ELISA TroubleShooting Guide

Problem	Possible Cause	Solution
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Incorrectly prepared, incomplete or wrong substrate	Make sure that the substrate selected is appropriate for the enzyme conjugate (such as pNPP for alkaline phosphatase and OPD or TMB for peroxidase; see substrate selection guide). Make sure that fresh H_2O_2 is added if necessary.
	Washes too stringent	Use an automated plate washer if available. Eliminate or reduce detergent concentration in washing buffer.
	Incubation times inadequate	Incubation times should be appropriate for the system. Typical substrate development times vary from 10-30 minutes.
	Substrate or conjugate is no longer active or is weak	Test conjugate and substrate for activity
	Enzyme inhibitor present	Sodium azide will inhibit peroxidase reactions.
	Plate reader settings not optimal	Verify the wavelength and filter settings in the plate reader
	Incorrect assay temperature (too cold)	Use recommended incubation temperature. Bring substrates to room temperature before use.
	Inadequate volume of substrate	Check to make sure that correct volume is delivered by pipette.
	Blocking protein included in the coating solution	Omit blocking protein from coating solution.
High background	Cross-Reactivity	Detection antibody cross-reacting with coating antibody. Run appropriate controls.
	Non-specific binding of antibodies	Use appropriate blocking buffer.
	Concentration of conjugated second antibody too high	Perform dilutions to determine optimal working concentration.
	Incorrect assay temperature	Check that the incubation temperature did not exceed 37°C.
	Inadequate washing	Ensure all wells are filling with wash buffer and are being aspirated completely. Use an automated plate washer if available.
	Contaminating enzymes present in sample	Test sample with substrate alone to check for contaminating enzyme activity.
Uneven color development,	Incomplete washing of wells	Ensure all wells are filling with wash buffer and are being aspirated completely. Use an automated plate washer if available.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps. Use an automated plate washer if available.
	Plates stacked during incubations	Keep plates separated if not rotating plates.
	Pipetting error, poor dilution series	Check pipetting technique and double-check calculations
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
	Poor or variable adsorption of reagents to plate	Check choice of coating buffer, usually PBS, pH 7.4 or carbonate-bicarbonate buffer, pH 9.6. Try extending incubating time. Consider different plates. Check homogeneity of samples.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.
	Technique problem	Proper mixing of reagents and wash steps are critical.
	Inappropriate ELISA plate used	If using fluorescence detection, appropriate plates must be used.