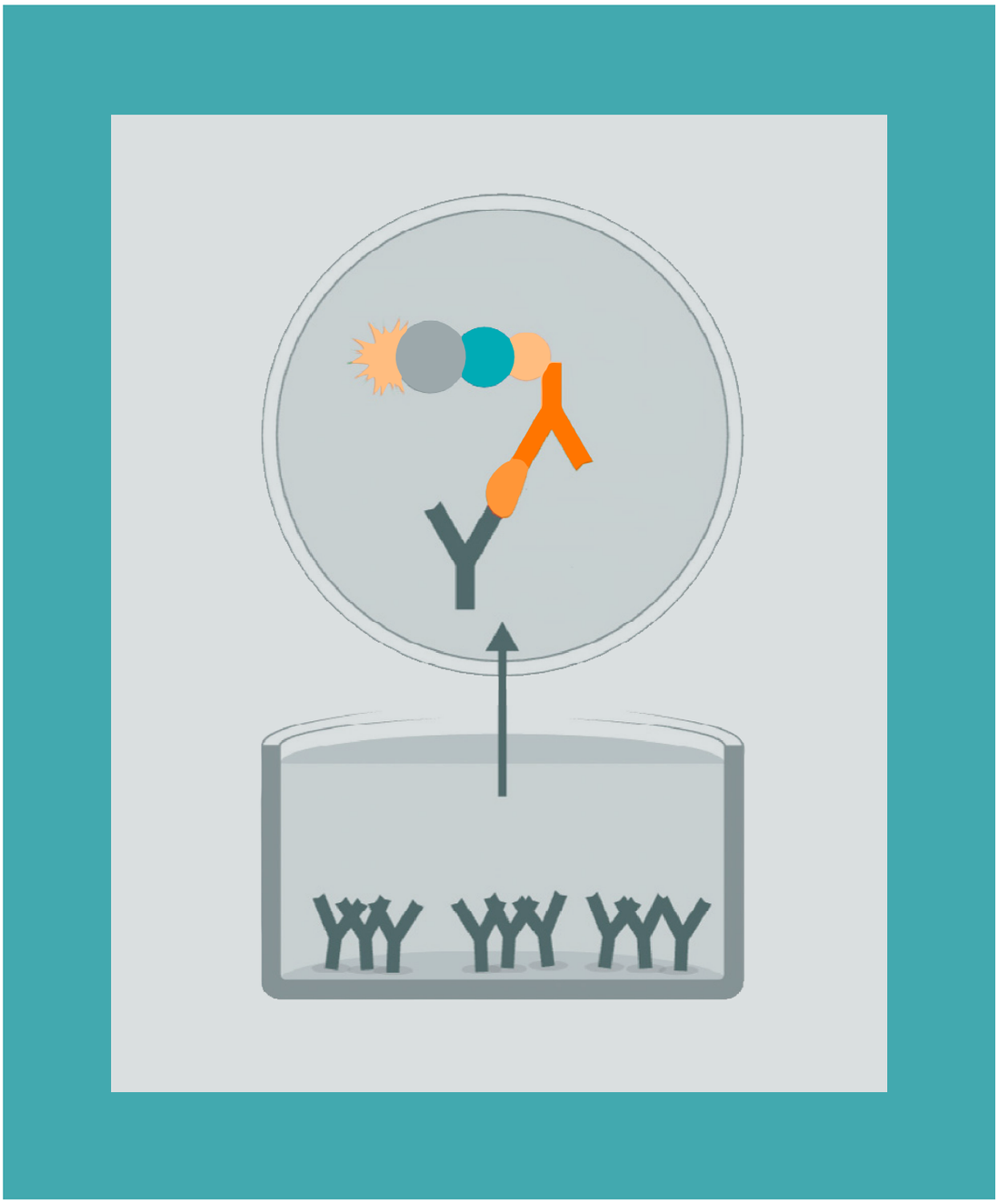
ELISA guide

Everything you need to perform your ELISA experiments



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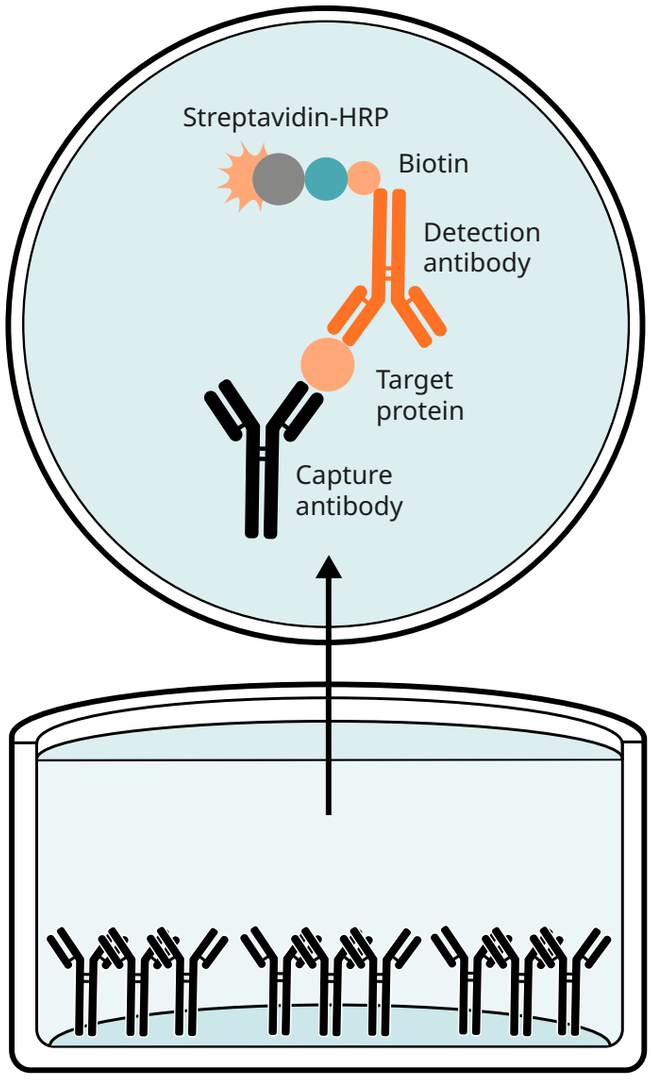
Principles of ELISA

ELISA stands for enzyme-linked immunosorbent assay, also often referred to as enzyme immunoassay (EIA). An ELISA, like other types of immunoassays, relies on antibodies to detect a target antigen using highly specific antibody-antigen interactions. In an ELISA assay, the antigen must be immobilized to a solid surface. This is done either directly or via the use of a capture antibody itself immobilized on the surface. The antigen is then complexed to a detection antibody conjugated with a molecule amenable for detection such as an enzyme or a fluorophore.

An ELISA is typically performed in a multi-well plate (96- or 384-wells), which provides a solid surface to immobilize the antigen. The immobilization of analytes facilitates the separation of the antigen from the rest of the components in the sample, making ELISA one of the easiest assays to perform on multiple samples simultaneously.



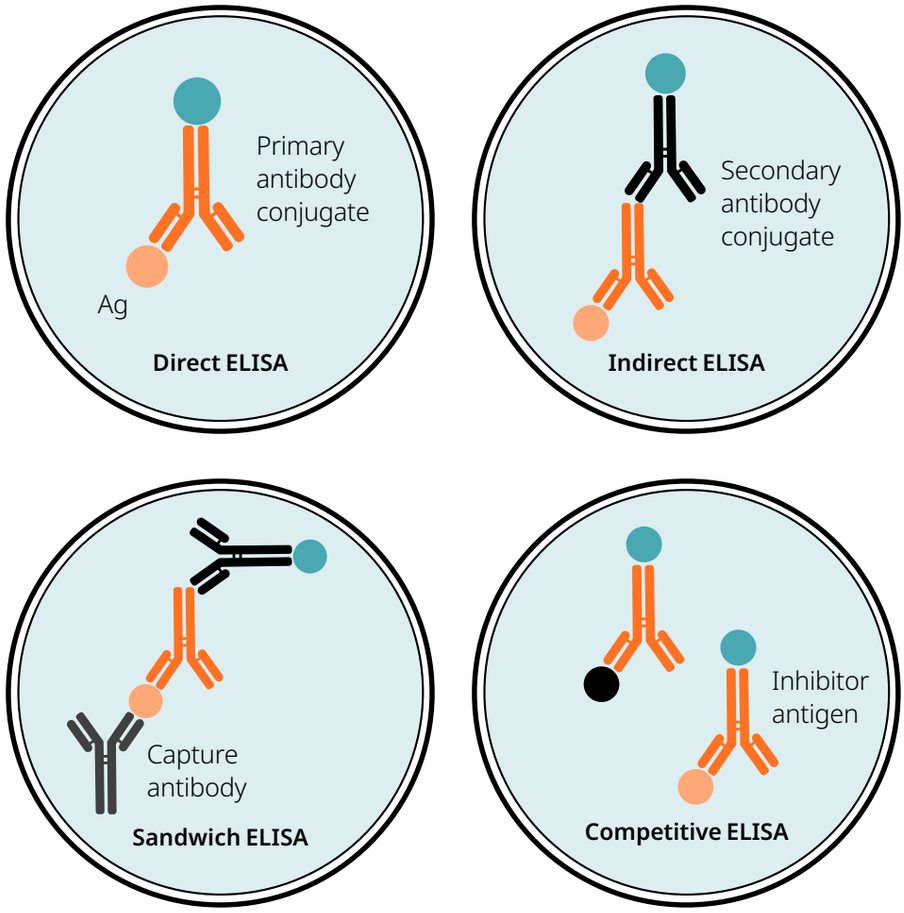
Advantages and disadvantages of ELISA

Advantages	Disadvantages
High sensitivity and specificity: it is common for ELISAs to detect antigens at the picogram level in a very specific manner due to the use of antibodies.	Temporary readouts: detection is based on enzyme/substrate reactions and therefore readout must be obtained in a short time span.
High throughput: commercial ELISA kits are normally available in a 96-well plate format. However, the assay can be easily adapted to 384-well plates.	Limited antigen information: information limited to amount or presence of the antigen in the sample.
Easy to perform: protocols are easy to follow and involve little hands-on time.	
Quantitative: it can determine the concentration of antigen in a sample.	
May be used to test various sample types: serum, plasma, cellular and tissue extracts, urine, and saliva, among others.	

These are the general ELISA advantages and disadvantages. There are other advantages and disadvantages depending on the type of ELISA used as explained in the next section.

Types of ELISA

ELISAs come in different formats, each one with its own advantages and disadvantages. The diagram below illustrates the four main different types of ELISA.



Direct ELISA

The antigen is immobilized on the surface of the multi-well plate and detected with an antibody specific to the antigen that is directly conjugated to HRP or another detection molecule.

Indirect ELISA

Similar to direct ELISA assays, the antigen is immobilized on the surface of the multi-well plate. However, a two-step process is required for detection whereby a primary antibody specific for the antigen binds to the target, and a labeled secondary antibody against the host species of the primary antibody binds to the primary antibody for detection.

This method can also be used to detect specific antibodies in a serum sample by substituting the serum for the primary antibody.

See all indirect ELISA kits at **www.abcam.com/indirect-ELISA-kits**

Sandwich ELISA

Sandwich ELISA (or sandwich immunoassay), the most commonly used ELISA format, requires two antibodies specific to different epitopes of the antigen. These two antibodies are commonly referred to as matched antibody pairs. One of the antibodies is used to coat the surface of the multi-well plate where it serves as a capture antibody to facilitate the immobilization of the antigen. The other antibody is conjugated and facilitates the detection of the antigen.

See all sandwich ELISA kits at **www.abcam.com/sandwich-ELISA-kits**

SimpleStep ELISA® Kits

Our SimpleStep ELISA® kits provides improved speed and performance while retaining the familiar process and standard data output of a traditional ELISA kit. SimpleStep ELISA® kits reduce the number of wash steps by enabling the sandwich complex formation in one step rather than sequentially.
Total time required is less than two hours.

See all SimpleStep ELISA® kits at **www.abcam.com/simplestep-ELISA-kits**

Competitive ELISA

Also known as inhibition ELISA or competitive immunoassay, this assay measures an antigen's concentration by detecting signal interference. Each of the previous formats can be adapted to the competitive format. The sample antigen competes with a reference antigen for binding to a specific amount of labeled antibody. The reference antigen is pre-coated on a multi-well plate. The sample is added to the plate wells with a labeled antibody. This assay orientation is referred to as "antigen down." Depending on the amount of antigen in the sample, more or fewer free antibodies will be available to bind the reference antigen. This means that the more of the antigen there is in the sample, the less reference antigen will be detected and the weaker the signal will be.

Some competitive ELISA kits use labeled antigens instead of labeled antibodies, an assay orientation referred to as "antibody down". In this orientation, the labeled antigen and the sample antigen (unlabeled) compete for binding to the primary antibody, which is immobilized on the plate. The lower the amount of antigen in the sample, the stronger the signal due to more labelled antigen in the well.

See all competitive ELISA kits at **www.abcam.com/competitive-ELISA**

Competitive SimpleStep ELISA® Kits

Our Competitive SimpleStep ELISA® Kits provide improved speed and performance while retaining the familiar process and standard data outputs of a traditional competitive ELISA® kit. Competitive SimpleStep ELISA® kits reduce the number of wash steps by enabling the antibody analyte complex formation in one step rather than sequentially.

The total time required is less than two hours.

See all competitive SimpleStep ELISA® kits at **www.abcam.com/simplestep-ELISA-kits**

Advantages and disadvantages of the different types of ELISA

	Advantages	Disadvantages
Direct ELISA	Short protocol: saves time and reagents. No cross-reactivity from secondary antibody.	Potential high background: all proteins in the sample bind to the surface. No signal amplification. Low flexibility: the primary antibody must be conjugated.
Indirect ELISA	Signal amplification: several secondary antibodies will bind to the primary antibody. High flexibility: the same secondary antibody may be used for several primary antibodies.	Long protocol if compared to direct ELISA. Potential cross-reactivity from secondary antibody.
Sandwich ELISA	High specificity: involves two antibodies detecting different epitopes on the same antigen. Suitable for complex samples. High flexibility and sensitivity: both direct and indirect methods can be used.	Demanding design: finding two antibodies against the same target that recognize different epitopes and work well together can be challenging at times.
Competitive ELISA	Suitable for small molecules and short peptides. Matrix effects are minimized: only one antibody used.	The competing antigen must be conjugated. Demanding design: potential cross-reactivity from antibodies with other small molecules. Protocols can be more complicated and time-consuming than standard ELISA Lower-concentration samples can be more sensitive to variance in OD between replicates.

SimpleStep ELISA® kits

Save time without compromising on performance. Upgrade your ELISA for specific and sensitive results with a single-wash protocol.

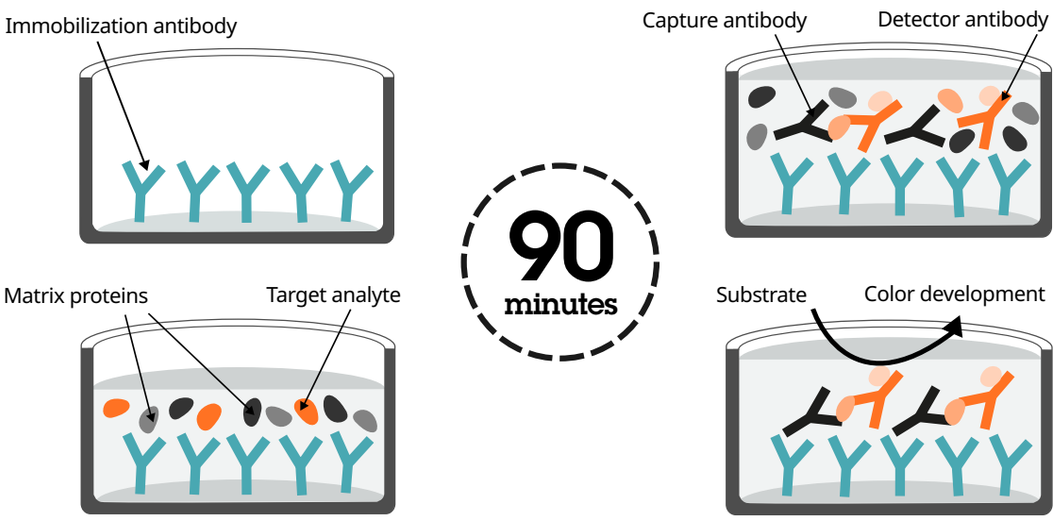
- Single-wash protocol reduces assay time to 90 minutes or less
- Comparable or better sensitivity than competitor assays
- Fully validated in biological samples
- No special equipment required

Your ELISA upgrade:

	Conventional ELISA kits	**SimpleStep ELISA® kits**	
Sensitivity	✓✓	✓✓✓✓	Liquid-phase reaction system drives higher sensitivity
Reproducibility	✓✓	✓✓✓✓	Recombinant antibodies provide better reproducibility
Full validation	✓	✓✓✓✓	Validated on several biological samples to ensure reaction with the endogenous protein
Fast and easy protocol		✓✓✓✓	One-wash 90-minute protocol for fewer handling steps

One-wash 90-minute protocol

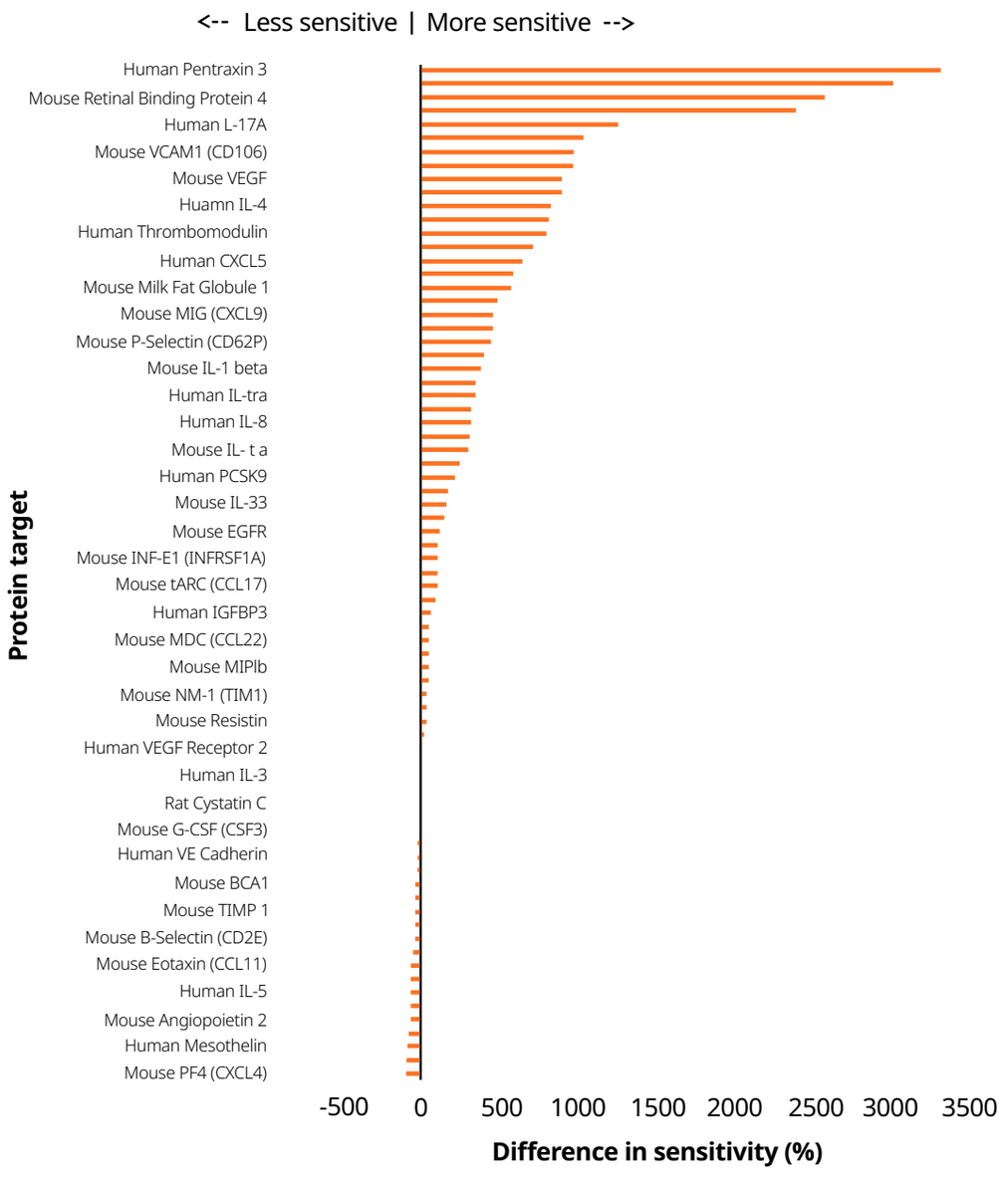
With SimpleStep ELISA® kits, an analyte-capture and detector antibody sandwich complex is formed in solution. In just one incubation and wash step, the complete sandwich complex forms in the well and is anchored to the plate with an immunoaffinity tag



Uncompromising on sensitivity

SimpleStep ELISA® kits drastically reduce assay times without compromising on performance. We compared the sensitivity of SimpleStep ELISA® kits to the most popular competitor brand of ELISA kits for 75 popular human and mouse proteins. SimpleStep ELISA® kits show superior sensitivity in 56 out of 75 cases.

Sensitivity: SimpleStep ELISA® vs competitor



Tested in biological samples

Every SimpleStep ELISA® kit is validated using multiple biological samples for assay specificity. All secreted serum or plasma-based targets are tested and fall within World Health Organization blood reference ranges. When available, the SimpleStep ELISA® kits are calibrated against known NIBSC international standards and includes a conversion factor for data comparison.

Wide range of analytes

We currently have SimpleStep ELISA® kits for almost 600 targets, including popular proteins like PD-L1, GFP, and fibrinogen, and are continually adding more to the catalog.

384-well SimpleStep ELISA® kits

Same principle, same results, higher throughput. Our SimpleStep ELISA® kits are now available in a 384-well plate so you can easily scale up your work. They use the same antibody pairs as our 96-well kits, and guarantee the same sensitivity, specificity, and reproducibility so you can transition to high-throughput with confidence. You can also get more done with less, running up to 4.5x the number of samples with only a quarter of the analyte.

CatchPoint® SimpleStep ELISA® kits

CatchPoint SimpleStep ELISA® kits have been developed using a fluorescent substrate to provide improved linearity over an extended dynamic range when compared to colorimetric ELISA kits. The extended dynamic range provides better quantification at both the lower and the upper end of the curve.

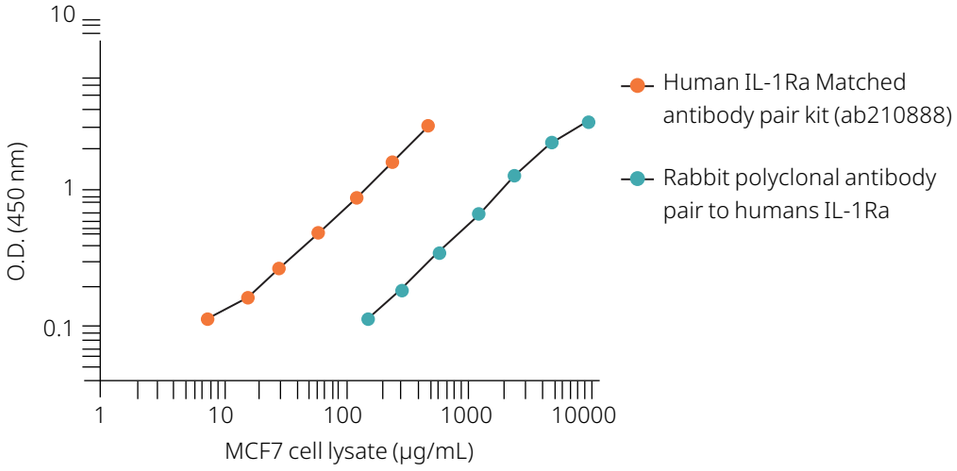
Find out more at **https://www.abcam.com/kits/simplestep-elisa-kits**

Matched antibody pair kits

Create your own ELISA with high-performing antibody pairs.

Matched antibody pair kits and reagents deliver consistent, specific, and sensitive results.

- Batch-to-batch consistency: only recombinant monoclonal antibodies are used in our matched antibody pairs.
- Specificity: antibody pairs are screened in plasma and serum to ensure specificity in complex samples.



- Sensitivity: benchmarked against commercially available antibody pairs to ensure equivalent or superior performance compared with the competition.

Matched antibody pair kits include a titrated capture and biotinylated detector antibody pair and a

calibrated protein standard. Kits are available in two sizes, with enough reagents for either 2 or 10 x 96-well plates using a standard sandwich ELISA.
The human IL-1Ra matched antibody pair kit (ab210888) is 10-fold more sensitive than a rabbit polyclonal antibody pair when measuring human IL-1Ra standard protein using a sandwich ELISA.

See all our matched antibody pairs at **www.abcam.com/matched-antibody-pairs**

Choose the right ELISA kit

Careful evaluation of assay performance is an important first step in choosing a new ELISA kit. Important parameters include sensitivity, dynamic range and precision, which are reported for most ELISA kits. Other parameters are more predictive of ELISA performance for typical sample types. Percent (%) recovery and linearity of dilution measure the target protein in real samples, such as plasma, serum or cell culture media.

This chapter explains how to interpret the different parameters to help you find the right ELISA kit for your sample.

Summary of parameters to look for when choosing an ELISA kit:

Parameter	Acceptance criteria
Sensitivity	Depends on the target protein*
Dynamic range	
CV (%) for intra-assay precision	≤ 10%
CV (%) for inter-assay precision	≤ 15%
Specificity	Check reactivity with highly homologous proteins
% Recovery	≥ 80%
Linearity of dilution	≤ 20% difference from undiluted sample

*It is important to know what levels of the target protein are expected in a sample to check that the sensitivity and dynamic range of a given ELISA kit are appropriate. Samples with high concentrations of the target protein can be diluted so that the raw signal falls within the dynamic range of the assay.

ELISA sensitivity

Sensitivity is the lowest amount of protein that the antibody pair used in the ELISA kit can detect. It should always be less than the lowest point of the respective standard curve.

ELISA dynamic range

The dynamic range is defined as the upper and lower concentrations of the target protein that the assay can accurately quantify.

Reported values for those two parameters can be misleading because they are often determined by using a standard protein in simple buffers. This may not reflect the kinetic of detection of an endogenous protein in a biological sample.

ELISA CV

The CV (%) or the coefficient of variability shows how consistent the assay is.

The CV is generally calculated to evaluate the inter-assay precision or plate-to-plate consistency and the intra-assay precision or consistency between duplicates run in the same experiment.

- Inter-assay % CVs of less than 15 are generally acceptable.
- Intra-assay % CVs should be less than 10.

ELISA specificity

It is important that the antibodies used in the ELISA kit do not cross-react with non-target proteins. These can be proteins of high homology across species.

For instance, Human Factor IX SimpleStep ELISA® Kit (ab188393), is specific of human Factor IX and does not react with mouse, rat, rabbit, goat, guinea pig, hamster, cow, dog, or pig sample.

ELISA percent recovery

Percentage recovery is determined by spiking a known amount of purified target protein into a biological sample type (also called sample matrix). For secreted proteins such as cytokines, typical sample matrices are plasma and serum; for intracellular proteins such as kinases, the samples used are cell culture lysates.

The spiked sample is measured in the ELISA, and the concentration is calculated from the standard curve. This calculated concentration is compared to the known concentration of the protein and is expressed as a percentage. For example, 100% recovery means that the observed concentration was the same as the actual concentration of spiked protein in the sample. This can be used to determine whether other proteins and molecules in the sample interfered with the quantification of the target protein.

If the percentage recovery measures less than 80% for a specific sample type, then a different ELISA kit should be selected for quantification.

ELISA linearity of dilution

Linearity of dilution is a good companion to percentage recovery because it measures the native rather than spiked protein in biological samples. Poor linearity of dilution indicates that the natural sample matrix, the sample diluent, and/or the standard diluent affect the detection of the protein differently.

Linearity of dilution is determined by measuring multiple dilutions of known positive samples by ELISA. The concentration of the target protein is determined by multiplying the dilution factor by the calculated concentration.

For the best results, the concentration of the samples should be similar for all dilutions. If a difference of more than 20% from the undiluted sample is observed, then a different ELISA kit should be chosen for accurate quantification.

Standard curve

The standard curve is used to determine the concentration of the sample used. A poor standard curve, such as one that is low or flat, may be indicative of an antibody that did not bind properly or did not capture the protein standard.
In general, a good standard curve should have an R^2 value above 0.99, a blank well optical density lower than 0.25, and a maximum absorbance value higher than 0.8.

Biological samples

Testing an ELISA with a biological sample ensures sensitivity to the endogenous protein, not just the recombinant protein.

Species cross-reactivity

Species cross-reactivity measures the extent to which an antibody pair binds to non-target proteins. This measure should ideally be below 5%.

International standard

Calibration to an international control enables cross-comparison between data sets. This helps you switch from one ELISA supplier to another one for the same ELISA kit.

ELISA sample preparation

Tips for preparing your sample before running an ELISA

Please refer to the protocol included with your kit for product-specific details regarding sample preparation and compatible sample types.

These guidelines are intended to be an educational resource for preparing commonly tested samples for use in ELISA assays. Optimum sample preparation procedures will vary depending on the target and assay of interest. When developing a new assay, it is always good practice to consult the literature for experimental examples similar to your own.

Sample preparation methods

Cell culture supernatant

- Pipette cell culture media into a centrifuge tube and centrifuge at 300 x g for 10 min at 4°C.
- Immediately aliquot supernatant and store samples at -80°C. Minimize freeze/thaw cycles.

Cell extract

- Place tissue culture plates on ice.
- Aspirate medium and gently wash cells once with ice-cold PBS.
- Aspirate PBS and add 0.5 mL complete extraction buffer per 100 mm plate.
- Scrape cells and tilt the plate to collect the cells, and place the cells in a pre-chilled tube.
- Vortex briefly and incubate on ice for 15–30 min.
- Centrifuge at 15,000-17,000 x g for 10 min at 4°C to pellet insoluble contents.
- Aliquot supernatant (this is the soluble cell extract) into clean chilled tubes on ice and store samples at -80°C. Minimize freeze/thaw cycles.

Conditioned medium

- Place cells in complete (serum-containing) growth medium and allow cells to proliferate to the desired level of confluence.
- Remove growth medium and wash very gently with warm PBS. Repeat the wash step at least twice.
- Remove any PBS and gently add serum-free growth medium.
- Incubate for 1-2 days.
- Pipette medium into a centrifuge tube and centrifuge at 300 x g for 10 min at 4°C.
- Immediately aliquot supernatant and store samples at -80°C. Minimize freeze/thaw cycles.

Milk

- Collect samples and centrifuge at 10,000 x g for 2 min at 4°C.
- Aliquot supernatant and store samples at -80°C. Minimize freeze/thaw cycles.

Plasma

- Collect whole blood into an anti-coagulant containing tube or add 0.1 M sodium citrate to 1/10 final volume.
- Centrifuge at 720 x g for 10 min at 4°C.
- Immediately aliquot supernatant (plasma) and store samples at -80°C. Minimize freeze/thaw cycles.

Urine

- Collect samples and centrifuge at 10,000 x g for 2 min at 4°C.
- Aliquot supernatant and store samples at -80°C. Minimize freeze/thaw cycles.

Saliva

- Collect samples and centrifuge at 10,000 x g for 2 min at 4°C.
- Aliquot supernatant and store samples at -80°C. Minimize freeze/thaw cycles.

Serum

- Collect whole blood in an untreated test tube or, for example, an anti-coagulant-free tube.
- Incubate undisturbed at room temperature for 20 min.
- Centrifuge at 720 x g for 10 min at 4°C.
- Immediately aliquot supernatant (serum) and store samples at -80°C. Minimize freeze/thaw cycles.

Tissue extract

- Dissect the tissue of interest with clean tools, preferably on ice and as quickly as possible to prevent degradation by proteases.
- Place the tissue in round bottom microfuge tubes and immerse the tubes in liquid nitrogen to snap freeze. Store samples at -80°C for later use or keep on ice for immediate homogenization.
- For a ~5 mg piece of tissue, add ~300 µL complete extraction buffer (see cell/tissue extraction buffer recipe) to the tube and homogenize with an electric homogenizer.
- Rinse the blade twice using 300 µL complete extraction buffer for each rinse, then maintain constant agitation for 2 h at 4°C (eg place on an orbital shaker in the cold room).
- Centrifuge for 20 min at 15,000-17,000 x g at 4°C. Place on ice, aliquot supernatant (this is the soluble protein extract) to a fresh, chilled tube and store samples at -80°C. Minimize freeze/thaw cycles.

Tip: The volumes of lysis buffer must be determined in relation to the amount of tissue present. The typical concentration of the final protein extract should be >1 mg/ml.

Cell/tissue extraction buffer recipe

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

- 0.5% Sodium deoxycholate

Additional reagents required to produce complete extraction buffer.

- Phosphatase inhibitor cocktail
- Protease inhibitor cocktail
- PMSF

Tip: Supplement the cell extraction buffer with phosphate and protease inhibitor cocktails as described by the manufacturer and add PMSF to a final concentration of 1 mM immediately before use.

General recommendations

- Recommended protein extract concentration is at least 1–2 mg/mL.
- Typically, serum, plasma, cell, and tissue extracts are diluted by 50% with binding buffer.
- Prior to use after thawing, centrifuge samples at 10,000 x g for 5 min at 4°C to remove any precipitate.

Control samples required for ELISA

Running the appropriate controls helps you to accurately separate true positive results from potentially false results. Positive and negative controls will also be useful if you ever need to troubleshoot your protocol. Here we explain the various types of control samples you should use when running an ELISA.

Positive control

Use either an endogenous soluble sample known to contain the protein you are detecting or a purified protein or peptide known to contain the immunogen sequence for the antibody you are using. A positive result from the positive control, even if the samples are negative, will indicate the procedure is optimized and working. It will verify that any negative results are valid.

We recommend checking the antibody datasheet, which will often provide a suggested positive control. If no control is suggested, we recommend the following:

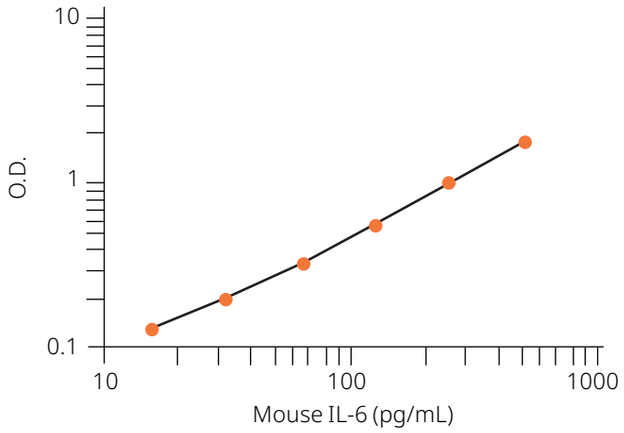
- Check to see if there are any Abcam product reviews for the antibody. Any tissues, cells, or lysates that have been used successfully can be considered a suitable positive control.
- Try looking at the Swiss-Prot or Omnigene database links on the datasheet. These databases often have a list of tissues where the protein is expressed. These can also be considered suitable positive controls.
- Check the GeneCards entry for the protein. This will usually provide you with relative levels of expression in various tissues.
- If you still have difficulty finding a suitable control, we recommend doing a quick literature search on PubMed to see which tissues/cells express the protein of interest.

Negative control

This is a sample that does not express the protein you are detecting, which allows you to check for non-specific binding and false positive results. Each plate you use should contain a negative control sample to validate the results.

Non-specific binding control (competitive ELISA)

If background subtraction is desired for competitive ELISA, you can test for non-specific signals by adding only the detector component of the assay (for example, the HRP-conjugated antibody or analyte), and then bringing the well to the final volume with assay buffer. This will identify any signal arising from the non-specific binding of the detection component to the plate. You can then subtract this value from your data to obtain the background-corrected OD.



Standard

This is a sample that contains a known concentration of the target protein, from which the standard curve can be obtained. For example, below is a typical standard curve from our Mouse IL-6 ELISA kit (**ab46100**) with concentration ranging from 15.6 to 500 pg/mL. A poor standard curve means the antibody didn't bind properly or doesn't capture the protein standard. The R2 value of the trend line should be >0.99.

Standard in sample matrix (spike) control

When testing serum samples in ELISA, include a standard in normal diluent buffer as usual. But we also recommend the inclusion of a standard diluted serum from the species you are testing. The two can then be compared to ensure there is no effect on the standard curve from other proteins in the serum. This is known as a spike control and tells you that a target protein is recoverable after being spiked into a matrix. Acceptable results are 80–120%.

Endogenous positive control

We recommend including an endogenous positive control if you are testing a recombinant protein sample. This should be an essential component of your experiment.

There are inherent difficulties with antibody detection of recombinant proteins that need to be considered. Folding of the recombinant protein may be different from the endogenous native form and may prevent antibody access to the epitope. This is particularly the case with tagged proteins. Always ensure tags are placed on the N- or C-terminal end of the recombinant protein.

Most importantly, always ensure the recombinant protein includes the immunogen sequence of the antibody you are using. An endogenous positive control is important to validate the results, as well as to indicate how well the reagents (eg antibodies) and procedure are working.

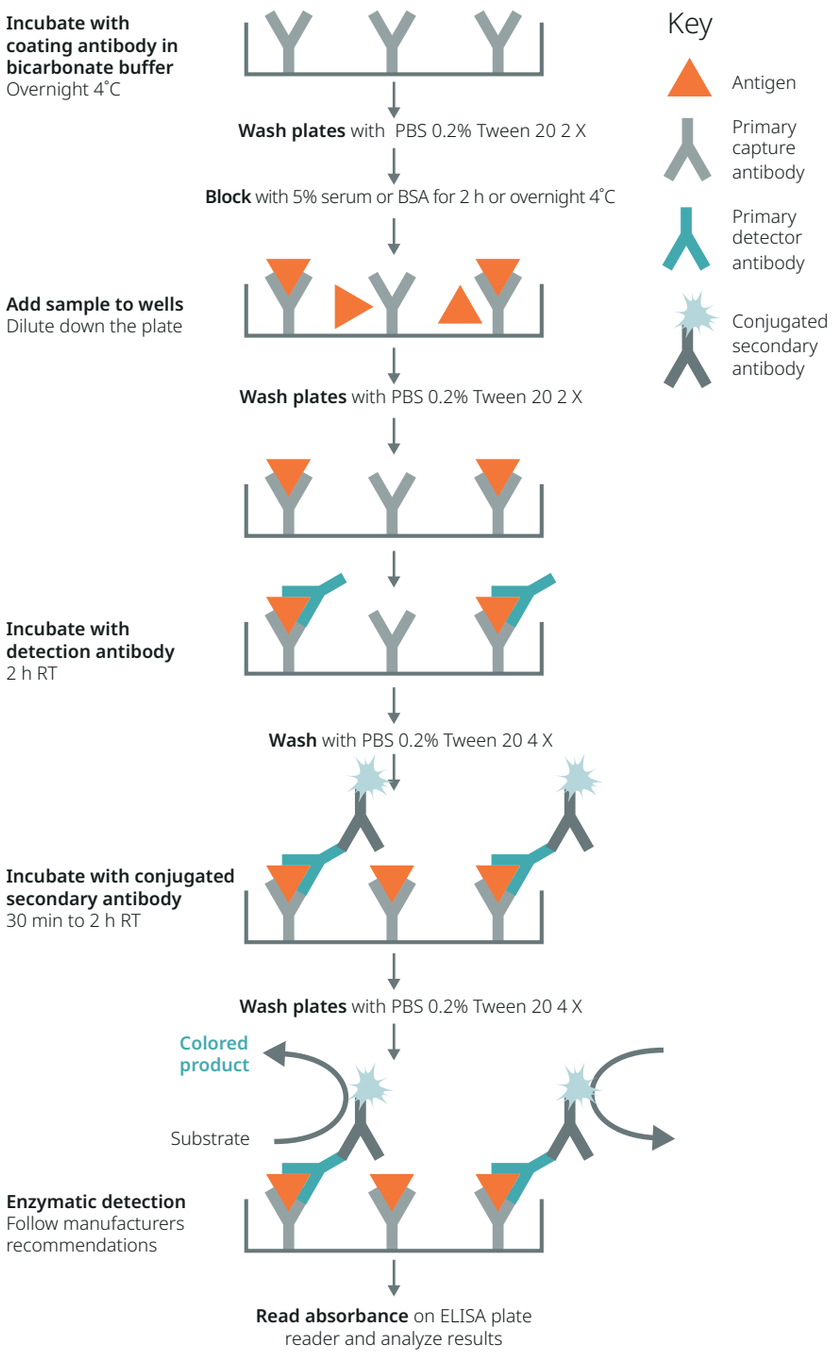
Sandwich ELISA protocol

Introduction

A sandwich ELISA measures the levels of an antigen using two different antibodies (a capture and a detector antibody). The target antigen must contain at least two antigenic sites capable of binding to antibodies.

Monoclonal or polyclonal antibodies can be used as the capture and detection antibodies in sandwich ELISA systems. Monoclonal antibodies recognize a single epitope that allows quantification of small differences in antigen. A polyclonal is often used as the capture antibody to pull down as much of the antigen as possible.

Sandwich ELISAs remove the sample purification step before analysis and enhance sensitivity (2–5 times more sensitive than direct or indirect ELISAs).



Sandwich ELISA procedures can be difficult to optimize, so tested matched antibody pairs should be used. This ensures the antibodies are detecting different epitopes on the target protein and do not interfere with the other antibody binding. We are unable to guarantee the performance of our antibodies in sandwich ELISA unless they have been specifically tested.

Always review antibody datasheets for tested applications information.

Coating with capture antibody

1. Coat the wells of a PVC microtiter plate with the capture antibody at 1–10 µg/mL concentration in carbonate/bicarbonate buffer (pH 9.6).

   Unpurified antibodies (eg ascites fluid or antiserum) may require increased concentration of the sample protein (try 10 µg/mL) to compensate for the lower concentration of the specific antibody.

2. Cover the plate with adhesive plastic and incubate overnight at 4°C.
3. Remove the coating solution and wash the plate twice by filling the wells with 200 µL PBS. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.

Blocking and adding samples

1. Block the remaining protein-binding sites in the coated wells by adding 200 µL blocking buffer (5% non-fat dry milk/PBS) per well.
2. Cover the plate with adhesive plastic and incubate for at least 1–2 h at room temperature or overnight at 4°C.
3. Wash the plate twice with 200 µL PBS.
4. Add 100 µL of the diluted sample to each well. Always compare the signal of an unknown sample against a standard curve. Run standards (duplicates or triplicates) and a blank with each plate. Incubate for 90 min at 37°C.

   Ensure concentration of standards span the most dynamic detection range of antibody binding. You may need to optimize the concentration range to obtain a suitable standard curve. Always run samples and standards in duplicate or triplicate.

5. Remove samples and wash the plate twice with 200 µL PBS.

Incubation with detection and secondary antibody

1. Add 100 µL of diluted detection antibody to each well.

   Check that the detection antibody recognizes a different epitope on the target protein to the capture antibody. This prevents interference with antibody binding. Use a tested matched pair whenever possible.

2. Cover the plate with adhesive plastic and incubate for 2 h at room temperature.
3. Wash the plate four times with PBS.
4. Add 100 µL of conjugated secondary antibody, diluted in blocking buffer immediately before use.
5. Cover the plate with adhesive plastic and incubate for 1–2 h at room temperature.
6. Wash the plate four times with PBS.

Detection

Horseradish peroxidase (HRP) and alkaline phosphatase (ALP) are the two most widely used enzymes for detection in ELISA.

Consider that some biological materials have high levels of endogenous enzyme activity (such as high ALP activity in alveolar cells or high peroxidase activity in red blood cells) that may result in a nonspecific signal. If necessary, perform an additional blocking treatment with levamisol (for ALP) or 0.3% H_2O_2 in methanol (for peroxidase).

ALP substrate
P-Nitrophenyl-phosphate (pNPP) is the most widely used substrate for most applications. Measure the yellow color of nitrophenol at 405 nm after 15–30 min incubation at room temperature and stop the reaction by adding equal volume of 0.75 M NaOH.

HRP chromogenes
The substrate for HRP is hydrogen peroxide. Cleavage of hydrogen peroxide is coupled to oxidation of a hydrogen donor which changes color during the reaction.

TMB (3,3',5,5'-tetramethylbenzidine)
Add TMB solution to each well, incubate for 15–30 min, add an equal volume of stopping solution (2 M H_2SO_4) and read the optical density at 450 nm.

OPD (o-phenylenediamine dihydrochloride)
The end product is measured at 492 nm. The substrate is light sensitive, so store and use it in the dark.

ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline-6 sulfonic acid] diammonium salt)
The end product is green and the optical density can be measured at 416 nm.

Always handle with care and wear gloves as some enzyme substrates are considered hazardous (potential carcinogens).

Data analysis

Prepare a standard curve from the serial dilutions data, with concentration on the x-axis (log scale) vs. absorbance on the y-axis (linear). Interpolate the sample's concentration from this standard curve.

ELISA analysis

ELISA assays can be classified as follows according to the type of data obtained:

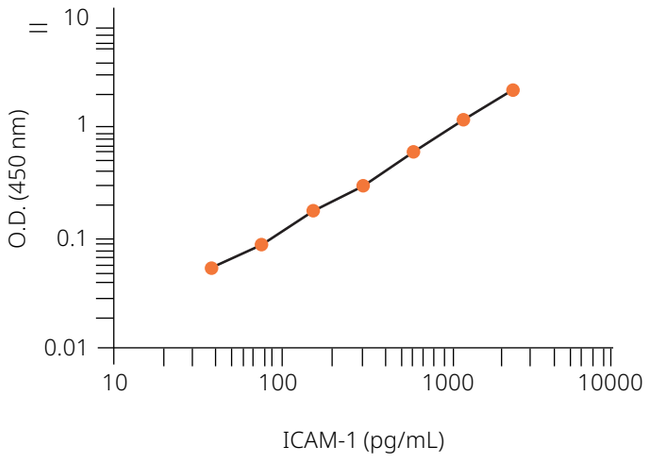
- Qualitative ELISA: only determines whether the antigen is present or not in the sample. It requires a blank well containing no antigen or an unrelated control antigen.
- Semi-quantitative ELISA: allows the relative comparison of the antigen levels between the samples.
- Quantitative ELISA: allows calculating the amount of antigen present in the sample. It requires comparison of the values measured for the samples with a standard curve prepared from a serial dilution of a purified antigen in a known concentration. This is the most commonly reported ELISA data.

ELISA standard curve

The standard or calibration curve is the element of the quantitative ELISA that will allow calculating the concentration of antigen in the sample.

The standard curve is derived from plotting known concentrations of a reference antigen against the readout obtained for each concentration (usually optical density at 450 nm).

Most ELISA plate readers come with software for curve fitting and data analysis. The concentration of the antigen in the sample is calculated by extrapolation of the linear portion of the standard curve.



Example of a quantitative ELISA standard curve using a Human ICAM1 SimpleStep ELISA® Kit (**ab174445**).

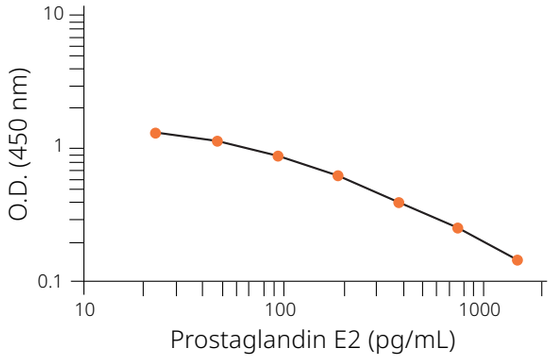
Curve fitting software allows the use of different models to plot your data.

- **Linear plot:** presents the concentration of the antigen in one axis and the readout in the other. R2 values are normally used here to determine fitting, with values higher than 0.99 representing a very good fit. However, linear plots tend to compress data points on the lower end of the curve resulting in decreased resolution at lower values.
- **Semi-log plot:** this helps counter the compression at the lower end caused by linear plots. Semi-log plots use the log of the concentration against the readout. This method commonly results in a sigmoidal curve that distributes the data points more evenly.
- **Log/log plot:** provides good linearity for the low to medium range of the concentrations. The higher end of the range tends to lose linearity.
- **4- or 5-parameter logistic (4PL or 5PL) curves:** they are more sophisticated methods that take into account other parameters such as maximum and minimum and therefore require more complex calculations. 4PL assumes symmetry around the inflection point while 5PL takes asymmetry into account, which normally is a better fit for immunoassays.

If your software allows it, 4-PL and 5-PL will fit most ELISA calibration standard curves. If not, the best option is to use a semi-log or a log/log plot.

Competitive ELISA standard curve

For competitive ELISA, the antigen concentration is determined from the standard curve in the same manner as a conventional ELISA. The only difference is that the standard curve is inverted, with the highest concentration corresponding to the lowest OD value and vice versa, as seen below.



For competitive ELISA, the binding ratio, B/Bo, is a useful metric for optimizing the range of the standard curve. To He the binding ratio, simply divide B, the OD at a given concentration of analyte, by Bo, the OD when no sample analyte is present (i.e., the maximum OD for the assay.) If the binding ratio for a point on the standard curve is lower than 5% or higher than 95%, consider shifting the standard range as this may improve the interpolated values at the high or low ends of the curve.

Calculating and evaluating ELISA data

Calculation of results from ELISA data and recommended guidelines on statistical assay validation.

Calculating results

Always run ELISA samples in duplicate or triplicate. This will provide enough data for statistical validation of the results. There are several pieces of software available to help process ELISA results in this manner.

Calculate the average absorbance values for each set of duplicate standards and duplicate samples. Duplicates should be within 20% of the mean.

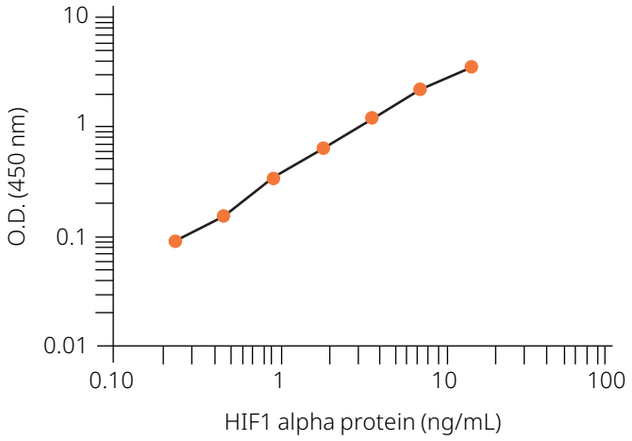
Standard curve

Create a standard curve for the target protein by plotting the mean absorbance (y-axis) against the protein concentration (x-axis). Draw the best-fit curve through the points in the graph (we suggest using suitable software for this).

We recommend including a standard on each ELISA plate to provide a standard curve for each plate used.

The figure below shows a representative standard curve from the human HIF1 alpha SimpleStep ELISA® Kit (**ab171577**). Each point on the graph represents the mean of the three parallel titrations.

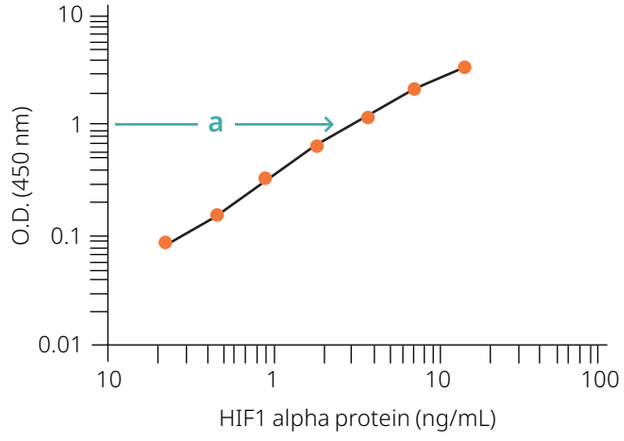
We recommend using a sample of known concentration as a positive control. The concentration of the positive control sample should be within the linear range of the standard curve to obtain valid and accurate results.



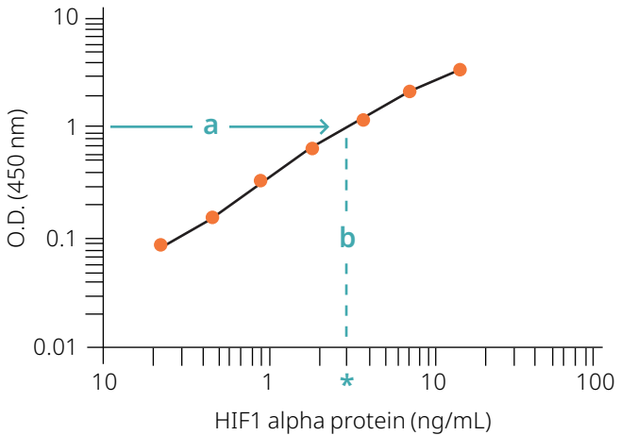
Concentration of the target protein in the sample

To determine the concentration of target protein concentration in each sample, first find the mean absorbance value of the sample. From the y-axis of the standard curve graph, extend a horizontal line from this absorbance value to the standard curve.

For example, if the absorbance reading is 1, extend the line from this absorbance point on the y-axis (a):



At the point of intersection, extend a vertical line to the x-axis and read the corresponding concentration (b).



Samples that have an absorbance value falling out of the range of the standard curve

For samples that have an absorbance value falling outside of the range of the standard curve, to obtain an accurate result, these samples should be diluted before proceeding with the ELISA staining. The concentration obtained from the standard curve when analyzing the results should be multiplied by the dilution factor.

Calculating the coefficient of variation

The coefficient variation (CV) is the ratio of the standard deviation σ to the mean μ:

$$C_v = \frac{\sigma}{\mu}$$

This is expressed as a percentage of variance from the mean and indicates any inconsistencies and inaccuracies in the results. A larger variance indicates greater inconsistency and error. Certain software can calculate CV values based on the results of an ELISA.

High CV can be caused by

– Inaccurate pipetting; ensure pipette tips are sealed to the pipette before use so they draw up to the correct volume of liquid.

– Splashing of reagents between wells.

– Bacterial or fungal contamination of either screen samples or reagents.

– Cross contamination between reagents.

– Temperature variations across the plate; ensure the plates are incubated in a stable temperature environment away from drafts.

– Some of the wells are drying out; ensure the plates are always covered during the incubation steps.

Spike recovery

Spike recovery determines the effect sample constituents have on the antibody's detection of the antigen. For example, the many proteins contained in tissue culture supernatant may hinder antibody binding and increase the signal-to-noise ratio, resulting in an underestimation of the target concentration.

Known protein concentrations are spiked into both the sample matrix and a standard diluent. The spiked protein is quantified using the assay, and the results from the sample matrix and the standard diluent are compared.

If the results are identical, then the sample matrix is considered to be valid for the assay procedure. If the recovery is different, then components in the sample matrix are interfering with the analyte detection.

What if a spike recovery experiment indicates that the sample matrix is affecting the results?

We recommend producing the standard curve using standard diluted in the sample matrix. Any effects on the results from the sample matrix will also be present in the standard, and therefore comparison between the standard curve and the samples are more accurate. Many of our ELISA kits contain a standard serum diluent for this purpose.

Another solution is to alter the sample matrix. For example, if neat biological sample is used, try diluting this in standard diluent. However, with this option, you will need to ensure that the dilution factor is taken into account when analyzing the results and that the concentration stays within the linear section of the standard curve.

How to interpret the dynamic range of an ELISA

The dynamic range of an ELISA is the range of antigen concentrations that can be measured accurately by the assay. This article explains how dynamic range is determined and how to interpret it for different sample types.

How is the dynamic range determined?

The dynamic range can be determined by plotting a standard curve of antigen concentrations against the detection signal from the assay.

Measurements that are considered accurate, and therefore within the dynamic range, must show:

- Low standard deviation between replicates of the standards
- Strong correlation between the antigen concentration and the detection signal

Our ELISA kit datasheets and protocols usually detail the dynamic ranges of experimental samples that have been shown to be measured accurately by the assay. These dynamic ranges are generally presented one of two ways: either as concentrations of the protein being detected, or as dilutions (more common for samples like plasma or serum).

Examples

More details can be found in the examples below.

Presented as a range of concentrations

The protocol of Abcam's Human Frataxin ELISA Kit (**ab176112**) includes a table that lists the ranges of total protein concentrations of lysates of various cell lines that we expect to be within the dynamic range. Those concentrations were within the linear range of the standard curve when they were tested.

For HeLa cell lysates:

- At the low end of the dynamic range, the ELISA could accurately detect a total frataxin concentration of 0.5 µg/mL
- At the high end of the dynamic range, the ELISA could accurately detect a total frataxin concentration of 100 µg/mL
- The dynamic range is therefore presented as 0.5 – 100 µg/mL

Presented as a range of dilutions or percentages

The dynamic range of the concentrations in other sample types, such as serum and plasma, is usually presented as dilutions, either as fractions or as percentages.

That is the case for Abcam's TNF alpha ELISA Kit (**ab208348**). The protocol includes a table of ranges of plasma dilutions that are within the dynamic range of the assay.

For plasma collected in heparin:

- At the low end of the dynamic range, the ELISA kit could accurately detect the protein in dilutions equivalent to 1/16 (6.25/100, or 6.25 %).
- At the high end of the dynamic range, the ELISA kit could accurately detect the protein in undiluted plasma (100/100 =1, or 100%)
- The dynamic range is therefore presented as 6.25 – 100%

ELISA troubleshooting tips

Poor standard curve

Cause	Solution
Improper standard solution	Confirm dilutions are made correctly.
Standard improperly reconstituted	Briefly spin vial before opening; inspect for undissolved material after reconstituting.
Standard degraded	Store and handle standard as recommended.
Curve doesn't fit the scale	Try plotting using different scales, eg log-log, 5 parameter logistic curve fit.
Pipetting error	Use calibrated pipettes and proper pipetting technique.

No signal

Cause	Solution
Incubation time too short	Incubate samples overnight at 4°C or follow the manufacturer guidelines.
Target concentration present below the detection limits of the kit	Decrease dilution factor or concentrate samples.
Incompatible sample type	Detection may be reduced or absent in untested sample types. Include a sample in which the assay is known to detect a positive control.
Recognition of epitope impeded by adsorption to plate	To enhance detection of a peptide by direct or indirect ELISA, conjugate the peptide to a large carrier protein before coating it onto the microtiter plate.
Assay buffer compatibility	Ensure the assay buffer is compatible with the target of interest (eg enzymatic activity retained and protein interactions retained).
Not enough detection reagent	Increase the concentration or amount of detection reagent, following manufacturer guidelines.
Sample prepared incorrectly	Ensure proper sample preparation/dilution. Samples may be incompatible with the microtiter plate assay format.
Insufficient antibody	Try different concentrations/dilutions of the antibody.

Cause	Solution
Incubation temperature too low	Ensure the incubations are carried out at the correct temperature. All reagents, including plates should be at room temperature or as recommended by the manufacturer before proceeding.
Incorrect wavelength	Verify the wavelength and read plate again.
Plate washings too vigorous	Check and ensure the correct pressure is used in the automatic wash system. If you are manually washing with a pipette, apply the wash buffer gently.
Wells dried out	Do not allow wells to become dry once you have started the assay. Cover the plate using sealing film or tape during all incubation steps.
Slow color development of enzymatic reaction	Prepare substrate solution immediately before use. Ensure the stock solution has not expired and is not contaminated. Allow longer incubation periods.

Large coefficient of variation (CV)

Cause	Solution
Bubbles in wells	Ensure no bubbles are present before using the plate reader.
Wells not washed equally/thoroughly	Check that all ports of the plate washer are unobstructed. Wash wells as recommended.
Incomplete reagent mixing	Ensure all reagents are mixed thoroughly.
Inconsistent pipetting	Use calibrated pipettes and proper technique to ensure accurate pipetting.
Edge effects	Ensure the plate and all reagents are at room temperature.
Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (eg minimize freeze/thaw cycles).

High background

Cause	Solution
Wells are insufficiently washed	Wash wells as per protocol recommendations.
Contaminated wash buffer	Prepare buffer using fresh filtered water.
Too much detection reagent	Ensure the reagent has been diluted properly or decrease the recommended concentration of the detection reagent.
Blocking buffer ineffective (eg detection reagent binds blocker; wells not completely blocked)	Try a different blocking reagent and/or add the blocking reagent to the wash buffer.
Salt concentration of incubation/wash buffers	Increasing salt concentrations may reduce non-specific and/or weak off-target interactions.
Waiting too long to read plate after adding stop solution	Read plate immediately after adding stop solution.
Non-specific binding of antibody	Use suitable blocking buffers, eg BSA or 5-10% normal serum – from the same species as the primary antibody if using a directly conjugated detection antibody or the same species as the secondary antibody if using a conjugated secondary antibody. Ensure wells are pre-processed to prevent non-specific attachment.
High antibody concentration	Try different dilutions for optimal results.
Substrate incubation carried out in light	Substrate incubations should be carried out in the dark or as recommended by the manufacturer.
Precipitate formed in wells upon substrate addition	Increase the dilution factor of the sample or decrease the concentration of substrate.
Dirty plate	Clean the bottom of the plate or use a different plate.

Low sensitivity

Cause	Solution
Improper storage of ELISA kit	Store all reagents as recommended. Please note that all reagents may not have identical storage requirements.
Not enough target	Concentrate sample or reduce sample dilution.
Inactive detection reagent	Ensure the reporter enzyme/fluorophore has the expected activity.
Plate reader settings incorrect	Ensure the plate reader is set to read the correct absorbance wavelength or excitation/emission wavelengths for fluorescent detection.
Assay format is not sensitive enough	Switch to a more sensitive detection system (eg colorimetric to chemiluminescence/ fluorescence). Switch to a more sensitive assay type (eg direct ELISA to sandwich ELISA). Lengthen incubation times or increase temperature.
Target poorly adsorbs to microtiter plate	Covalently link target to the microtiter plate.
Not enough substrate	Add more substrate.
Incompatible sample type (eg serum vs cell extract)	Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect as a positive control.
Interfering buffers or sample ingredients	Check reagents for any interfering chemicals. For example, sodium azide in antibodies inhibits HRP enzyme activity, and EDTA, when used as an anticoagulant for plasma collection, inhibits enzymatic reactions.
Mixing or substituting reagents from different kits	Avoid mixing components from different kits.

Matrix effect

ELISA quantification of plasma and serum occasionally encounters problems, which are caused by the matrix effect. The matrix effect can arise from several matrix components including, but not limited to, the interaction between endogenous biological components, such as phospholipids, carbohydrates, and endogenous metabolites (bilirubin), or an interaction between the analyte of interest and the matrix, such as covalent binding to plasma proteins. This results in erroneous sample readings.

Simply diluting the samples by 2–5 fold reduces the matrix effect; when diluting the samples, remember to use the same diluent as used for the standard curve.

Competitive ELISA-specific troubleshooting tips

Cause	Solution
Non-specific background signal is too high	Test to see of the detector conjugate is binding non-specifically to the plate.
B/B_0 ratio for the end of the standard curve point is >95% or <5%	Increase/decrease the amount of standard by one dilution factor to shift the standard curve.
Standard curve reaches a plateau prior to the bottom of the standard curve	Make sure that the standard curve was pipetted and diluited properly.
Standard improperly constituted	Standard curve concentration is too low for the dynamic range of the ELISA.
No signal for the standard curve; signal for zero standard is normal	Concentration of the standard is too high; decrease the amount of standard to within the dynamic range of the assay.
OD without standard present is too low	Increase antibody concentration.
High CVs at the bottom of the standard curve	Higher ODs (ie lower concentrations) are more likely to demonstrate variance in replicates. If the variance in replicates. If the variance is too high, consider increasing the concentration of standard by one.

ELISA terms glossary

Definition of the terms commonly used when running or developing an ELISA.

Accuracy
The closeness of the mean test results to the true concentration of the analyte.

Affinity
Antibody affinity is typically represented by the equilibrium dissociation constant (K_d), a ratio of K_{off}/K_{on} between the antibody and its antigen, where a lower K_d value suggests a higher affinity relationship.

Assay precision: intra-assay and inter-assay coefficient variability (CV)
Repeatability between samples ran on multiple assay plates (inter-assay CV) or the measure of each sample in duplicate for each analyte (intra-assay CV).
The inter-assay CV is an expression of plate-to-plate consistency that is calculated from the mean values for the high and low controls on each plate.
The intra-assay CV reported in these studies is an average value calculated from the individual CVs for all of the duplicates, even if the total number of samples requires the use of multiple assay plates.
• Inter-assay CVs of less than 15% are generally acceptable.
• Intra-assay CVs should be less than 10%.

Batch-to-batch (lot-to-lot) variability
The change in antibody or conjugate lots can dramatically affect assay performance
The calibration of the capture antibodies and the detector antibodies ensure consistent results over time. Polyclonal antibodies can be difficult to calibrate. When available and suitable for the assay development, recombinant monoclonal antibodies are favored and provide greater lot-to-lot consistency.

Calibrator
The protein standard used to interpolate the concentration of a target protein in the sample. The best protein standard will mimic the natural protein in a biological sample. Calibration to an international control (NIBSC, WHO, or ARUP) will enable cross-comparison between data sets.
For example, the total Human PSA SimpleStep ELISA kit is calibrated against a highly purified human prostate specific antigen (PSA) protein. The NIBSC/WHO unclassified purified human PSA preparation 96/670 was evaluated in this kit. To convert sample values obtained with the SimpleStep human total PSA kit to approximate NIBSC ng/mL units, use the equation below.
NIBSC (96/670) approximate value (ng/mL) = 1.6857 x total PSA SimpleStep ELISA value (ng/mL)

Cross-reactivity
The extent to which an antibody pair reacts against non-target proteins. Generally acceptable results are less than 5% cross-reactivity. Testing is typically completed for protein families of high homology or if antibody pairs are required for use in a multiplex assay platform.

Dynamic range
The upper and lower concentrations of the target protein that the assay can accurately quantify.
The dynamic range extends from the lowest point of a standard curve to the highest point.
For targets abundantly present in a biological sample, the sample should be diluted so that the raw signal for the sample falls within the dynamic range of the assay.

Heterophilic interference
Heterophilic antibodies in human samples are anti-animal antibodies (HAAA) that arise through exposure to animal antigens, such as those that might be encountered by laboratory workers or in people living in close proximity to mice or other animals. In particular, human anti-mouse antibodies (HAMA) commonly result in false positive signals by complexing with both capture and detection antibodies, and generating

a signal whether or not analyte is present. Estimates of the frequency of human samples with heterophilic antibodies at concentrations high enough to interfere with an immunoassay range is up to 5%.

Interference
Unintended specific interactions between biological sample and assay components that affect the accuracy of the assay. Interference is tested for by spiking a sample with a known concentration of target protein and additional inhibitory protein and measuring target protein recovery. Generally acceptable results should be less than 5% interference as determined by raw signals.

International units
International units (IU) are assigned to reference materials to allow the assessment of 'biologicals' in a consistent internationally agreed manner when the physico-chemical determination of the standard units (eg mass) is not possible.

Linearity of dilution
A measure of parallelism between the dilution of the standard protein and native signal. If both the standard protein and native signal dilute proportionally, interpolated sample values will have an identical value at all doses tested once corrected for dilution. Linearity of dilution experiments are used to determine what concentrations of a given biological matrix are compatible with the assay and define the linear range of the assay for that biological sample.

Linearity of dilution is determined by measuring multiple dilutions of known positive samples by ELISA. The concentration of the target protein is determined by multiplying the dilution factor by the calculated concentration.

Lower limit of quantification (LLOQ)
The lowest level of protein concentration that can be detected and reliably quantified with a specific degree of confidence. To determine the LLOQ, 10 times the standard deviation of a series of blanks is added to the mean value of the blank measurements; this sum is then interpolated on the standard curve and should be less than 15% of the CV. Together with the ULOQ, they define the range of quantification of the assay.

Matched antibody pairs
Set of co-developed antibodies that detect different epitopes on the same protein and are capable of detecting both recombinant and endogenous proteins in a sandwich ELISA. Matched antibody pairs can be two monoclonal antibodies, two polyclonal antibodies, or a combination of monoclonal and polyclonal.

Matrix effects
The combined effect of all components in the sample, other than the analyte, on the measurement of the specific analyte. The matrix effect often results in a reduction in assay performance. If a specific component can be identified as causing an effect, then this is referred to as interference.

Examples of matrix effects
- Matrix proteins that non-specifically bind to the CDR regions
- Mineral or pH imbalance that alters antibody affinity to target protein
- Direct interference by cellular components, eg growth factor and receptor

Minimal detectable dose (MDD) or detection limit / sensitivity
The lowest signal that can be statistically distinguished from the background measurement. To determine the MDD, two times the standard deviation of a series of blanks is added to the mean of the blanks; this sum is then interpolated on the standard curve.
The MDD is a measure of the variability in the blank measurements and as such is best calculated when the number of blanks used for the calculation is high (eg >16 wells for ELISA). The MDD should always be less than the lowest point of the standard curve.

Specificity
Indication of an antibody binding to a unique epitope of a particular protein and species.

Spike and recovery
A test to determine if a target protein is recoverable after being spiked into a biological matrix. It identifies unknown factor(s) within the biological matrix that can interact with the target protein in a way that affects antibody binding. More or less apparent signals may be recovered if factors affecting the binding are present.
Generally, acceptable results should fall within ± 20% variation (80–120%) in recovery. Results should be compared to the recovery of an identical spike in standard diluent: if the recovery observed for the spike is identical in both the biological matrix and the standard diluent, the sample matrix is considered valid for the assay procedure (Table 1).

Supported biological samples
Sample types that have been tested with the product, but either no signal is detected or the signal is weaker than the lowest point on the standard curve. These samples are tested for compatibility with the product by spiking the standard protein into the biological sample.
The expectation for assays validated in this manner is that if you use the assay with a native sufficiently high signal (eg, a sample from a patient with a specific disease with levels in the dynamic range of assay) then the assay is suitable for this sample type.

Upper limit of quantification (ULOQ)
The highest protein concentration that can be detected and reliably quantified based on a specific degree of confidence. To determine the ULOQ, 10 times the standard deviation of the highest data point on the straight part of the standard curve is added to the mean; this sum is then interpolated on the standard curve and should be less than 15% of the CV. Together with the LLOQ, they define the range of quantification of the assay.

Validated biological samples
Sample types that have been tested with the product and the native signal is measurable.

Sample type	Average % recovery
50% conditional media	91%
50% human serum	84%
25% human plasma (sodium citrate)	87%
25% human plasma (EDTA)	87%
25% human plasma (heparin)	83%
0.1% human urine	101%

Table 1. Three concentrations of total purified human PSA were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the total PSA SimpleStep ELISA kit.

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