ELISA Troubleshooting Guide

Poor Precision Between Replicates for Standards and Samples (High CV Values)

Inadequate plate washing	For Automated Washers: Check nozzles for clogs, verify dispense volume, and perform regular cleaning to prevent and remove any buildup. Refer to owner's manual for additional maintenance information. For Manual Washing: Ensure that all wells are being forcefully overfilled with wash buffer. Refrain from using a multi-channel pipette for washing. Consider switching from a wash bottle to a hand-held manifold or automated plate washer, particularly when running **chemiluminescence assays**. A video displaying proper washing technique is available **here**. Do not skip wash or soak steps.
Problem with multi-channel pipette during the addition of the conjugate or substrate	Check multi-channel pipette calibration. Ensure all tips are affixed securely and drawing the same volume of liquid each time tips are changed. Watch for bubbles and consider a reverse pipetting technique to ensure air bubbles do not interfere with pipetting accuracy. **See video on forward and reverse pipetting.**
Pipetting technique	Check single pipette calibration. Watch for bubbles and consider a reverse pipetting technique to ensure air bubbles do not interfere with pipetting accuracy. **See video on forward and reverse pipetting.**
Bubbles in wells	Be sure to forcefully tap plates dry after each wash step to minimize residual wash buffer in the wells. Bubbles remaining in the wells after addition of assay reagents, particularly substrate, should be removed prior to plate incubation and plate reading. A clean pipette tip can be used to remove bubbles.

Poor Precision Between Replicates for **Samples Only** (High CV Values)	
Inadequate sample mixing	Vortex each sample prior to pipetting to ensure samples are homogeneous.
Pipetting technique	Draw sample in and out of tip twice to coat the inside of the tip before transferring the first sample replicate to the plate. Ensure no sample droplets are adhering to the outside of the tip when transferring sample. If volume permits, consider a reverse pipetting technique to ensure air bubbles do not interfere with pipetting accuracy. **See video on forward and reverse pipetting**.
Inadequate plate washing	For Automated Washers: Check nozzles for clogs, verify dispense volume, and perform regular cleaning to prevent and remove any buildup. Refer to owner's manual for additional maintenance information. For Manual Washing: Ensure that all wells are being forcefully overfilled with wash buffer. Refrain from using a multi-channel pipette for washing. Consider switching from a wash bottle to a hand-held manifold or automated plate washer, particularly when running **chemiluminescence assays**. A video displaying proper washing technique is available **here**. Do not skip wash or soak steps.
Carry over from high and low samples	Ensure tips are changed between each sample. Use a fresh plate sealer for each incubation.
Wrong sample dilution buffer	If samples require dilution, confirm the appropriate dilution buffer is used.

Poor Standard Curve (Does Not Match Previous Results or Certificate of Analysis)	
Inadequate plate washing	For Automated Washers: Check nozzles for clogs, verify dispense volume, and perform regular cleaning to prevent and remove any buildup. Refer to owner's manual for additional maintenance information. For Manual Washing: Ensure that all wells are being forcefully overfilled with wash buffer. Refrain from using a multi-channel pipette for washing. Consider switching from a wash bottle to a hand-held manifold or automated plate washer, particularly when running **chemiluminescence assays**. A video displaying proper washing technique is available **here**. Do not skip wash or soak steps.

Poor Standard Curve (Does Not Match Previous Results or Certificate of Analysis)	
Pipetting technique	Check pipette calibration. Watch for bubbles and consider a reverse pipetting technique to ensure air bubbles do not interfere with pipetting accuracy. **See video on forward and reverse pipetting**.
Inadequate mixing of reagents	Allow reconstituted reagents to sit for the length of time indicated in the manual before use. Use appropriate equipment (vortex mixer, etc.) to ensure reagents are homogeneous before use. Confirm the correct volumes and diluent were used if mixing kit reagents to make a "working" component for the assay.
Insufficient shaking of plate	Incorrect shaking motion can cause insufficient mixing and antibody/analyte interaction. Shake plate at the speed recommended in the instructions for use. Shakers with an orbital motion with a very small radius are recommended. **See example**.
Wrong volume of reagents added to the well	Check residual volumes to confirm correct volume of reagents were added to the wells.
Storing of prepared reagents	Ensure all reagents are stored properly and used within the stated expiration. For **chemiluminescence assays**, advance preparation of luminol substrate can result in reduced signal.
Low or inconsistent ambient temperature	Follow incubation temperatures as indicated in the protocol.
Differences in chemiluminescence instrumentation	Signal, or Relative Light Units (RLUs), can vary significantly from one **chemiluminescence plate reader** to another. Signal presented in a "typical" standard curve or Certificate of Analysis may be very different from observed signal.

All Wells Turned Bright Blue for Colorimetric Assay	
Inadequate plate washing	For Automated Washers: Check nozzles for clogs, verify dispense volume, and perform regular cleaning to prevent and remove any buildup. Refer to owner's manual for additional maintenance information. For Manual Washing: Ensure that all wells are being forcefully overfilled with wash buffer. Refrain from using a multi-channel pipette for washing. Consider switching from a wash bottle to a hand-held manifold or automated plate washer, particularly when running **chemiluminescence assays**. A video displaying proper washing technique is available **here**. Do not skip wash or soak steps. Failing to wash between SA-HRP and substrate steps will result in all wells showing very high signal.
Contamination of the substrate with enzyme conjugate	Ensure all containers are clean and clearly marked. Check TMB for blue coloring. If TMB has turned blue, do not use in your assay and **contact product support**. Do not expose substrate to light before use. Use a new reagent reservoir for addition of each component.

High Background (Blank or 0 Standard Values are Too High)	
Inadequate plate washing	For Automated Washers: Check nozzles for clogs, verify dispense volume, and perform regular cleaning to prevent and remove any buildup. Refer to owner's manual for additional maintenance information. For Manual Washing: Ensure that all wells are being forcefully overfilled with wash buffer. Refrain from using a multi-channel pipette for washing. Consider switching from a wash bottle to a hand-held manifold or automated plate washer, particularly when running **chemiluminescence assays**. A video displaying proper washing technique is available **here**. Do not skip wash or soak steps.
Old or contaminated wash buffer	Wash buffer stored for too long or left in automated washer tubing can cause contamination. Prepare fresh wash buffer as needed and clean automated washing equipment regularly.

High Background (Blank or 0 Standard Values are Too High)	
Contamination of the substrate with enzyme conjugate	Ensure all containers are clean and clearly marked. Check TMB for blue coloring. If TMB has turned blue, do not use in your assay and **contact product support**. Do not expose substrate to light before use. Use a new reagent reservoir for addition of each component.
Wrong conjugate dilution	A higher than normal concentration may result in an elevated background. Check residual volumes and confirm conjugate was prepared correctly.
Wrong filter used in the plate reader	Confirm the correct filter indicated in the protocol is being used when reading the plate. Include recommended subtraction of blanks or OD readings as indicated in the instructions for use.

No Color Development or Very Low OD Readings for Standards and Samples	
Wrong filter used in the plate reader	Confirm the correct filter indicated in the protocol is being used when reading the plate.
Error in reagent preparation	Ensure all containers are clearly marked and appropriate concentrate and diluent are added to the correct vial(s) to make each “working” component for the assay.
Inadequate mixing of reagents	Allow reconstituted reagents to sit for the length of time indicated in the manual before use. Use appropriate equipment (vortex mixer, etc.) to ensure reagents are homogeneous before use.
Insufficient shaking of plate	Incorrect shaking motion can cause insufficient mixing and antibody/analyte interaction. Shake plate at the speed recommended in the instructions for use. Shakers with an orbital motion with a very small radius are recommended. **See example**.
Mix-up in reagents	If running more than one assay at a time, keep reagents separated to avoid mix-ups. Double-check reagent labels before adding reagents to the assay plate.

No Color Development or Very Low OD Readings for Standards and Samples	
Reagents contaminated or added in the wrong order	Verify appearance, storage conditions and expiration dating for all assay components. Follow the steps as instructed in the protocol. Ensure each component was added in the proper sequence.
Alternate component lot or expired component used	Some components are highly lot specific. Always verify the use of an alternate component lot with the manufacturer. Do not use reagents beyond the expiration date.
Assay incubation times not followed correctly	Follow the incubation times and temperatures as indicated in the protocol. Reducing either factor will affect the assay kinetics and may cause low signal. If the protocol lists a time range for TMB incubation, ensure the assay is being incubated for the maximum suggested time.
Delayed reading of plate	If running a **chemiluminescence ELISA**, the plate should be read within a short period of time. Consult protocol for kit-specific timing. Delayed reading of the plate can result in reduced signal.

No Color Development or Very Low OD Readings for **Samples Only**	
Error in experimental design	Investigate sample origin. If possible, test samples using an alternate method to confirm that samples should be positive for the biomarker of interest in the working assay range. When stimulating biomarker production, it may be necessary to adjust experimental design.
Incorrect sample handling	Ensure samples have not been exposed to room temperature or refrigeration for an extended period of time. Verify that samples have not undergone repeated freeze-thaw cycles. When possible, aliquot and store samples at ≤ -70°C.
Error in sample dilution calculations	Verify that sample dilutions are correct. If necessary and if within kit specifications, re-test samples at a higher concentration (lower dilution).
Non-validated sample type	A non-validated sample type may not be compatible with assay buffers or the assay range for the kit.
Insufficient shaking of plate	Incorrect shaking motion can cause insufficient mixing and antibody/analyte interaction. Shake plate at the speed recommended in the instructions for use. Shakers with an orbital motion with a very small radius are recommended. **See example**.

Control Out of Range	
Incorrect reagent handling	Ensure reagents have been stored and handled properly and are within expiration. Note that some reagents have limited shelf-life following reconstitution.
Poor standard curve	Verify the appropriate standard curve was used and check results against values for previous assays and values given in the certificate of analysis. See “Poor Standard Curve” section for additional information. Note: For **chemiluminescence assays**, RLUs can vary significantly from one chemiluminescence reader to another.
Error in reconstitution of standards and/or controls	See protocol and lot-specific Certificate of Analysis for correct volume and buffer for reconstitution of standards/calibrators and controls. Allow reconstituted reagents to sit for the length of time indicated in the manual before use. Use appropriate equipment (vortex mixer, etc.) to ensure reagents are homogeneous before use.
Wrong curve fit used	Use the curve fit recommended in the protocol. If more than one recommendation is provided, use the regression method that best fits the standard data points. The use of a software program with options for 4-parameter and 5-parameter regression is highly recommended.

Unexpected Results for Samples	
Improperly prepared sample	Follow method in protocol for appropriate sample preparation. Ensure samples have not been exposed to room temperature or refrigeration for an extended period of time. Verify that samples have not undergone repeated freeze-thaw cycles. When possible, aliquot and store samples at ≤ -70°C.
Sample IDs mixed up	Review sample IDs to confirm they were labeled and added to the plate correctly.
Error in sample dilution	Verify that sample dilutions are correct and that extrapolated sample values were multiplied by the correct dilution factor, if necessary.
Discrepancy in reported units	Verify that the unit of measure being reported is consistent with the unit of measure for the assay. Perform any necessary conversion calculations. When comparing values obtained in different kits, be sure to correct for any differences in assay calibration if a reference standard is available.

Unexpected Results for Samples	
Error in experimental design	Investigate sample origin. If possible, test samples using an alternate method to confirm expected sample values. When stimulating biomarker production, it may be necessary to adjust experimental design.
Interfering substances	Consider potential interfering substances related to sample matrix (e.g. anticoagulant used), clinical diagnoses (e.g. rheumatoid factor), or treatments.
Specificity	Recognition of natural protein and recombinant proteins expressed in different systems (e. coli, yeast, mammalian cell lines) can vary due to differences in glycosylation and protein folding and antibody specificity. Recognition may vary from one vendor's kit to another.

Assay Drift: Difference in Values from Beginning to End of Plate	
Delays due to reconstitution or dilution	Have standards, controls, and diluted samples prepared prior to start of assay workflow.
Reagents not at temperature	Ensure all required reagents are at room temperature as stated in the instructions for use prior to the start of assay.
Extended time between addition of reagent to first well and last well	If possible, prepare standards and controls in a dilution plate or microtiter tubes, then transfer to the assay plate using a multi-channel pipette. Use a reagent reservoir and multi-channel pipette to add common reagents to the assay plate.

Inter-assay Variability (Poor Precision for Controls or Samples Tested on Different Plates)	
Inter-operator variability	Watch for differences in reagent preparation, pipetting technique, and assay timing.
Differences in pipette calibration	Differences in pipette calibration can introduce variability, particularly when pipetting small volumes.

Inter-assay Variability (Poor Precision for Controls or Samples Tested on Different Plates)	
Differences in instrumentation	When testing samples or controls on more than one plate on the same day or different days, the same plate shaker, plate washer, and plate reader should be used at the same settings.
Variation in environmental conditions	Be aware of any differences in temperature. Keep in mind that general room temperature, proximity to windows (especially on a very cold or very sunny day), and proximity to instrumentation that may be giving off heat can affect assay temperature.
Differences in sample handling	Additional freeze-thaw cycles or increased time at room temperature or in the refrigerator may affect sample results.