

# ELISA TROUBLESHOOTING TIPS

Creative Diagnostics

## Abstract

ELISA is a popular format of "wet-lab" type analytic biochemistry assay that uses a solid-phase enzyme immunoassay (EIA) to detect the presence of a substance, usually an antigen, in a liquid sample or wet sample.

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## ELISA troubleshooting tips

Solve your ELISA problems with these troubleshooting tips, covering common causes of poor standard curve, no signal or weak signal, high background, large coefficient of variation and more.

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#### ➤ **Poor standard curve**

Possible causes and corresponding solutions

##### 1. **Improper standard solution**

Confirm dilutions are made correctly.

##### 2. **Standard inappropriately reconstituted or stored**

Reconstitute standard according to protocol. Briefly spin vial before opening; inspect for undissolved material after reconstituting.

Store reconstituted standard in appropriate vials. Store reconstituted standard at -70°C.

##### 3. **Standard degraded**

Use new standard. Store and handle standard as recommended.

##### 4. **Curve doesn't fit scale**

Try plotting using different scales e.g. log-log, 5 parameter logistic curve fit.

##### 5. **Reagents added to wells with incorrect concentrations**

Check for pipetting errors and correct reagent volume. Use calibrated pipettes and proper pipetting technique.

#### ➤ **No signal or Weak signal**

Possible causes and corresponding solutions

##### ■ **Operation problems**

##### 1. **Incorrect assay procedure**

Review protocol. Repeat assay using a positive control.

2. [Incubation time too short](#)

Incubate samples overnight at 4°C or follow the manufacturer guidelines.

3. [Incubation temperature too low](#)

Optimize the incubation temperature for your assay. Ensure the incubations are carried out at the correct temperature. All reagents including plate should be at room temperature before proceeding or as recommended by the manufacturer.

4. [Plate washings too vigorous](#)

Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually.

5. [Wells dried out](#)

Do not allow wells to become dry once the assay has started. Cover the plate using sealing film or tape for all incubations.

6. [Plate reader has the wrong settings](#)

Check plate reader for wavelength, filters, gain etc. and read plate again.

■ Sample problems

1. [Sample prepared incorrectly](#)

Ensure proper sample preparation/dilution. Samples may be incompatible with microtiter plate assay format.

2. [Target present below detection limits of assay](#)

Decrease dilution factor or concentrate samples.

3. [Incompatible sample type](#)

Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect a positive control.

■ Reagents problems

1. [Assay buffer compatibility](#)

Ensure assay buffer is compatible with target of interest (e.g. enzymatic activity retained, protein interactions retained).

2. [Not enough detection reagent](#)

Increase concentration or amount of detection reagent, following manufacturer guidelines.

3. [Detection reagent too old, contaminated or used at the wrong pH](#)

Use fresh detection reagents at the correct pH.

4. [Wash buffer contains sodium azide](#)

Avoid sodium azide in the Wash Buffer.

■ Antibody problems

1. [Insufficient antibody](#)

Increase concentration of antibody.

2. [Incorrect secondary antibody used](#)

Retrace steps. Repeat assay using the correct secondary antibody.

3. [Antibody stored at 4°C for several weeks or subjected to repeated freeze/thaw cycles](#)

Use a fresh aliquot of antibody that has been stored at -20°C or below.

■ Other Possible causes

1. Recognition of epitope impeded by adsorption to plate

To enhance detection of a peptide by direct or indirect ELISA, conjugate peptide to a large carrier protein before coating onto the microtiter plate.

2. Antigen not coated properly

Try longer coating times, different coating buffers, or avidin plates with biotinylated antigen

➤ **Color developing slowly or no color development**

Possible causes and corresponding solutions

1. Plates and reagents are at wrong temperature

Ensure plates are at room temperature and that the reagents are at room temperature before use

2. Detection reagent too old, contaminated or used at the wrong pH

Use fresh detection reagents at the correct pH. Reagents presence of contaminants, such as sodium azide and peroxidase can affect the substrate reaction. Avoid using reagents containing these preservatives.

3. Contamination of solutions

Make fresh solutions.

4. Reagents were used in the wrong order or an assay step, reagent or antibody was omitted.

Check the package insert for the assay protocol and repeat the assay.

5. Conjugate too weak

Prepare the substrate solutions immediately before use. Ensure the stock solutions are in date and have been stored correctly, and are being used at the correct concentration. Ensure the reagents are used as directed, at the correct concentration.

6. Wrong conjugate was used, conjugate was prepared incorrectly or has deteriorated.

Be sure that the conjugate came with the kit. All conjugates are kit- and lot-specific. If preparation of a working conjugate is needed, be sure that the concentrate and diluent are mixed in correct volumes. Prepare the working solution just before use and do not save any unused portion for future use or return it to the stock bottle. If no conjugate preparation is necessary, be sure to pour out only the amount required for immediate use.

7. Wash buffer contains sodium azide.

Wash buffer contains sodium azide will cause no color development. Make sure wash buffer without sodium azide.

➤ **Large coefficient of variation (CV)**

Possible causes and corresponding solutions

1. Inaccurate multichannel pipette used

Calibrate pipettes and operate correctly to ensure accurate pipetting.

2. Having bubbles in wells

Try remove bubbles prior to reading plate.

3. **Inadequate or uneven plate washing**

Check that all ports of the plate are washed clean. Make sure pipette tips are tightly secured and all reagents are removed completely in all wash steps.

4. **Non-uniform reagents or samples**

Ensure all reagents and samples are mixed thoroughly.

5. **Samples have high particulate matter**

Remove the particulate matter by centrifugation.

6. **Insufficient plate agitation**

The plate should be agitated during all incubation steps using an ELISA plate shaker at a constant speed with solutions in wells not splashing.

7. **Cross-well contamination**

When reusing plate sealers check that no reagent has touched the sealer.

Care should be taken when using same pipette tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

➤ **Positive results in negative control**

Possible causes and corresponding solutions

1. **Contamination of reagents or samples**

Reagents or samples contaminated, or cross contamination from splashing between wells. Use fresh reagents and pipette carefully.

2. **Insufficient washing of plates**

Ensure wells area washed adequately by filling the wells with wash buffer. Ensure all residual antibody solutions are removed before washing.

3. **Too much antibody used leading to non-specific binding**

Check the recommended amount of antibody suggested. Try using less antibody.

4. **Sandwich ELISA – Detection antibody is detecting coating antibody.**

Check the correct coating antibody and detection antibodies are being used and that they will not detect each other.

➤ **High background**

Possible causes and corresponding solutions

■ **Operation problems**

1. **Wells are insufficiently washed or contaminated**

Increase number of washes or wash wells as protocol recommendations. Increase soaking time between washes prior to addition of substrate solution.

Avoid cross-well contamination by using the sealer appropriately. Use multichannel pipettes without touching the reagents on the plate.

2. **Waiting too long to read plate after adding stop solution**

Read plate immediately after adding stop solution.

3. **Substrate incubation carried out in light**

Substrate incubations should be carried out in the dark or as recommended by manufacturer.

4. **Reaction not stopped**

Use stop solution to prevent overdevelopment.

5. **Wrong settings on the plate reader**

Check settings and adjust as needed.

6. **Incubation temperature too high**

Optimize the incubation temperature for your assay.

7. **Plates stacked during incubations leading to uneven temperature distribution**

Avoid stacking plates.

8. **Uneven evaporation of solution from wells during incubation**

Always incubate with a lid on the plate.

■ **Antibody problems**

1. **Non-specific binding of antibody**

Use suitable blocking buffers e.g. BSA or 5-10% normal serum - species same as primary antibody if using a directly conjugated detection antibody or same as secondary if using conjugated secondary.

Use affinity-purified antibody.

Ensure wells are pre-processed to prevent non-specific attachment.

2. **Too much antibody used**

Try different dilutions and reduce antibody concentration for optimal results.

■ **Reagents problems**

1. **Contaminated wash buffer**

Prepare fresh water buffer.

2. **Too much detection reagent**

Ensure the reagent has been diluted properly or decrease the recommended concentration of detection reagent.

3. **Blocking buffer ineffective (e.g. detection reagent binds blocker; wells not completely blocked)**

Try different blocking reagent and/or add blocking reagent to wash buffer.

4. **Salt concentration of incubation/wash buffers**

Increasing salt concentrations may reduce non-specific and/or weak off-target interactions.

5. **TMB Substrate Solution was contaminated**

TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.

6. **Insufficient amount of Tween® in the buffers**

Use PBS containing 0.05% Tween®.

7. **Reagents were not mixed properly**

Before pipetting solutions into wells, make sure all reagents and samples have been thoroughly mixed.

■ **Other possible causes**

1. **Precipitate formed in wells upon substrate addition**

Increase dilution factor of sample or decrease concentration of substrate.

2. **Dirty plate**  
Clean the plate bottom carefully and reread.
3. **Matrix used has endogenous analyte or interference**  
Check the matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium).

➤ **Low sensitivity**

Possible causes and corresponding solutions

1. **Inappropriate storage**  
Different reagents may not have identical storage requirements. Store all reagents as recommended.
2. **Detection reagent was inactive**  
Ensure reporter enzyme/fluor has the expected activity.
3. **Not enough target**  
Concentrate sample or reduce sample dilution.
4. **Plate reader settings incorrect**  
Ensure plate reader is set to read the correct absorbance wavelength or excitation/emission wavelengths for fluorescent detection.
5. **Assay format not sensitive enough**  
Switch to a more sensitive detection system (e.g. colorimetric to chemiluminescence / fluorescence). Switch to a more sensitive assay type (e.g. direct ELISA to sandwich ELISA). Lengthen incubation times or increase temperature.
6. **Target poorly adsorbs to microtiter plate**  
Covalently link target to microtiter plate.
7. **Not enough substrate**  
Add more substrate.
8. **Incompatible sample type (e.g. serum vs. cell extract)**  
Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect as a positive control.
9. **Interfering buffers or sample ingredients**  
Check reagents for any interfering chemicals. For example, sodium azide in antibodies inhibit HRP enzyme and EDTA used as anticoagulant for plasma collection inhibits enzymatic reactions.
10. **Mixing or substituting reagents from different kits**  
Avoid mixing components from different kits.

➤ **Poor Reproducibility**

- Replicates within a 96-well plate show poor reproducibility
1. **Taking excessive time to add samples controls or reagents to the assay plate**  
Have all materials set up and ready to use quickly. Use a multichannel pipette to add reagents to multiple wells simultaneously. Rack controls with samples and dispense them onto the plate at the same time as the samples.
  2. **Inconsistent washing or washer system malfunctioning**  
Make sure the performance of the washer system. Have the system repaired if there

is any problem.

3. **Multichannel pipette malfunctioning**

Confirm pipette calibration and check tips on tight. Be sure all channels of the pipette draw and dispense equal volumes.

4. **Poor distribution of antibody in the sample.**

Mixing samples prior to dilution. Diluted samples also need to be mixed prior to adding them to the plate.

■ **Poor reproducibility plate to plate**

1. **Kit controls and samples were at different temperatures**

Be sure to allow sufficient time for sample diluent, samples and kit controls to come to room temperature by removing them from the kit box. Larger volumes will require longer equilibration time. If using a water bath to hasten equilibration, make sure it is maintained at room temperature; do not use a warm water bath for controls, samples or diluent.

2. **Inconsistent washing occurred from plate to plate**

Use the same number of washes for each plate. Be sure the performance of the washer system. Have the system repaired if any ports drip, dispense or aspirate poorly.

3. **Inconsistent incubation times occurred from plate to plate**

Time each plate separately to ensure that plates have consistent incubation periods.

4. **Reagents were being used from different kit lots**

If running two different kit lots at the same time, make sure to label reagent trays, etc., so all reagents within a lot are used with the corresponding plates.

➤ **Matrix effect**

ELISA quantification of plasma and serum occasionally encounters problems which are caused by the matrix effect. The matrix effect can arise from a number of matrix components including, but not limited to: interaction between endogenous biological components such as phospholipids, carbohydrates and endogenous metabolites (bilirubin) or an interaction between the analyte of interest and the matrix, such as covalent binding to plasma proteins. This results in erroneous sample readings.

Simply diluting the samples by 2 or 5 folds reduces the matrix effect, when diluting the samples remember to use the same diluent as used for standard curve.