Frequently Asked Questions about ELISAs

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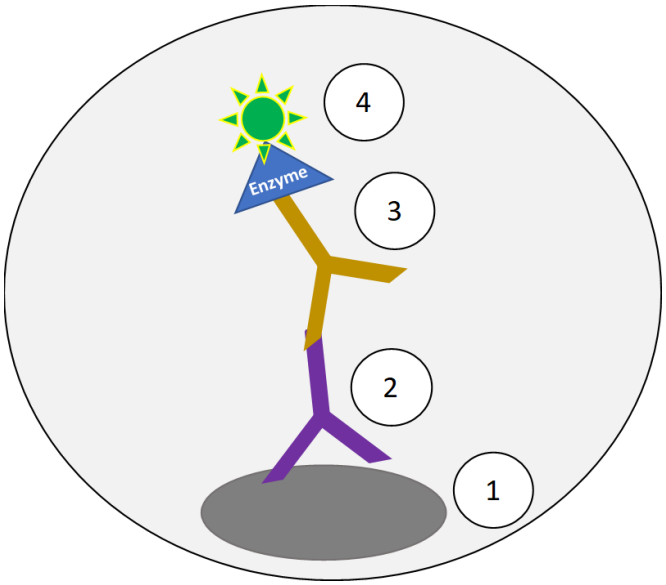
BACKGROUND

Enzyme Linked Immunosorbent Assays (ELISA) are a type of immunoassay commonly used to quantify levels of a specific analyte within a sample, typically cell culture supernatant, cell lysate, serum, plasma, saliva, or urine. This assay methodology was initially reported by Engvall and Perlmann in 1972 and revolutionized the quantitative method based on antigen-antibody reactions. Engvall and Perlmann substituted the radioisotopes in the radioimmunoassay technique with enzymes, making the assay safe and easy to use (1-3). The ELISA method relies on the antigen or anti-antigen antibody adhering to a solid phase such as a rigid polymer of beads or plates. Currently the most common solid phase for the ELISA is a plastic 96-well ELISA plate (2,3). Standards, controls, and samples are added to the wells and the plate is incubated for a set time. This incubation allows for the analyte of interest from the samples to bind with the immobilized antigen or antibody in the wells. Next, a detection antibody, usually conjugated with enzymes such as horse radish peroxide (HRP) or alkaline phosphatase (AP) is added to the well and incubated for a specific time to allow binding of the detection antibody to analytes. The assay is finalized by adding a substrate/chromogen, such as 3,3',5,5'-tetramethylbenzidine (TMB) or o-phenylenediamine dihydrochloride (OPD) which reacts with the conjugated enzyme and elicits a signal that is proportional to the amount of analyte bound in the assay.

There are several ELISA formats, including direct, indirect, sandwich, and competitive (3). Each ELISA type should be considered carefully as they each have their own benefits and pitfalls. Below is a description of the main ELISA types offered by Chondrex, Inc.

INDIRECT ELISA

The aim of an indirect ELISA is to quantify the levels of antibodies specific for an antigen in samples. Therefore, the plate wells are ***coated with the antigen*** and a positive signal means the samples contain antibodies against the antigen. Chondrex, Inc provides important information in an article regarding false positive and negative results typically obtained in indirect ELISAs (4,5).



Indirect ELISA

1. Antigen of interest is coated on the plate
2. Sample containing antibody of interest is added. If the antibody of interest has an affinity for the antigen of choice, the antibody should bind the antigen bound on the plate.
3. Secondary antibody against immunoglobulin of the sample species is added. This secondary antibody has a conjugated enzyme which will react to the substrate.
4. Substrate is added which makes quantification of the protein possible.

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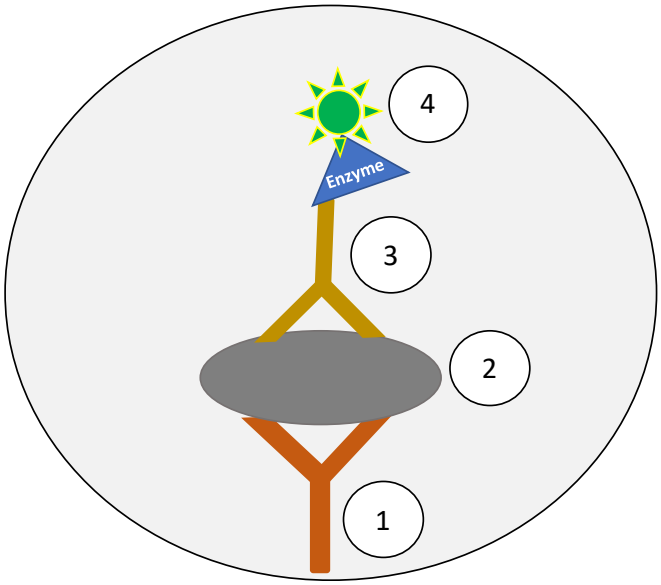
Pros of Indirect ELISA	Cons of Indirect ELISA
Great signal amplification as multiple antibodies in the sample can bind to the antigen.	Cross reactivities of secondary antibody against different immunoglobulin subtypes or subclasses.
High flexibility as secondary antibody can be used for several samples	Immunoglobulin in samples may non-specifically bind to the plate, causing false positive results.
	Need high purity antigens for coating plates to detect antigen-specific antibodies.
	Different subclass immunoglobulins in samples may compete for the same antigen, resulting in false negative results

SANDWICH ELISA

The goal of a sandwich ELISA is to quantify the levels of a specific antigen in samples. For this ELISA type, the plate wells are ***coated with antibodies (capture antibody) specific to the antigen of interest***. A positive signal indicates the sample contains the antigen of interest.



Sandwich ELISA

1. Capture antibody is coated on the plate.
2. Sample containing antigens of interest is added. If antigens are present, the antibody will "capture" the antigens.
3. Detection antibody is added. This antibody usually recognizes a different epitope than the capture antibody on the same antigen. This antibody has a conjugated enzyme which will react to the substrate.
4. Substrate is added which makes quantification of the antigen possible.

Pros of Sandwich ELISA	Cons of Sandwich ELISA
High specificity as it involves two antibodies detecting different epitopes on the same antigen.	Assay sensitivities and specificities depend on the quality of the antibodies used in ELISA.
High sensitivity and flexibility	The antigen of interest must be large enough so that two different antibodies can bind to it at different epitopes. If the antigen forms a dimer, the same antibody may work for both the capture and/or detection antibody.
	Samples may contain antibodies against the antibody subclass of the antibodies in assays, such as human anti-mouse IgG antibodies. These antibodies can cause false positive results.

ELISA TROUBLESHOOTING

Scientists at Chondrex, Inc. provide educational and convenient protocols with each of our ELISA kits. Sometimes common issues can be experienced while running an ELISA and here we provide a table describing those common issues. If the suggested troubleshooting does not improve assay results, please contact Chondrex Inc. for further assistance.

Customer and Technical Support Email: support@chondrex.com
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Problem: High Background or Signal

Possible Cause	Suggestion Solution
Non-specific binding due to insufficient plate blocking	Immunoglobulins or antigens in samples may non-specifically bind to the wells. The plate must be blocked with appropriate blocking agents that mask antigen/antibody non-coated space in wells. If developing your own assay, appropriate blocking agents and sample dilution buffers are necessary. Chondrex, Inc. recommends using ChonBlock™ ELISA Buffer (Cat # 9068) as a blocking agent, as this buffer was developed to substantially reduce background interference in indirect ELISAs.
Residual solution in well plates after washing	At the end of each washing step, invert plate on absorbent tissue and allow to completely drain, tapping forcefully to remove any residual fluid.
Insufficient Wash Buffer volume was used	Make sure wells are filled to the brim during each wash. This is important as the top half of the wells usually do not receive blocking reagents and these areas must be washed in each step. On a 96 well-plate, 300 µL or more per well/per wash is recommended.
Insufficient salt concentration was used in Wash Buffer	Salts in Wash Buffer help reduce hydrophilic non-specific binding of sample components to the plate. Crystals may form in the 20X wash buffer when stored at cold temperatures. If crystals have formed, warm the wash buffer by placing the bottle in warm water until crystals are completely dissolved. Then dilute to 1X in the proper solution.
Longer incubation times than recommended	Unnecessarily longer incubation times increase background levels without increasing assay sensitivity. Follow incubation times according to protocol instructions.
Sample components non-specifically bind well plates	Components in serum samples can bind non-specifically causing false positive results in indirect ELISAs. Chondrex, Inc. recommends running antigen non-coated wells in addition to antigen-coated wells and subtracting the positive signals of the non-specific background levels from the values determined in the assay. Please refer to the following article for more information.
Background wells were contaminated	Do not re-use pipette tips for different samples in wells. Change tips frequently and if possible, use filter tips. Avoid cross-well contamination by carefully placing and removing the plate sealer.

Antigen in samples were too concentrated	Dilute samples further. Chondrex, Inc recommends a pilot study using three wide ranges of sample dilutions to obtain an appropriate sample dilution.
Incorrect pipetting technique	When pipetting reagents, carefully distribute reagents to the bottom of the well. If pipet tips touch the top half of the wells which tend to not receive blocking reagents, they may increase non-specific reactions of the analytes or chromogens.
Dry wells	Do not allow wells to dry out once the assay has started. Dried wells cause non-specific binding of analytes or loss of reagent activity. After wash steps, immediately load reagents into wells. Cover the plate using sealing films, plate lids, or food wrap for all incubations. Do not reuse the plate sealer for the next assay as that can propagate sample contamination.

Problem: Low or No Signal

Possible Cause	Suggestion Solution
Plate coating	Immediately distribute diluted coating solution to wells.
Coated plates stored incorrectly	Depending on the purchased kit, please follow protocol for plate coating (if needed), short-term, and long-term storage.
Reagents not at room temperature	Assay temperature may be critical in some ELISA. It is recommended that all reagents be brought to room temperature before starting the assay. Allow reagents to sit for 30 minutes or longer to reach room temperature. If reagent is light sensitive, store properly.
Reagents lost activity due to incorrect reagent storage	New and partially used reagents should be kept at –20°C, unless otherwise stated in the protocol. If the reagent is light sensitive, store properly. Depending on the purchased kit, follow protocol for plate coating (if needed), short-term, and long-term storage.
Incorrect reagents were added/prepared	Ensure reagents were added in the proper order and prepared to the correct dilution. Note: OPD and TMB can oxidize easily, hence preparing these chromogen solutions immediately prior to use is important.
Incorrect pipetting technique	Pipetting technique is essential for a successful assay. Check that the pipette is capturing the correct pre-set volume. Chondrex, Inc. provides extra volume in all reagents to help with measurement error.
Wells were scratched with pipette tips or washing tips	Use caution when dispensing into and aspirating out of wells. Automated plate washers may need to be calibrated to prevent tips from touching the bottoms of the wells.
Plates were read at incorrect wavelength	Manufactured kits have optimized protocols. Make sure to use the recommended wavelength/filter. Ensure the plate reader is accurately set for the type of substrate being used.
Dry wells	Do not allow wells to dry out once the assay has started. Dried wells cause non-specific binding of analytes or loss of reagent activity. After wash steps, immediately load reagents into wells. Cover the plate using sealing

	films, plate lids, or food wrap for all incubations. Do not reuse the plate sealer for the next assay as that can propagate sample contamination.
Plate washing is too vigorous	Check the correct pressure in the automatic plate washer. If washes are done manually, pipette wash buffer gently.

Problem: Poor Standard Curve

Possible Cause	Suggestion Solution
Coated plates stored incorrectly	Depending on the purchased kit, follow protocol for plate coating (if needed), short-term, and long-term storage.
Standard was poorly mixed or was incorrectly stored	Reconstitute standard according to the protocol and make sure resuspension is complete. Partially used reagents should be kept at –20°C, unless otherwise stated in the protocol.
Incorrect pipetting technique	Pipetting technique is essential for a successful assay. Check that the pipette is capturing the correct pre-set volume. Chondrex, Inc. provides extra volume in all reagents to help with measurement error.

Problem: Inconsistent Results Within Assay or Assay-to-Assay

Possible Cause	Suggestion Solution
Plate coating	Immediately distribute diluted coating solution to wells.
Coated plates stored incorrectly	Depending on the purchased kit, follow protocol for plate coating (if needed), short-term, and long-term storage.
Incorrect pipetting technique	Pipetting technique is essential for a successful assay. Check that the pipette is capturing the correct pre-set volume. Chondrex, Inc. provides extra volume in all reagents to help with measurement error.
Non-homogenous samples	Thoroughly mix samples before pipetting. Serum or plasma samples may contain insoluble materials or fats and these components may prevent antigen-antibody reactions. The samples must be centrifuged, and the supernatant used for assays.
Residual solution in well plates after washing	At the end of each washing step, invert plate on absorbent tissue and allow to completely drain, tapping forcefully to remove any residual fluid.
Plate sealers were not used or reused	Dried wells tend to have higher signal. Cover the plate using sealing films, plate lids, or food wrap for all incubations. Do not reuse the plate sealer for the next assay as that can propagate sample contamination.
Bubbles present	Bubbles prevent antibody-antigen reactions. Pipette carefully and ensure no bubbles are present prior to reading the plate.
Dry wells	Do not allow wells to dry out once the assay has started. Dried wells cause non-specific binding of analytes or loss of reagent activity. After wash steps, immediately load reagents into wells. Cover the plate using sealing films, plate lids, or food wraps for all incubations. Do not reuse the plate sealer for the next assay as that can propagate sample contamination.
Inconsistent plate incubation and placement	When several plates are assayed at the same time, make sure all plates have the same incubation conditions and avoid stacking plates.
Inconsistent light source through assays	Incubations should take place in subdued light or in the dark.

REFERENCES

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