ELISA - The Essentials

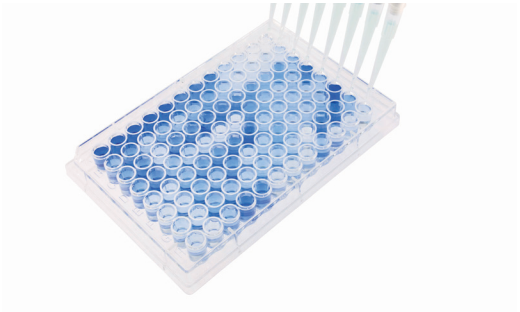
Pocket Guide to ELISA

BIO-RAD

Table of Contents

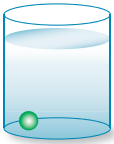
1. Overview

The enzyme-linked immunosorbent assay (ELISA) measures the concentration of an analyte, usually a protein, in solution. ELISAs start with immobilization of the analyte in the wells of a microtiter plate. Next a detection antibody is added to measure the amount of analyte. The readout comes from substrate catalyzed by enzyme conjugated detection antibody or secondary antibody. An analyte can be immobilized by direct adsorption or capture antibody. The main ELISA types to detect analytes are: direct, indirect, sandwich, and competitive.



Typical ELISA output. Darker wells indicate higher levels of analyte in the original sample.

2. Analyte Capture



Direct adsorption

Capture antibody

The first step is to determine how the analyte will be captured. This will depend on reagent availability and assay expectations.

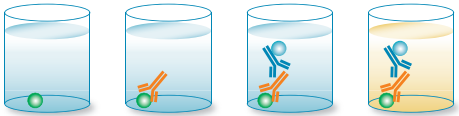
	Direct Adsorption	**Capture Antibody**
Key points	Adsorption to the plate's surface	Analyte specific capture antibody
Advantages	No antibody Fewer steps Less error prone	Higher specificity Higher sensitivity in sandwich ELISA
Disadvantages	Immobilization is not specific Higher background noise may be observed	More steps Additional specific antibody required

If the analyte is adsorbed to the well surface, proceed to direct or indirect ELISA; if a capture antibody is used go to the sandwich ELISA section.

Find out more at **bio-rad-antibodies.com/elisa**

3. ELISA Types
Direct and indirect ELISA

Direct ELISA

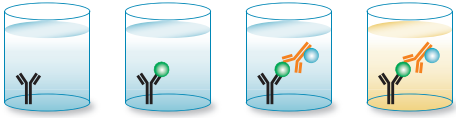


Indirect ELISA

The analyte is adsorbed to the well surface and can then be detected either directly (direct ELISA) or indirectly (indirect ELISA).

	Direct ELISA	**Indirect ELISA**
Key points	Detection antibody is enzyme conjugated	Analyte specific detection antibody
Advantages	Only one antibody Faster than other ELISAs Less prone to error	High sensitivity due to multiple binding sites Fewer enzyme conjugated antibodies needed Greater flexibility to change detection antibody/enzyme
Disadvantages	Each target needs its own enzyme conjugated detection antibody No signal amplification	Extra antibody needed Possibility of background Longer protocol

Sandwich ELISA



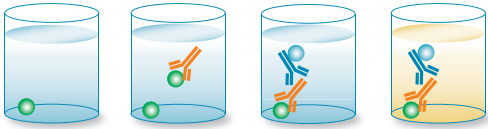
Direct sandwich ELISA

Sandwich ELISAs deliver higher specificity and sensitivity; they can be run with direct or indirect detection. The key difference is the application of a capture antibody.

	Sandwich ELISA
Key points	Analyte specific capture antibody immobilizes analyte followed by specific detection antibody. Detection step can be run as direct ELISA or indirect ELISA type
Advantages	High sensitivity - 2 to 5 times more sensitive than direct or indirect ELISA High specificity - two antibodies are involved in capture and detection Flexibility - both direct and indirect detection can be used
Disadvantages	Antibody optimization can be difficult - cross-reactivity may occur between the capture and detection antibodies Needs a standardized ELISA kit or tested antibody pair
See **Direct and Indirect ELISA** section for detection benefits and drawbacks	

Find out more at **bio-rad-antibodies.com/elisa**

Competitive ELISA



Direct adsorption based competitive ELISA

Competitive ELISAs are suited to quantification, especially of small analytes. A key difference in the readout of the competitive ELISA, to the preceding ELISA types, is that increasing the amount of analyte decreases levels of signal output, as the labeled reagent gets ‘competed out’.

	Competitive	**Non-competitive**
Key points	The reference analyte is bound to the solid phase, the sample analyte competes with it for binding to a limited amount of labeled detection antibody in solution In the capture antibody based protocol labeled reference analyte competes with sample analyte for binding to a limited amount of capture antibody	Analyte specific capture and/or detection antibodies present in excess, binding all the available analyte
Advantages	More suited to quantification Used for small analytes that are too small for sandwich ELISA Robust, complex analyte mixture can be analyzed with single antibody Can cope with cross-reactivity	Higher sensitivity Suitable for larger multi epitope analytes
Disadvantages	Sub-saturating antibody concentration needs optimizing	Not suitable for small analytes

4. Antibodies

The antibodies used in ELISA assays can be monoclonal, polyclonal, or a combination of both. Each antibody type offers distinct advantages in the development of ELISAs.

Polyclonal Antibodies	Monoclonal Antibodies
Quicker and easier to make	Take longer to establish
Multiple isotypes	Single isotype
More stable over a range of pH and salt concentrations	Less stable to matrix condition changes
Polyspecific, can recognize several epitopes	Monospecific, can distinguish small changes in molecular structure
Can have cross-reactivity, higher background	Generally exhibit low background
Batch differences	Constant antibody secreted from hybridoma

Antibody labeling

Unlabeled detection antibody, for all ELISA types, can be conjugated to horseradish peroxidase (HRP) or to alkaline phosphatase (AP) with the Bio-Rad's LYNX Rapid Antibody Conjugation Kits. Find out more at **bio-rad-antibodies.com/lynx**

For more antibodies and ELISA tips and tricks, please visit **bio-rad-antibodies.com/elisa-hints**

Antibody optimization

To find the optimal antibody concentrations generate a standard curve by performing a sandwich ELISA with direct detection. Set up as follows:

- High (H) and low (L) concentration of your analyte and a blank (0)
- Capture antibody dilutions at 0.5, 1, 2 and 5 µg/ml
- Detection antibody at 1:200, 1:1,000, 1:5,000 and 1:25,000

Then follow the sandwich ELISA protocol using the plate layout shown below.

Antibody concentration optimization plate layout.

Detection Antibody	Capture Antibody											
	5 µg/ml			2 µg/ml			1 µg/ml			0.5 µg/ml		
1:200	H	L	O	H	L	O	H	L	O	H	L	O
	H	L	O	H	L	O	H	L	O	H	L	O
1:1,000	H	L	O	H	L	O	H	L	O	H	L	O
	H	L	O	H	L	O	H	L	O	H	L	O
1:5,000	H	L	O	H	L	O	H	L	O	H	L	O
	H	L	O	H	L	O	H	L	O	H	L	O
1:25,000	H	L	O	H	L	O	H	L	O	H	L	O
	H	L	O	H	L	O	H	L	O	H	L	O

Find the capture and detection antibody combination with the maximum signal-to-noise ratio, i.e. the largest difference between low and high analyte concentrations. Select that one as the starting point for generating the optimum standard curve in the sandwich ELISA type. Finally, adapt as necessary for other ELISA formats, e.g. omit capture antibody for direct adoption based analyte immobilization.

5. Controls

Control samples with analyte concentration validated by another method should be included in all ELISA types. Control samples, in the matrix used for the test samples, help to keep track of assay performance over time. A spiked sample, where a known amount of analyte is added to a subgroup of samples, is an alternative way to obtain calibration points in the ELISA.

Find out more at **bio-rad-antibodies.com/elisa**

6. Buffers and Substrate

A basic ELISA needs buffers for the following protocol steps:

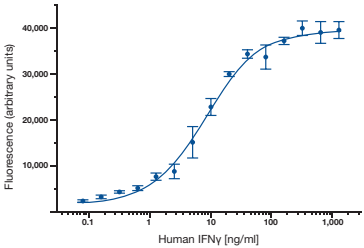
- Dilution of antibodies and controls/standards
- Coating
- Several washing steps
- Detection

Bio-Rad supplies a range of coating, blocking, and washing buffers. TMB (3,3´,5,5´-tetramethylbenzidine) substrates for HRP and p-nitrophenyl phosphate (pNPP) solution for AP enzyme based detection systems can be obtained as optimized ready-to-use reagents.

Find optimized buffers for these steps at:
bio-rad-antibodies.com/elisa-buffers

7. ELISA Results

ELISA experiments can be set up to deliver qualitative, semi-quantitative, and quantitative results. ELISA data is generally presented as a plot of optical density vs log concentration, producing a sigmoidal curve (see graph below). Known concentrations of analyte are used to generate the standard curve. The linear portion of the sigmoidal curve is used to measure the amount of analyte in the test samples.



A typical ELISA standard curve

To determine the concentration of an unknown sample:

- Locate its absorbance value on the Y axis
- Draw a horizontal line until it meets the standard curve
- Draw a vertical line down to the x axis
- Read off the concentration

Find out more at **bio-rad-antibodies.com/elisa**

8. Protocols
Reagents

Coating buffer
Na_2CO_2, 1.5 g
$NaHCO_3$, 2.93 g
Distilled water, 1 liter, pH to 9.6
For an alternative coating buffer, use ELISA Coating Buffer (cat. #BUF030)

Blocking buffer
Phosphate Buffered Saline (PBS) (#BUF036A) containing 1% w/v BSA
For an alternative blocking buffer, use either ELISA BSA Block (#BUF032), ELISA Ultrablock (#BUF033), or ELISA Synblock (#BUF034)

Wash buffer
PBS containing 0.05% v/v Tween-20
For an alternative wash buffer use ELISA Wash Buffer (#BUF031)

Recommended substrates and stop solutions
TMB Core+ (#BUF062), for use with HRP-conjugated antibodies. Stop with 2M H_2SO_4
pNPP (#BUF044), for use with alkaline phosphatase-conjugated antibodies. Stop with 1M NaOH

ELISA Methods

Direct ELISA with streptavidin-biotin detection

1. Coat microtiter plate with antigen/analyte, cover the plate and incubate overnight at 4˚C. Wash 3 times with wash buffer.
2. Add blocking buffer. Incubate for 60 min at 37˚C. Wash 4 times with wash buffer.
3. Add samples and standards to the relevant wells, preferably in triplicate. Incubate for 90 min at 37˚C or overnight at 4˚C. Wash 3 times with wash buffer.
4. Add biotin-conjugated detection antibody (in wash buffer) to each well. Incubate for 1 hr at 37˚C. Wash 3 times in wash buffer.
5. Add enzyme-conjugated streptavidin (in wash buffer). Incubate for 60 min at 37˚C. Wash 3 times in wash buffer.
6. Add substrate solution to each well. Incubate at room temperature until desired color change is attained.
7. Add stop solution and read absorbance values.

Find out more at **bio-rad-antibodies.com/elisa**

Indirect ELISA

1. Coat microtiter plate with antigen/analyte, cover the plate and incubate overnight at 4˚C. Wash 3 times with wash buffer.
2. Add blocking buffer. Incubate for 1 hr at 37˚C. Wash 4 times in wash buffer.
3. Add samples and standards to the relevant wells, preferably in triplicate. Incubate for 90 min at 37˚C or overnight at 4˚C. Wash 3 times with wash buffer.
4. Add detection antibody (in wash buffer) to each well. Incubate for 1 hr at 37˚C. Wash 3 times in wash buffer.
5. Add enzyme-conjugated secondary antibody to each well. Incubate for 1 hr at 37˚C. Wash 3 times in wash buffer.
6. Add substrate solution to each well. Incubate at room temperature until desired color change is attained.
7. Add stop solution and read absorbance values.

Sandwich ELISA with direct detection

1. Coat microtiter plate wells with coating antibody. Cover the plate and incubate overnight at 4˚C. Wash the plate 3 times in wash buffer.
2. Add blocking buffer. Incubate for 1 hr at 37˚C. Wash 4 times in wash buffer.
3. Add samples/analyte and standards to the relevant wells, preferably in triplicate. Incubate for 90 min at 37˚C or overnight at 4˚C. Wash 3 times with wash buffer.
4. Add enzyme-conjugated detection antibody. Incubate for 1 hr at 37˚C. Wash 3 times in wash buffer.
5. Add substrate solution to each well. Incubate at room temperature until desired color change is attained.
6. Add stop solution and read absorbance values.

Find out more at **bio-rad-antibodies.com/elisa**

Sandwich ELISA with streptavidin-biotin detection

1. Coat microtiter plate wells with coating antibody. Cover the plate and incubate overnight at 4˚C. Wash the plate 3 times in wash buffer.
2. Add blocking buffer. Incubate for 1 hr at 37˚C. Wash 4 times in wash buffer.
3. Add samples/analyte and standards to the relevant wells, preferably in triplicate. Incubate for 90 min at 37˚C or overnight at 4˚C. Wash 3 times with wash buffer.
4. Add biotin-conjugated detection antibody. Incubate for 1 hr at 37˚C. Wash 3 times in wash buffer.
5. Add enzyme-conjugated streptavidin. Incubate for 60 min at 37˚C. Wash 3 times in wash buffer.
6. Add substrate solution to each well. Incubate at room temperature until desired color change is attained.
7. Add stop solution and read absorbance values.

Competitive ELISA

1. Coat microtiter plate wells with antigen/analyte solution in coating buffer. Cover the plate and incubate overnight at 4˚C. Wash the plate 3 times in wash buffer.
2. Add blocking buffer to plate. Prepare the analyte antibody mixture for samples and standards. Incubate for 1 hr at 37˚C. Wash plate 4 times in wash buffer.
3. Add analyte and standard mixture to relevant wells. Incubate for 1 hour at 37˚C. Wash 3 times in wash buffer.
4. Add enzyme-conjugated secondary antibody to each well. Incubate for 1 hr at 37˚C. Wash 3 times in wash buffer.
5. Add substrate solution to each well. Incubate at room temperature until desired color change is attained.
6. Add stop solution and read absorbance values.

For comprehensive protocols, links to ELISA validated antibodies, supporting information to enhance your experiments, and details on ELISA accessory reagents, please visit:
bio-rad-antibodies.com/elisa

9. ELISA Troubleshooting

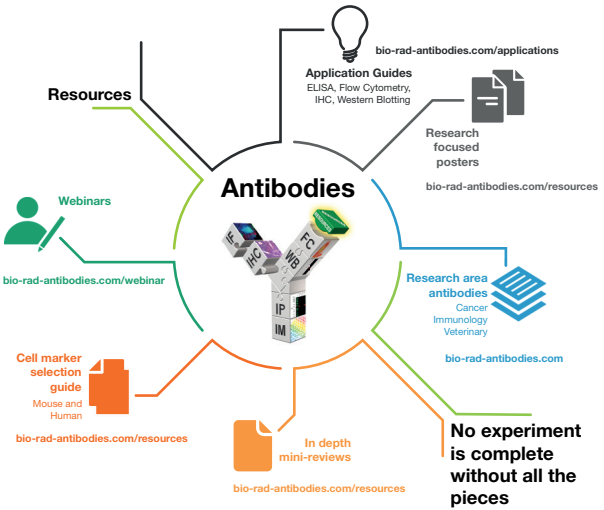
Problem	Possible Causes	Action/Solution
No signal	Incorrect secondary antibody used	Repeat with correct secondary antibody
	Not enough antibody used	Optimize antibody concentration
	Antigen not coated properly	Try longer coating times, different coating buffers, or avidin plates with biotinylated antigen
	Antibody at 4˚C for too long or freeze/thawed repeatedly	Use a fresh aliquot of antibody from -20˚C stocks
Weak signal	Insufficient amount of antigen was coated to microtiter plate	Use more antigen for coating or very coating buffer
	Not enough antibody used	Optimize antibody concentration
	Detection reagent too old, contaminated, or used at the wrong pH	Use fresh detection reagents at the correct pH
	Incubation temperature too low	Optimize the incubation temperature
High background signal	Too much antibody used	Optimize antibody concentrations
	Non-specific antibody binding	Use suitable blocking buffer or affinity-purified antibody
	Too much detection reagent used	Repeat with higher dilution
	Too few washing cycles	Increase washing cycles
	Contaminated blocking agent	Use fresh blocking agent
	Wrong concentration of blocking agent	Check the concentration of blocking agent
	Blocking buffer interferes with antibody binding	Wash off blocking buffer before adding antibody
	Reaction not stopped	Use stop solution to prevent overdevelopment
	Insufficient amount of Tween in the buffers	Use PBS containing 0.05% Tween

Problem	Possible Causes	Action/Solution
High background signal	Incubation with substrate carried out in the light	Perform substrate incubation in the dark
	Inconsistent washing of wells	Reduce variability of washes
	Uneven evaporation of solution from wells	Always incubate with a plate sealer
Slow color developmemt	Incubation temperature is wrong	Keep reaction at room temperature
	Contaminated solutions	Make fresh solutions
	Detection reagent too old, contaminated or used at the wrong pH	Use fresh detection reagents at the correct pH

9. Technical Support

For further help and expert advice contact our scientist staffed technical support department.
bio-rad-antibodies.com/technical

Think Antibodies. Think Bio-Rad.



Receive Antibody Updates Straight into Your Inbox
Sign up to our emailers and we will let you know when we launch new products and resources.
bio-rad-antibodies.com/sign-up

bio-rad-antibodies.com

BIO-RAD

Bio-Rad Laboratories, Inc.

Bulletin 7229 Ver A US/EG *1119 Sig 0119*