MABTECH

ELISA

Troubleshooting: tips and tricks

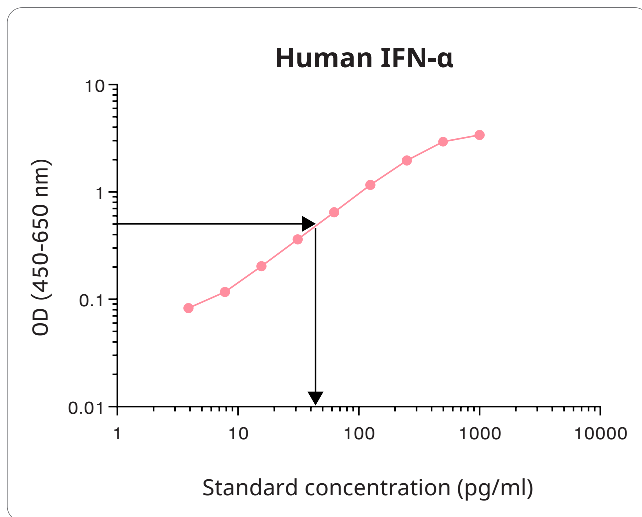
ELISA is a straightforward technique used to quantify proteins in solution. However, things don't always end up as they should. We're here to help you tackle ELISA pitfalls by breaking them down to their common causes.

This will save you time and frustration!

Problem with ELISA standard curve

The standard curve allows you to determine the concentration of your sample, making it a central component. Sometimes, things don't go as planned, but don't worry, we are here to help! Let's dive into some possible reasons behind a poor standard curve and how to overcome them.

Problem: Poor standard curve	
Potential cause	**Suggestion**
Standard reconstitution error	Check protocol for how to prepare the standard: Check whether the correct buffer and volumes were used for standard reconstitution. (Volumes might vary between batches.) Lyophilized (freeze-dried) standards: Resuspend and incubate typically for 5 minutes, then mix (for example, by vortexing), check for undissolved material.
Standard degraded	Check storage instructions and expiry date. After standard reconstitution, prepare aliquots, don't freeze-thaw aliquots.
Inaccurate standard curve	Check calculations of standard reconstitution and dilution series. (Luckily, Mabtech's protocols already include a dilution scheme.) Check for pipetting errors.
Plate development	Check development protocol, substrate, wavelength.



Example:
Mean standard curve for human IFN-α ELISA using TMB substrate.

No signal or weak signal

You are eagerly awaiting the results of your ELISA experiment, just to find out that there is no or a weak signal. Shoot! But don't despair. Go through our checklist to find out whether any of these reasons could explain your results.

Problem: No signal or weak signal	
Potential cause	**Suggestion**
Incorrect assay setup – reagent missing or added in the wrong order	Check the protocol.
Wrong substrate	Check the substrate.
The capture antibody did not bind properly to the plate	Use ELISA plates, not tissue culture plates. Check protocol for concentration and buffer. Tip: Avoid any coating doubts by using pre-coated plates.
Incorrect antibody pair	Review antibodies.
Analyte not present	Include appropriate positive controls.
Samples diluted too much	Dilute samples less. (However, dilute samples at least 2x.) Tip: Run a pilot experiment to identify reasonable dilutions when the expected concentration range is unknown.
Samples absorbed during dilution steps	Don't use ELISA plates for sample preparation. (They bind proteins.) Use low protein-binding plastics for sample preparation.
Reagents prepared too early	Dilute detection antibody and Streptavidin-enzyme conjugate just before use.
Enzyme inhibitors present, for example, sodium azide inhibits HRP	Prepare fresh buffers.
Plate coated for too long	We recommend coating overnight at +4 °C and using the plate the next day. Shorter or longer coating times may work, but this varies from antibody to antibody.
Incorrect temperature	Perform all incubation steps at room temperature. Bring all buffers, except substrate to room temperature before use.
Plate reading too late	Kits with HRP: Read the plate directly after adding the stop solution.
Incorrect reader settings	Check wavelength. Note that the correct wavelength depends on the substrate used. If possible, subtract a reference wavelength of 650nm. This corrects for errors in the plastic.
Antigen poorly bound or denatured, epitope hidden	This only applies when you run a direct/indirect ELISA (an ELISA where you coat the plate with antigen). Test different plates. Test different coating protocols. Tip: The right buffer (pH) can make all the difference. Consider reverse setup. Read more about detecting immunoglobulins with ELISA.

Signal too strong

If maximum OD values are seen in your samples, you won't be able to determine the concentrations correctly. In such cases, repeat the experiment and try to solve the issue by altering one or more parameters. It can be as easy as diluting the sample more or shortening the development time, but finding the cause behind an unspecific high signal might be trickier. Hopefully, the list below helps you speed up the troubleshooting process.

Problem: Too strong of a signal from samples	
Potential cause	**Suggestion**
Sample OD above range: samples not sufficiently diluted	Dilute samples more. Tip: Run a pilot experiment first to identify reasonable dilutions when the expected concentration range is unknown.
Heterophilic interference	If you're analyzing human plasma/serum samples, we recommend the use of our ELISA diluent. This buffer prevents false-positive results caused by heterophilic antibodies, commonly found in human blood. Plasma/serum from patients with autoimmune diseases can contain large amounts of interfering antibodies, which makes the ELISA diluent insufficient to block false-positive signals. For such samples, we recommend our ELISA PathRF kits.

Problem: High background	
Potential cause	**Suggestion**
Insufficient or inconsistent plate washing	If using an automated plate washer, check that the correct program is used. Also, check whether all ports of the ELISA washer are unblocked/functional. After each wash cycle, tap the plate against paper towels to remove as much liquid as possible. Check the wash steps in the protocol.
Contamination of reagents	Prepare fresh buffers. The substrate should be colorless.
Incomplete blocking	Check the protocol. Try different blocking reagents or different manufacturers of ELISA plates.
Unspecific binding **(for example, too much antibody used, detection antibody binds capture antibody; too much Streptavidin-enzyme conjugate used)**	Antibody: Check protocol for recommended antibody concentrations, confirm the specificity of antibody pair. Streptavidin-enzyme conjugate: Check protocol for recommended dilution.
Substrate incubation under light exposure	Incubate plates in the dark after adding the substrate.

Problem: Precipitates (black/green) after adding substrate	
Potential cause	**Suggestion**
Too concentrated analyte (target protein)	Dilute samples more.

Large intra-assay variation

The coefficient of variation (CV) is a statistical term describing the deviation between replicates. To be precise, CV is calculated by dividing the deviation by the mean, expressed in %. If you observe this in one plate, it's described as intra-assay variation. Observing a large spread between your data points could make you question your results, so you'd want to figure out what went wrong.

A rule of thumb would be that any CV above 10% for values in the standard range, not the negative control, should be considered unacceptable. You might encounter this problem, especially for samples with low OD values. (A detail to bear in mind is that some ELISA analysis software calculates the CV values for the standard curve differently than for unknown samples. For the CV of the standard curve, the software might use the OD values itself, while for the unknown samples, it performs a back calculation to the concentration.)

Problem: Large intra-assay variation	
Potential cause	**Suggestion**
Too few replicates	Use at least duplicates in ELISA, for the standard curve as well as the samples. A higher number of replicates leads to lower CV values.
Uneven evaporation	Use plate sealers during all incubation steps.
Contamination	Use plate sealers during all incubation steps, clean plastics, properly stored reagents, and a clean work environment.
Sample preparation	Prepare enough standard dilutions and sample dilutions; mix well.
Poor replicates	Check the protocol and your pipettes to avoid pipetting errors.
Bubbles during plate reading	Check for bubbles. And, if needed, pop with a pipette tip.
Dirty plate	Wipe the plate bottom before placing the plate into the ELISA reader.

Large inter-assay variation

Inter-assay variation is a statistical term describing the difference between separate experiments. A considerable inter-assay variation translates to poor reproducibility between different experiments. If you observe such an inter-assay CV increase, you might get suspicious about the reproducibility of your results. But before you doubt the method, go through this checklist to eliminate common causes.

Problem: Large inter-assay variation	
Potential cause	**Suggestion**
Differences in protocols and materials used	Use the same plates, antibody batches, reagents, buffers, temperatures, and protocol.
Reagent expired or incorrectly stored	Check your reagents and their storage conditions.
Standard not intact	Avoid freeze-thawing cycles.
Differences in plate coating	Use the same plates, antibody batch, and protocol. Tip: Our ELISA Pro kits include pre-coated plates and all required buffers. This supports reproducibility across experiments.

Do you have more questions regarding ELISA? Please contact us.

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