ELISA Experimental Problems and Solutions

Problem	Possible Source	Test or Action
High Background	Insufficient washing	Increase number of washes Add a 30 second soak step in between washes
No signal	Reagents added in incorrect order or incorrectly prepared	Repeat assay Check calculations and make new buffers, standards, etc.
	Contamination of HRP	Use fresh reagents
	Not enough antibody used	Increase concentration
	Standard has gone bad (if there is a signal in the sample wells)	Check that standard was handled according to directions. Use new vial.
	Buffer containing FCS used to reconstitute antibodies	Requalify your reagents of choice
	Capture antibody did not bind to plate	• Use an ELISA plate (not a tissue culture plate) • Dilute in PBS without additional protein
	Buffers contaminated	Make fresh buffers
Very low readings across the plate	Incorrect wavelengths	Check filters/reader
	Insufficient development time	Increase development time
	Coated plates are old and have gone bad	Coat new plates
	Capture antibody did not bind to the plate	• Use an ELISA plate (not a tissue culture plate) • Dilute in PBS without additional protein
	Buffer containing FCS used to reconstitute antibodies	Requalify your reagents of choice
Too much signal	Insufficient washing/washing step skipped - unbound peroxidase remaining	See washing procedure from our standard protocol
	Substrate Solution mixed too early and turned blue	Substrate Solution should be mixed and used immediately
	Too much streptavidin-HRP	Check dilution, titrate if necessary
	Plate sealers or reagent reservoirs reused, resulting in presence of residual HRP. This will turn the TMB blue non-specifically	Use fresh plate sealer and reagent reservoir for each step
	Buffers contaminated with metals or HRP	Make fresh buffers
Poor Duplicates	Insufficient washing	• See washing procedure from our standard protocol. • If using an automatic plate washer, check that all ports are clean and free of obstructions, add a 30 second soak step and rotate plate halfway through the wash
	Uneven plate coating due to procedural error or poor plate quality (can bind unevenly)	• Dilute in PBS without additional protein • Check coating and blocking volumes, times, and method of reagent addition. • Check plate used • Use an ELISA plate (not a tissue culture plate)
	Plate sealer reused	Use a fresh plate sealer for each step
	No plate sealers used	Use plate sealers
	Buffers contaminated	Make fresh buffers
	Insufficient washing	• See washing procedure from our standard protocol • If using an automatic plate washer, check that all ports are clean and free of obstructions, add a 30 second soak step and rotate plate halfway through the wash

Problem	Possible Source	Test or Action
Standard curve achieved but poor discrimination between points (low or flat curve)	Not enough streptavidin-HRP	Check dilution, titrate if necessary
	Capture antibody did not bind well to plate	• Use an ELISA plate (not a tissue culture plate) • Dilute in PBS without additional protein
	Not enough detection antibody	Check dilution, titrate if necessary
	Plate not developed long enough	• Increase Substrate Solution incubation time • Use recommended brand of Substrate Solution
	Incorrect procedure	Go back to General ELISA Protocol; eliminate modifications, if any
	Improper calculation of standard curve dilutions	Check calculations, make new standard curve
	Not enough streptavidin-HRP	Check dilution, titrate if necessary
No signal when a signal is expected, but standard curve looks fine	No cytokine in sample	• Use internal controls • Repeat experiment, reconsider experimental parameters
	Sample matrix is masking detection	Dilute samples at least 1:2 in appropriate diluent, or preferably, do a series of dilutions to look at recovery
Samples are reading too high, but standard curve looks fine	Samples contain cytokine levels above assay range	Dilute samples and run again
Poor assay to assay reproducibility	Insufficient washing	• See washing procedure from our standard protocol • If using an automatic plate washer, check that all ports are clean and free of obstructions
	Variations in incubation temperature	• Adhere to recommended incubation temperature • Avoid incubating plates in areas where environmental conditions vary
	Variations in protocol	Adhere to the same protocol from run to run
	Plate sealer reused, resulting in presence of residual HRP which will turn the TMB blue	Use fresh plate sealer for each step
	Improper calculation of standard curve dilutions	• Check calculations, make new standard curve • Use internal controls
	Buffers contaminated	Make fresh buffers
Edge Effects	Uneven temperatures around work surface	• Avoid incubating plates in areas where environmental conditions vary. • Use plate sealers
Drift	Interrupted assay set-up	Assay set-up should be continuous - have all standards and samples prepared appropriately before commencement of the assay
	Reagents not at room temperature	Ensure that all reagents are at room temperature before pipetting into the wells unless otherwise instructed in the antibody inserts