ELISA

Troubleshooting

ELISA Troubleshooting

- Though without that complicated protocols as IHC, immunoprecipitation etc, ELISA assay might generate many problems as what could occur in most of the immuno assays. This part provides troubleshooting of ELISA with means of cause-solution table with problems listed beforehand. Please note that the conditions described here may not pertain to every ELISA kit because performance requirements vary for individual assays. Be sure to check your package insert for specifications.

(1) If ELISA signal is extraordinarily high, and standard curves have saturated ODs, its possible causes and recommended troubleshooing are:

POSSIBLE CAUSES	RECOMMENDED TROUBLESHOOTING
▣ Standard reconstituted with less volume than required.	▣ Reconstitute lyophilized standard with correct volume of solution recommended in the protocol.
▣ Plate incubation was too long.	▣ Decrease incubation time.
▣ Detection antibody incubation time is too long.	▣ Decrease detection antibody incubation time.
▣ Substrate solution incubation time is too long.	▣ Decrease substrate solution incubation time.

(2) If sample readings are out of range, possible causes and troubleshooting are as follows.

POSSIBLE CAUSES	RECOMMENDED TROUBLESHOOTING
▣ Samples contain no or below detectable levels of analyte..	▣ If samples are below detectable levels, it may be possible to use higher sample volume. Check with technical support for appropriate protocol modifications.
▣ Samples contain analyte concentrations greater than highest standard point..	▣ Samples may require dilution and reanalysis..

High Background

POSSIBLE CAUSES

- Poor-quality water was used to wash plates or to prepare wash solution.
- Substrate solution has deteriorated.
- There was insufficient washing or poor washer performance.
- Washer system had microbial contamination.
- Wash system contained an alternate wash formulation.

RECOMMENDED TROUBLESHOOTING

- Check the water quality. If it is questionable, try substituting an alternate water source, such as bottled distilled water, to wash plates or prepare the wash solution.
- Make sure the substrate is colorless prior to addition to the plate.
- Try using the highest number of washes recommended for the assay. Make sure that at least 400 µL of wash solution is dispensed per well per wash. Verify the performance of the washer system. Have the system repaired if any ports drip, dispense or aspirate poorly.
- Clean out microbial contamination by flushing the system with a dilute solution of bleach (10% by volume) followed by a large amount of distilled or deionized water. Prime the system with the appropriate wash solution before use. The tubing may need to be changed if the contamination is heavy.
- Be sure each unique wash solution is properly labeled. Prime the system thoroughly when switching between solutions.

High Background

POSSIBLE CAUSES

- Reader was malfunctioning or not blanked properly; this is a possible cause if the OD readings were high and the color was not dark.
- Laboratory temperature was too high or too low.
- Reagents were intermixed, contaminated or prepared incorrectly.
- Wells of the plate were contaminated
- TMB substrate solution was contaminated

RECOMMENDED TROUBLESHOOTING

- Check the water quality. If it is questionable, try substituting an alternate water source, such as bottled distilled water, to wash plates or prepare the wash solution.
- Maintain the room temperature within 18–25°C. Avoid running assays near heat sources, in direct sunlight or under air vents.
- Ensure that the correct reagents were used, that working solutions were prepared correctly and that contamination has not occurred.
- Avoid cross-well contamination by using the sealer appropriately. Use multichannel pipettes without touching the reagents on the plate.
- TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.

Low OD Readings

POSSIBLE CAUSES

- Laboratory temperature was too low.
- Wash solution was prepared incorrectly or the wrong wash solution was used.
- Washer system had microbial contamination or contained an alternate wash formulation.
- Too many wash cycles were used.
- Incubation periods were too short.

RECOMMENDED TROUBLESHOOTING

- Maintain the room temperature within 18°C–25°C. Avoid running assays under air conditioning vents or near cold windows.
- Be sure to use the wash solution recommended for the kit and that it is prepared correctly. Label each unique wash solution to avoid using the wrong one.
- Clean out microbial contamination by flushing the system with a dilute solution of bleach (10% by volume) followed by a large amount of distilled or deionized water, then prime the system with the appropriate wash solution. Be sure each unique wash solution is properly labeled. Prime the system thoroughly when switching between solutions.
- Stay within the recommended range for the number of wash cycles. Try to use the lowest number of washes recommended for the assay.
- Follow protocol for incubation times. Time each plate separately to ensure accurate incubation periods.

Low OD Readings

POSSIBLE CAUSES

- Reagents and plates were too cold.
- Reagents were expired or intermixed from a different lot number.
- Wrong conjugate was used, conjugate was prepared incorrectly or has deteriorated.
- Assay plate read was at wrong wavelength, or reader was malfunctioning.
- Positive control was diluted (indirect format only).

RECOMMENDED TROUBLESHOOTING

- Make sure plates and reagents are at room temperature by taking them out of the refrigerator, and the kit components out of the kit box, at least 2–3 hours before starting the assay.
- Verify the expiration dates and lot numbers on the reagents.
- Be sure that the conjugate used is the one that came with the kit and 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 appropriate volumes. Do not prepare the working solution too far in advance, and do not save any unused portion for future use. If no conjugate preparation is necessary, be sure to pour out only the amount required for immediate use, and do not return any unused portion to the stock bottle.
- Verify the correct wavelength for the assay and read the plate again. Verify reader calibration and lamp alignment.
- Do not dilute controls unless specified in the package insert.

Low OD Readings

POSSIBLE CAUSES

- Excessive kit stress has occurred.
- Assay plates were compromised or previously used..
- Insufficient amount of antigen was coated to microtiter plate.
- Not enough antibody used.
- Detection reagent too dilute.

RECOMMENDED TROUBLESHOOTING

- Check records to see how many times the kit has cycled from the refrigerator. Check to see if the kit was left out on a loading dock or other area for too long or at extreme temperatures.
- Be sure to refrigerate plates in sealed bags with a desiccant to maintain stability. Prevent condensation from forming on plates by allowing them to equilibrate to room temperature while in the packaging. If partial plates are used, be sure to label used wells to prevent reuse; cover them with sealing tape and use the remaining wells as soon as possible. Do not store partially used plates with other plates. Include a desiccant in the storage bag.
- Use more antigen for coating
- Increase concentration of the primary and/or secondary antibody. Optimize antibody concentrations for your assay.
- Use a higher concentration of detection reagent.

No Color Development

POSSIBLE CAUSES

- Reagents were used in the wrong order or an assay step was omitted.
- Samples were not added to diluent (indirect format only).
- Wrong conjugate was used, conjugate was prepared incorrectly or has deteriorated.
- Incorrect or no detection antibody was added.
- Substrate solution was not added.
- Wash buffer contains sodium azide.

RECOMMENDED TROUBLESHOOTING

- Check the package insert for the assay protocol and repeat the assay.
- Verify that the samples were added to the diluent.
- Be sure that the conjugate used is the one that 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. Do not prepare the working solution too far in advance and do not save any unused portion for future use. If no conjugate preparation is necessary, be sure to pour out only the amount required for immediate use and do not return any unused portion to the stock bottle.
- Add appropriate detection antibody and continue.
- Add substrate solution and continue.
- Avoid sodium azide in the wash buffer.

Replicates Within a Plate Show Poor Reproducibility

POSSIBLE CAUSES

- Excessive time was taken to add samples controls or reagents to the assay plate.
- Multichannel pipette was not functioning properly.
- There was inconsistent washing or washer system malfunctioning.
- There was poor distribution of antibody in the sample.

RECOMMENDED TROUBLESHOOTING

- Be sure to 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.
- Verify pipette calibration and check that tips are on tight. Be sure all channels of the pipette draw and dispense equal volumes.
- Verify the performance of the washer system. Have the system repaired if any ports drip or dispense/aspirate poorly.
- If the sample was thawed or refrigerated, make sure it was mixed prior to dilution. Diluted samples also need to be mixed prior to adding them to the plate.

Poor Reproducibility Plate to Plate

POSSIBLE CAUSES

- Inconsistent incubation times occurred from plate to plate.
- Inconsistent washing occurred from plate to plate.
- Pipette was working improperly.
- Kit controls and samples were at different temperatures.
- Reagents were being used from different kit lots.

RECOMMENDED TROUBLESHOOTING

- Time each plate separately to ensure that plates have consistent incubation periods.
- Use the same number of washes for each plate. Verify the performance of the washer system. Have the system repaired if any ports drip or dispense or aspirate poorly.
- 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.
- Ensure that the correct reagents were used, that working solutions were prepared correctly and that contamination has not occurred.
- 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.

Poor standard curve

CAUSE

- Improper standard solution
- Standard improperly reconstituted
- Standard degraded
- Curve doesn't fit scale
- Pipetting error

SOLUTION

- Confirm dilutions are made correctly.
- Briefly spin vial before opening; inspect for undissolved material after reconstituting.
- Store and handle standard as recommended.
- Try plotting using different scales e.g. log-log, 5 parameter logistic curve fit.
- Use calibrated pipettes and proper pipetting technique.

No signal

CAUSE

- Incubation time too short
- Target present below detection limits of assay
- Incompatible sample type
- Recognition of epitope impeded by adsorption to plate
- Assay buffer compatibility

SOLUTION

- Incubate samples overnight at 4°C or follow the manufacturer guidelines.
- Decrease dilution factor or concentrate samples.
- Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect a positive control.
- To enhance detection of a peptide by direct or indirect ELISA, conjugate peptide to a large carrier protein before coating onto the microtiter plate.
- Ensure assay buffer is compatible with target of interest (e.g. enzymatic activity retained, protein interactions retained).

No signal

CAUSE

- Not enough detection reagent
- Sample prepared incorrectly
- Insufficient antibody
- Incubation temperature too low
- Incorrect wavelength
- Plate washings too vigorous
- Wells dried out
- Slow color development of enzymatic reaction

SOLUTION

- Increase concentration or amount of detection reagent, following manufacturer guidelines.
- Ensure proper sample preparation/dilution. Samples may be incompatible with microtiter plate assay format.
- Try different concentrations/dilutions of antibody.
- Ensure the incubations are carried out at the correct temperature. All reagents including plate should be at room temperature or as recommended by the manufacturer before proceeding.
- Verify the wavelength and read plate again.
- Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually.
- Do not allow wells to become dry once the assay has started. Cover the plate using sealing film or tape for all incubations.
- Prepare substrate solution immediately before use. Ensure the stock solution has not expired and is not contaminated. Allow longer incubation.

Large coefficient of variation (CV)

CAUSE	SOLUTION
Bubbles in wells	Ensure no bubbles are present prior to reading plate.
Wells not washed equally/thoroughly	Check that all ports of the plate washer are unobstructed. Wash wells as recommended.
Incomplete reagent mixing	Ensure all reagents are mixed thoroughly.
Inconsistent pipetting	Use calibrated pipettes and proper technique to ensure accurate pipetting.
Edge effects	Ensure the plate and all reagents are at room temperature.
Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (e.g. minimize freeze/thaw cycles).

High background

CAUSE

- Wells are insufficiently washed
- Contaminated wash buffer
- Too much detection reagent
- Blocking buffer ineffective (e.g. detection reagent binds blocker; wells not completely blocked)
- Salt concentration of incubation/wash buffers

SOLUTION

- Wash wells as per protocol recommendations.
- Prepare fresh water buffer.
- Ensure the reagent has been diluted properly or decrease the recommended concentration of detection reagent.
- Try different blocking reagent and/or add blocking reagent to wash buffer.
- Increasing salt concentrations may reduce non-specific and/or weak off-target interactions.

High background

CAUSE

- Waiting too long to read plate after adding stop solution
- Non-specific binding of antibody
- High antibody concentration
- Substrate incubation carried out in light
- Precipitate formed in wells upon substrate addition
- Dirty plate

SOLUTION

- Read plate immediately after adding stop solution.
- 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. Ensure wells are pre-processed to prevent non-specific attachment.
- Try different dilutions for optimal results.
- Substrate incubations should be carried out in the dark or as recommended by manufacturer.
- Increase dilution factor of sample or decrease concentration of substrate.
- Clean the plate bottom.

Low sensitivity

CAUSE

- Improper storage of ELISA kit
- Not enough target
- Inactive detection reagent
- Plate reader settings incorrect
- Assay format not sensitive enough
- Target poorly adsorbs to microtiter plate

SOLUTION

- Store all reagents as recommended. Please note that all reagents may not have identical storage requirements.
- Concentrate sample or reduce sample dilution.
- Ensure reporter enzyme/fluor has the expected activity.
- Ensure plate reader is set to read the correct absorbance wavelength or excitation/emission wavelengths for fluorescent detection.
- Switch to a more sensitive detection system (e.g. colorimeteric to chemiluminescence / fluorescence). Switch to a more sensitive assay type (e.g. direct ELISA to sandwich ELISA). Lengthen incubation times or increase temperature.
- Covalently link target to microtiter plate.
- Add more substrate.

Low sensitivity

CAUSE

- Not enough substrate
- Incompatible sample type (e.g. serum vs. cell extract)
- Interfering buffers or sample ingredients
- Mixing or substituting reagents from different kits

SOLUTION

- Detection may be reduced or absent in untested sample types. Include a sample that the assay is known to detect as a positive control.
- Check reagents for any interfering chemicals. For example, sodium azide in antibodies inhibit HRP enzyme and EDTA used as anticoagulent for plasma collection inhibits enzymatic reactions.
- Avoid mixing components from different kits.

References

- https://www.abcam.com
- https://www.sinobiological.com
- https://www.bio-rad
- https://www.thermofisher.com