Enzyme-linked immunosorbent assay (ELISA): the basics

Yalow and Berson (1960) were the first to describe a technique that used antibody-mediated detection linked to a radioactive signal (i.e. radioimmunoassay). However, because of the health risks methods not involving radioactivity were sought. The discovery that certain enzyme-substrate combinations produced quantifiable colour changes led to a shift in immunodetection. Enzyme-substrate combinations that were linked to antibodies, which in turn could detect specific analytes, were developed (Avrameas, 1969). In 1971, two independent research groups in Europe published papers that described the step-by-step process of performing an enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971).

The ELISA method is used to detect and quantify a specific substance, usually an antigen, in a sample. The antigen is immobilized in a microplate well either directly or by a specific antibody known as a 'capture antibody'. A 'primary detection antibody' is added, forming an antigen–antibody complex. The primary detection antibody is either directly labelled with an enzyme (i.e. direct ELISA) or is itself attached to a secondary antibody known as a 'secondary detection antibody' (i.e. indirect ELISA). Between each step the well is washed with a buffer solution. The addition of a substrate produces a colour signal indicating the presence of the antigen in the sample. The measurement of the optical density is proportional to the quantity of antigen in the sample.

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ELISAs are commonly used in at-home pregnancy testing, and in point-of-care testing, such as to diagnose HIV, hepatitis B and malaria in clinic.

Methodology

ELISAs are divided into different categories depending on how the antigen is immobilized and detected. *Table 1* lists the advantages and disadvantages of different types of ELISAs.

Direct or indirect ELISA

The direct ELISA represents the simplest series of steps used to detect antigens. A buffered solution containing the analyte is added to a 96-well plate, where it is allowed to adhere to the plastic for a variable length of time (*Figure 1a*). Usually a carbonate-bicarbonate coating buffer is used. This contains a solution of sodium carbonate, sodium bicarbonate and distilled water, and facilitates passive absorption of the antigens onto the plate. The buffer is maintained at a pH of at least 9, allowing the antigens to remain soluble and ensuring that they have an overall negative charge that can bind to the positively charged plate.

At the same time, standards, positive and negative controls are plated. Standards are samples usually provided by the ELISA kit that contain known concentrations of the analyte. A standard curve is prepared by making serial dilutions of one known concentration of the analyte across a range near the expected unknown concentration. When performing ELISA on serum samples it is usually recommended to have a second standard curve diluted in the serum to assess whether other proteins within the serum have affected detection (i.e. spike control). A positive control represents a soluble sample or a purified protein known to contain the analyte. A negative control is a sample known not to express the analyte detected.

Figure 1. Direct ELISA protocol.

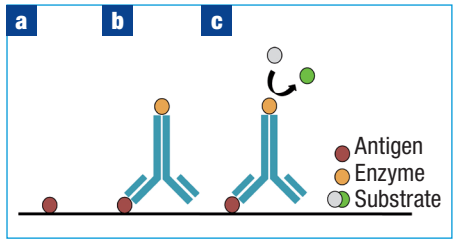


Table 1. Advantages and disadvantages of different types of enzyme-linked immunosorbent assays (ELISAs)

	Advantages	Disadvantages
Direct ELISA	■ Rapid ■ Secondary antibody cross-reactivity eliminated	■ Low sensitivity ■ Specific antibody for each ELISA; time-consuming and expensive
Indirect ELISA	■ High sensitivity ■ Cost-saving ■ Flexible; can use many primary antibodies	■ Risk of cross-reactivity between secondary antibodies
Sandwich ELISA	■ Minimal sample purification needed ■ High sensitivity and specificity	■ Must use 'matched pair' primary and secondary antibodies ■ Time consuming and expensive
Competitive ELISA	■ Minimal sample purification needed ■ Used to measure large range of antigens in a sample ■ Used for small antigens ■ Low variability	■ Low specificity so cannot be used in dilute samples

Figure 2. Indirect ELISA protocol.

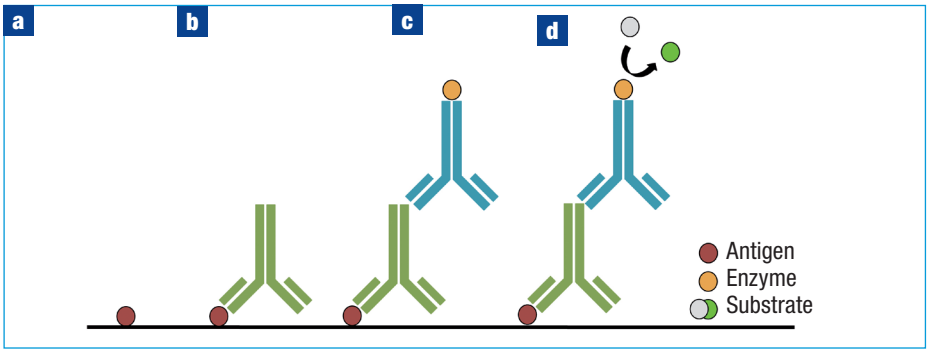


Figure 3. Point-of-care HIV testing.

Point-of-care HIV testing uses ELISAs by detecting HIV antibodies. The test stick is marked with HIV antigens and the blood sample is placed on the end of the stick. A buffer allows any antibodies to flow along the stick and they attach to the antigens. The stick is washed with antihuman antibody, the secondary antibody, labelled with a conjugate enzyme and then washed with a dye. A change in colour represents a positive result.

The controls are necessary to confirm that the assay is optimized and no contamination occurred. After allowing the antigen to bind, excess solution can be disposed of and a non-reacting protein (such as bovine serum albumin or casein) is added to cover the plastic surface that has not been coated by the antigen; this limits non-specific binding.

Between steps wells are washed with phosphate buffered saline at neutral pH, as directed by the kit. During the washing phase the wells are repeatedly filled with phosphate buffered saline and emptied. The washing buffer is used to remove any unbound assay components, or debris, from the wells. This minimizes background noise during detection and increases the specificity of the assay.

Once the plate is washed a blocking buffer such as bovine serum albumin is added. Blocking buffers are necessary to saturate unoccupied binding sites and thus minimize non-specific binding and non-specific protein–protein interactions. A standardized blocking buffer has not been identified as suitable for all assays. An ideal blocking buffer must have no cross-reactivity with other assay components, minimize denaturation and exhibit low enzyme activity. The two major classes of blocking buffers are proteins (e.g. bovine serum albumin, casein) and detergents (e.g. Tween 20, Triton X-100). Testing is required to choose the best blocking buffer and optimize the protocol. The blocking buffer choice is mainly influenced by the specific assay components and the plate surface chemistry. Following this step direct and indirect ELISAs deviate in protocol.

In a direct ELISA a primary detection antibody with a conjugated enzyme is added, which binds to the antigen that has coated the well (*Figure 1b*). The plate is incubated for sufficient time to permit antigen–antibody binding and washed with phosphate buffered saline to remove excess antibody. A substrate for the enzyme is then added, time is allowed for the enzyme–substrate interaction in a dark environment and the reaction is stopped with a specific solution. The enzyme–substrate interaction leads to colour formation that can be detected by a microplate reader (*Figure 1c*). For analysis, the sample readings are compared to the standard curve.

In an indirect ELISA following antigen immobilization (*Figure 2a*), blocking and washing, a primary antibody is added which binds to the antigen (*Figure 2b*). The primary antibody is attached to the plate using the carbonate-bicarbonate coating buffer. The plate is then incubated and washed with a washing buffer followed by addition of a blocker and a secondary antibody with a conjugated enzyme (*Figure 2c*). Similarly to the direct ELISA, the plate is then incubated, washed, a substrate is added and a microplate reader is used to scan the wells (*Figure 2d*). The secondary antibody can also be labelled with a fluorophore, and the result quantified under ultraviolet light using a fluorometer. This array is known as a fluorescence-linked immunosorbent assay (FLISA). Indirect ELISAs are used for point-of-care HIV testing and give near-instant results in a clinic setting (*Figure 3*).

Direct ELISA is a fast diagnostic tool. However, it is unable to allow signal amplification and therefore has a low sensitivity. It is best used when there is a high proportion of the antigen being assessed in the sample. The development of avidin/streptavidin-biotin complexes (ABC) has allowed samples with a low proportion of antigen to be analysed. Biotin tags linked to fluorophores are used to label secondary antibodies in indirect ELISAs. Avidin or streptavidin molecules are able to bind up to four biotin tags each, allowing multiple ABCs to be attached to one secondary antibody. This allows stronger signal detection and increases the sensitivity of the assay. However, if the ABC is too large it may be unable to penetrate certain tissues.

The main disadvantage of direct ELISAs is that a specific antibody must be used for each ELISA assay. There is no flexibility in the choice of antibody, and labelling each antibody is time consuming and can be expensive. Indirect ELISAs allow many different primary antibodies from one species to be used. All of the primary antibodies can be labelled with the same secondary antibody. A large range of secondary antibodies is commercially available. However, cross-reactivity may occur with the secondary antibody. This results in a non-specific signal and can be quantified using calibration curves to compare the assay relative to the analyte being detected.

Sandwich ELISA

More complex solutions are analysed by sandwich ELISA. The name of the assay arises from the setup being able to 'sandwich' an antigen between two antibodies (*Figure 4*). The coating buffer solution containing a capture antibody is added to a well plate and is allowed to adhere (*Figure 4a*). Following incubation, the plate is washed and the blocking buffer is added to block any remaining binding sites on the well. The sample is then added to each well and incubated for a specific length of time (*Figure 4b*).

To ensure the accuracy of results, a standard sample (positive control) and a blank sample (negative control) must be run with each plate. A detection antibody is then added to each well (*Figure 4c*), following which a conjugated secondary antibody and blocking buffer are added (*Figure 4d*). Between each step the wells are washed with phosphate buffered saline. A substrate solution is added and a microplate reader is used to detect the result (*Figure 4e*). Sandwich ELISAs are commonly used in point-of-care tests, such as in home pregnancy kits (*Figure 5*).

Figure 4. Sandwich ELISA protocol.

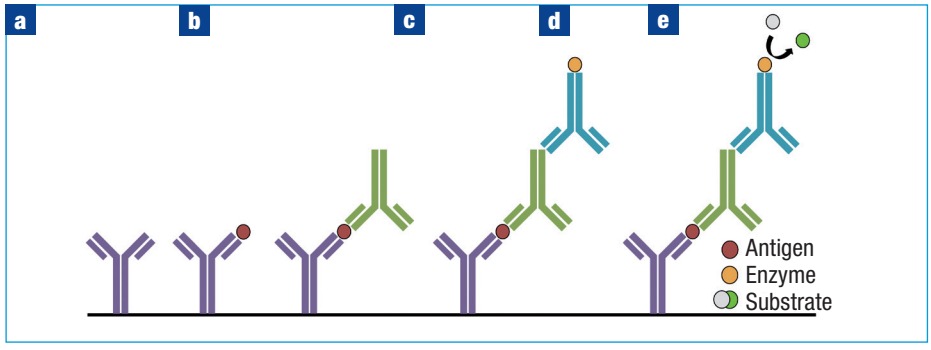
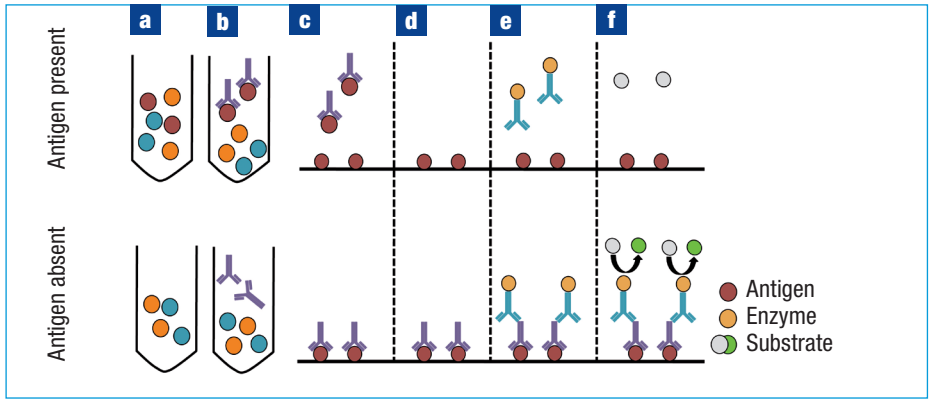


Figure 6. Competitive ELISA protocol.



The advantage of a sandwich ELISA is its higher specificity for analyte detection. The capture antibody immobilizes the specific antigen in the sample, so the sample does not need to be purified beforehand, unlike in direct and indirect ELISAs. By using two antibody recognition steps the antigen is effectively sandwiched between the primary and secondary antibodies. The main disadvantage of a sandwich ELISA is that particular primary and secondary antibodies must be used. The primary and secondary antibodies bind to different epitopes on the antigen and must be validated to work together as 'matched pairs' to prevent competition for antigen binding sites. A sandwich ELISA is also more time consuming and more expensive than direct and indirect ELISAs.

Competitive ELISA

A competitive ELISA is different to direct, indirect and sandwich ELISAs as it uses a competitive binding process. The primary antibody is incubated with an unpurified sample (*Figure 6a*) and binds to any antigen present in the sample (*Figure 6b*). The more antigens present in the sample, the more antigen–antibody complexes are formed. The antigen–antibody complexes are then added to a 96-well plate that is pre-coated with the antigen. Any unbound antibodies will bind to the antigen in the well (*Figure 6c*). The more antigen–antibody complexes are formed the fewer antibodies will be available to bind to the antigen in the well. Hence, there is competition between the antigens in the sample and those pre-coated in the well for the antibodies. The plate is incubated for a specific period of time and then washed with phosphate buffered saline to remove any unbound antibody and a blocking buffer is added (*Figure 6d*). A secondary antibody conjugated with an enzyme is added and binds to the primary antibody (*Figure 6e*). Between steps the plate must be washed. Finally, a substrate is added and a microplate reader is used to detect a colour change (*Figure 6f*). Competitive ELISA produces an inverse curve, such that a high amount of antigen in the sample yields a lower signal. Competitive ELISAs can be used to detect anti-drug antibody levels in the serum of patients being treated with anti-tumour necrosis factor (TNF) drugs for rheumatoid arthritis and inflammatory bowel disease (*Figure 7*) (Hock et al, 2015).

Competitive ELISAs are used for unpurified samples that often require no more than centrifuging to remove particulates, and can measure a larger range of antigens in a sample than a sandwich ELISA. Competitive ELISAs are used to detect small molecules that do not have multiple epitopes. However, competitive ELISAs have a low specificity, so should not be used for dilute samples. Sandwich ELISAs would be more appropriate in this situation.

Figure 5. Point-of-care pregnancy testing.

At home pregnancy kits use a sandwich ELISA to give rapid, patient-adjacent results. The test strip contains anti-beta-human chorionic gonadotropin (anti-βhCG) and conjugated antibodies that react with βhCG present in the urine sample. The strip is washed with tap water to remove any unbound sample and incubated with the substrate reagent for a few minutes. The presence and intensity of the colour signal is proportional to the amount of βhCG in the sample and can be used to estimate the gestational age.

Figure 7. Monitoring patients receiving anti-TNF therapy.

Patients with rheumatoid arthritis and inflammatory bowel disease often receive treatment with anti-tumour necrosis factor (anti-TNF) such as adalimumab and infliximab. However, there are limited methods to monitor the serum level of both drug and anti-drug antibodies in these patients. Hock et al (2015) developed a competitive ELISA that measured both drug and anti-drug antibody levels using an assay that allowed competitive inhibition between anti-drug antibodies and drug binding to solid phase TNF in vitro. It was tested in patients with rheumatoid arthritis and inflammatory bowel disease. The assay was unaffected by rheumatoid factor and was specific for anti-drug antibodies. It is hoped that this sort of testing could be used when monitoring patients taking adalimumab and infliximab.

Optimization

ELISAs can be optimized by changing the assay parameters to increase the sensitivity or specificity, generate results faster and increase the signal strength. Optimization ensures that no component or variable of the ELISA is present in a limiting concentration, and aims to increase the sensitivity of the assay. More than one variable of the assay can be optimized (e.g. capture antibody concentration, antigen concentration and detection antibody

Table 2. Troubleshooting

Problem	Possible source	Action
Low signal (samples and standard curve)	Old coated plates or reagents	Prepare new plates and reagents (check pH). Store appropriately
	Plate washings too vigorous	Pipette gently
	Wells dried out	Cover plate using sealing film
	Insufficient development	Optimize development temperature and time
	Incorrect wavelengths	Check filters and software on microplate reader
Low signal (samples only)	Target below level of detection	Decrease dilution factor or concentrate samples
	Incompatible sample type	Ensure compatibility using a positive control
Low/poor signal (standard curve only)	Standard inappropriately reconstituted or stored	Reconstitute new standard, store at -70ºC
	Standards added incorrectly	Check for pipetting errors
	Incorrect calculation of dilutions	Check calculations
Positive result in negative control	Sample contamination	Use fresh reagents and pipette carefully
	Inadequate washing	Increase washing steps
	Detection antibody interacts with coating antibody (in sandwich ELISA)	Ensure the antibodies do not cross-react
High background	Background wells contaminated	Pipette carefully, use multichannel pipettes
	Inadequate washing	Increase washing steps
High signal	Samples contain antigen above assay range	Dilute samples
	Inadequate washing	Increase washing steps
	Oversaturated samples	Decrease incubation time or temperature
High variation	Bubbles in wells	Ensure no bubbles present before reading
	Inconsistent pipetting	Use calibrated pipettes and careful pipetting
	Edge effect (i.e. signal near edge statistically different to central wells)	Ensure wells have the same temperature and humidity, use plate sealers and shaking
	Non-homogeneous samples	Thoroughly mix samples before pipetting
	Stacked plates	Do not stack plates

concentration) by titrating one component in each dimension of the well (e.g. X-axis: antigen, Y-axis: antibody). A confirmatory test, e.g. western blot, can be used to validate ELISA optimization.

Troubleshooting

Table 2 outlines possible problems which can be faced when performing an ELISA and their solutions.

Future developments

During the 2014 Ebola outbreak, ELISA was used to diagnose the disease within days of symptom onset. However, accurate results relied on an antigen detection threshold, limiting the effectiveness of ELISA testing in a disease that required immediate diagnosis and management (Kaushik et al, 2016). The commercialization of newer ELISA techniques will allow the production of kits that will be able to overcome problems like these.

de la Rica and Stevens (2012) developed an ELISA that used nanoparticle detection of ultralow antigen concentrations (1 x 10^{-18} g/ml). They could detect (but not quantify) both prostate-specific antigen and the p24 HIV antigen at concentrations undetectable by standard tests. Murdock et al (2013) have eliminated the need for microplate readers by using automated image analysis on Windows and Android mobile technology, making ELISAs more tractable in the field.

KEY POINTS

- Enzyme-linked immunosorbent assays (ELISAs) can detect analytes using a combination of antibodies and enzyme-substrate complexes.
- Measurement of the optical density can be used to quantify analyte concentrations in the sample.
- Different types of ELISAs are available with distinct advantages and disadvantages.
- Optimization ensures that no component of the assay is present in a limiting concentration.
- ELISAs are used in laboratory, point-of-care and at-home pregnancy testing.

Conclusions

ELISA is a diagnostic test used to detect analytes from an array of samples. It can detect and quantify biological processes and can rapidly provide accurate results in an increasingly cost-efficient manner. **BJHM**

Conflict of interest: none.

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