ELISA Troubleshooting Guide

When trouble shooting, prepare all fresh buffers. Sterile filter buffers containing BSA and allow all reagents to reach room temperature prior to use.

Problem	Possible Source	Suggestion
High Background/Non-specific Color Development	Non-specific binding of antibody	Modify blocking buffer used (e.g. substitute casein for BSA). Do not wash after blocking step; dump, blot & go directly to the next step.
	Plate not washed sufficiently	Be sure all wells are filled with buffer during every wash step. Be sure washing apparatus is working properly. Wash 3-5x between steps. Do not add additional washes. After final wash, blot plate forcefully on paper towel to remove residual buffer. Be sure the correct amount of Tween that was added to the wash solution (0.01-0.1% recommended).
	Contaminated Buffers	Prepare all buffers fresh (within 7 days of use) and sterile filter daily.
	Incubation temperature too high	Incubate at room temperature (25˚C) throughout whole procedure.
	Incubation time too long	Reduce incubation time.
	Contamination of the substrate with the conjugate or contamination of blank wells with positive control	Change pipette tips between reagents and use separate reservoirs. Ideally, wash buffer should be aspirated (pouring/dumping may lead to cross contamination).
	Concentration of detection antibody or avidin-HRP/streptavidin-HRP is too high	Check calculations or try further dilutions.
	Substrate exposed to light prior to use	Keep substrate in the dark until ready to dispense into wells.
	Wrong filter used when taking readings	Wavelength should be 405nm with a 650nm wavelength correction for ABTS, or 450nm with a 620nm wavelength correction for TMB, when using recommended plates.
	Read beyond time needed for development	Read at recommended time or time intervals in order to monitor blank O.D. readings.
Weak/No Color Development	A reagent or a step of the procedure omitted by mistake	Check protocol and follow steps carefully.
	Detergent concentration in wash buffer too high	Remove or decrease detergent (0.01-0.1% recommended).
	Plate washed inadequately	Decrease number of washes between steps, especially if not using automated or semi-automated washer. 1-2 washes may suffice.

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Problem	Possible Source	Suggestion
Weak/No Color Development (continued)	Enzyme inhibitor present in samples	Sodium azide inhibits peroxidase activity.
	Wrong incubation time or temperature	Check and follow protocol recommendations. Place plates in an incubator during incubation periods to avoid temperature fluctuations (set to 25˚C).
	Wrong substrate volume added to wells	Check pipette; use calibrated pipettes.
	Wrong enzyme-substrate system used	Check manufacturer specifications: avidin-HRP + ABTS or streptavidin-HRP + TMB.
	Conjugate or substrate inactive	Test activity in another system and check expiration dates.
	Incorrect storage of components	Store all components as recommended on data sheet.
	Wrong filter used when taking readings	Wavelength should be 405nm with a 650nm wavelength correction for ABTS, or 450nm with a 620nm wavelength correction for TMB, when using the recommended plates.
Poor Standard Curve	Incorrect preparation of standard	Reconstitute standard as suggested on data sheet. Dilute only with recommended diluent buffer unless alternative buffer has been optimized.
	Dilutions made too early	Prepare reagents immediately prior to use and add to wells promptly.
	Capture Antibody did not bind	Use ELISA-suitable plates (i.e. Nunc MaxiSorp Prod. # 439454 or Corning Prod. # 3590); not tissue culture plates.
	Inefficient washing	Be sure wash apparatus is working properly (i.e. distributing even volumes into each well). Be sure wells are empty after aspiration, yet be sure to fill wells in a timely manner.
	Pipetting error	Dispense quickly and identically into the side of each well. Use calibrated pipettes.
	Read beyond time needed for development	In general, reliable standard curves are obtained for ABTS Kits when O.D. readings do not exceed 0.2 for blanks or 1.2 for the highest standard concentration and for TMB Kits when O.D. readings do not exceed 0.15 for blanks.
	Incorrect storage of components	Store all components as recommended on data sheet. Do not allow reconstituted reagents to stay at room temperature for excess time.
Poor Precision	Insufficient mixing of reagents	Ensure adequate mixing of reagents before pipetting.
	Inefficient washing	Be sure wash apparatus is working properly (i.e. distributing even volumes into each well). Be sure wells are empty after aspiration, yet be sure to fill wells in a timely manner.
	Pipetting error	Dispense quickly and identically into the side of each well. Use calibrated pipettes.

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Problem	Possible Source	Suggestion
Poor Precision (continued)	Reused materials	Change pipette tips between samples, reservoirs between reagents, and plate sealers between incubation periods.
	Wells have been scratched by pipette tips or washing apparatus	Use caution when dispensing and aspirating.
	Precipitate in samples	Centrifuge vials prior to use.
	Unclean wells or debris	Inspect wells and remove debris prior to use. Wipe bottom of plate to remove any debris or fingerprints prior to reading.
Edge Effects	Evaporation	Use plate sealers between each step.
	Uneven temperature	Place plates in an incubator during incubation periods to avoid temperature fluctuations (set to 25˚C). Do not stack plates.