Overview

ELISA Troubleshooting

Several common issues can occur when running an ELISA. These are detailed in the following table, along with suggested solutions.

No signal or weak signal	
Capture antigen or antibody may not have adhered to the microplate	Check the binding capacity of the microplate according to the manufacturer's description Try using a different coating buffer Increase the incubation time for plate coating
Concentration of analyte-specific antibodies may be too low	Increase the concentration of the capture antibody and/or the analyte-specific detection antibody Increase the incubation time for analyte-specific antibody binding
Analyte-specific detection antibody and secondary antibody may be incompatible	Confirm that the host species of the analyte-specific detection antibody is compatible with the secondary antibody (for example, if the analyte-specific detection antibody was raised in rabbit, an anti-rabbit secondary antibody is required for detection)
Capture and detection antibodies may be competing for the same epitope (sandwich ELISA)	Confirm that the capture and detection antibodies recognize different epitopes Switch to using a matched antibody pair if available Consider using a different ELISA format (e.g. try using an indirect ELISA that requires only one analyte-specific antibody rather than a sandwich ELISA)
Fluorophore-conjugated antibodies may have been compromised by exposure to light	Ensure fluorophore-conjugated antibodies are stored correctly Protect fluorophore-conjugated antibodies from light when adding them to, and incubating them in, the microplate wells
If only the standard wells (and not the sample wells) are affected, the standard may have degraded	Try using a fresh vial of standard Check that the standard has been prepared and stored correctly
Azide (often added to antibody storage buffers as a preservative) may be inhibiting HRP activity	Ensure sufficient washing to remove any residual traces of azide

No signal or weak signal	
Sample material may contain only low levels of the target analyte	Obtain more concentrated samples
	Spike samples with a known amount of analyte to check the sample matrix is not a source of interference
ELISA kits / kit components may have been stored incorrectly	Check the manufacturer's instructions for storage
Plate may have been read at an incorrect wavelength	Check the reader settings are compatible with the chosen detection method
High Background	
Washing may be inadequate	Increase the number and/or duration of wash steps
	Try adding detergent (e.g. 0.01-0.1% Tween-20) to wash buffers
Blocking may be insufficient	Try using a more concentrated blocking solution
	Increase the incubation time for blocking
	Switch to using a different blocking buffer
Sample may be too concentrated	Try diluting the sample
Antibody concentration(s) may be too high	Decrease the concentration of the capture antibody, analyte-specific detection antibody, or secondary antibody
	Decrease the antibody incubation time
Colorimetric substrates may have been prepared too early	Always prepare substrates such as TMB immediately prior to use to avoid unwanted color development
Microplates may have sat around after the addition of stop solution (colorimetric detection)	Read colorimetric assays as soon as the stop solution has been added
Consumables such as pipette tips, reservoirs or buffers may have introduced contamination	Use fresh plasticware for each step
	Prepare fresh buffers for each assay
Incubation times may have been too long	Always follow the protocol and be consistent with reagent additions and timing
Poor reproducibility between plates	
Plates may have been coated unevenly	Ensure all solutions are thoroughly mixed before coating the plates
	Seal plates after adding the coating solution to prevent evaporation; such effects can especially be noticeable in edge wells
	Check pipettes have been calibrated and are performing as expected
Washing may be inadequate	Increase the number and/or duration of wash steps
	Confirm wells are fully emptied between washes
Wells may contain bubbles	Centrifuge microplates briefly prior to reading
Plate seals may be a source of cross-contamination	Always use fresh plate seals between incubations

	Poor reproducibility between runs
Reagents may have degraded	Prepare fresh reagents (including buffers) for each assay Check that the standard has been prepared and stored correctly
Samples may have been handled incorrectly	Always store and handle samples with care and avoid repeat freeze-thawing
Assay conditions may be inconsistent	Ensure all protocol steps are performed reproducibly Always run ELISAs under stable environmental conditions
	Edge effects / plate drift
The plate reader may be misaligned	Read the plate, then rotate it by 180o and read again; if the effect remains in the same position, the reader may need to be repaired by a qualified service engineer
Solutions may be cold	Ensure all solutions are at room temperature upon addition to the microplate unless otherwise stated in the protocol
Delays may have occurred during reagent addition	Prepare suitable quantities of reagents (including dead volumes) for the assay to avoid running out part-way across a plate
Volumes may be uneven across the microplate	Seal plates between reagent additions to prevent evaporation Only use calibrated pipettes
Plates may have cross-contaminated one another	Avoid stacking plates during incubations

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