TROUBLESHOOTING ELISA

Troubleshooting *This troubleshooting document gives the problem, possible cause and suggested solution for problems during the ELISA application:*

Problem: Poor standard curve

The standard stock solution has been improperly prepared	Dilute the lyophilized standard with the suggested diluent buffer as is suggested.
The standard stock solution has been improperly diluted	Check your calculation for the dilution.
Freezing/thawing of the standard	Use fresh standard.
The reagents are used beyond expiration date	Make sure that the antibodies have not exceeded their date of expiration, use fresh reagents.
Use of non-calibrated external recombinant protein preparation as standard	Calibrate against reference preparation standard.

Problem: Positive results in negative control

The reagents/samples are contaminated	The reagents or samples may be contaminated, or there is cross contamination from splashing between wells. Use fresh reagents and pipette carefully.
Sandwich ELISA – The detection antibody is detecting the coating antibody	Check if the correct coating antibody and detection antibodies has being used and that they will not detect each other.
Insufficient washing of plates	Ensure that wells are washed adequately by filling the wells with wash buffer. Make sure that all residual antibody solutions are removed before washing.
There is too much antibody used leading to non-specific binding	Check the amount of antibody suggested. Try using less antibody.

Problem: High Background

Wrong blocking component	Use a blocking buffer with a higher protein content.
Incubation times is too long	Reduce incubation time.
Substrate solution or stop solution is not fresh	Use fresh substrate solution. Stop solution should be clear.
Incubation temperature is too high	Antibodies will have optimum binding activity at the correct temperature. Incubation temperature may require some optimization.
Evaporation of fluid during incubation (37°C)	Cover the plated during incubation.
Concentration of biotinylated detection antibody and/or Streptavidin-HRP is too high	Check dilution of conjugate, use it at the recommended dilution. Stop the reaction using stop buffer as soon as the plate has developed enough for absorbance.
Inadequate washing after the Streptavidin-HRP step	Verify function of automated plate washer. Use enough wash buffer.
Non-specific binding of antibody	Ensure a block step is included. Ensure wells are pre-processed to prevent non specific attachment. Use an affinity purified antibody, preferably pre-absorbed.
Contamination of substrate with metal ions of oxidizing agents	Always use distilled water.
Contaminants from laboratory glassware	Ensure reagents are fresh and prepared in clean glassware. Sterilize glassware beforehand.
Substrate exposed to light	Substrate incubation should be carried out in the dark. Check for unusual appearance in all the components.
Degraded Streptavidin-HRP	Check for unusual appearance in all the components.
The reaction has not stopped	Color will keep developing if the substrate reaction is not stopped by stopsolution.
The plate is left too long before reading on the plate reader	Color will keep developing.

Problem: Weak/no color development:

No color has developed for the positive controls or for the samples	Check all the reagents for dating, concentration, and storage conditions. Check the integrity of the antibody reagent.
Plate has dried out between incubations	Keep plate covered during all incubations; perform pipetting steps in a timely manner to avoid excess exposure.
Reagents are not at room temperature (18-25 °C) at the start of assay	Allow all reagents to warm to room temperature prior starting the assay.
The coating concentration is too low	Titrate the coating antibody concentration.
The substrate solutions is not fresh or is combined incorrectly or not at the correct pH	Prepare the substrate solutions immediately before use. Ensure that the stock solutions are in date and they have been stored correctly. They need to be used at the correct concentration and pH. Make sure that the reagents are used as directed at the correct concentration.
One of the reagents is contaminated	Check for unusual appearances
Incorrect chromogen/stop solution used ***Stop solution has not been added***	Use the chromogen/stop solution which are recommended in the protocol. The addition of the stop solution increases the intensity of the color reaction and stabilizes the final color reaction.
Buffer contains azide which is not compatible with HRP	The use of azide in the assay should be avoided.
Plate has been read after a half-hour of stopping the reaction	Read the assay within 30 minutes of adding the stop solution.
The wells are scratched with pipette of washing tips	Restart the assay using new wells.
There has been mixing of reagents from different kits	Do not mix reagents from different kits.
The positive controls and the test samples have developed very little color	Check the dilution of the enzyme labeled antibody, and the concentration of the substrate.
Color has developed for the test samples but not the positive or negative controls	Check the source of the positive controls, their expiration date and their storage. If they are stored in a dilute form, the antigen may have adhered to the surface of the storage vessel.
The target protein is not expressed in the sample used/ There is a low level of target protein expression in sample used	Check the expression profile of the target protein to ensure it will be expressed in your samples. If there is a low level of target protein expression, increase the amount of sample used, or change to a more sensitive assay. Make sure you are using a positive control within the detection range of the assay.
Insufficient antibody	Check whether the recommended amount of antibody is being used. The concentration of antibody may require increasing for optimization of results.
The incubation time is not long enough	The incubation time may require increasing for optimization of results.
The incubation temperature is too low	Antibodies will have an optimum binding activity at the correct temperature. The incubation temperature may require some optimization. Ensure all reagents are at room temperature before proceeding.
Color can be seen, but the absorbance is not as high as expected	Check the wavelength setting.

Problem: High absorbance values for samples and/or positive control

The absorbance does not go down as the sample is diluted down the plate	The concentration of samples or positive control is too high and out of range for the sensitivity of the assay. Restart the assay you are using or reduce the concentration of samples and control by dilution before adding to the plate. Take the dilution into account when calculating the resulting concentrations.

Problem: Poor precision

Incorrect volume of reagents dispensed	Follow protocol for reagents dispensing volumes.
Errors in pipetting the standards, sample or subsequent steps	Check pipette for calibration and leaking.
The bottom of the microplate is scratched with pipette tip or washing tips	Repeat the assay using new wells.
Particulates or precipitates are found in the samples prior to dispensing into the assay	Remove any particulates/precipitates by centrifugation.
Improper washing	Verify the proper function of the washing device.
Liquid might be transferred from one well to the other by shaking too vigorously when shaking required	Check for correct rotator RPM.
Unequal evaporation of fluids	Cover the plate during incubation.
Tips are used repetitively for several samples or different reagents	Use fresh tips for each sample of reagents transfer.
The negative controls are giving positive results	There may be contamination of the substrate solution, or contamination of the enzyme-labeled antibody, or of the controls themselves.

Problem: Inconsistent absorbances across the plate

Plates are stacked during incubations	Stacking of plates does not allow even distribution of temperature across the wells of the plates. Avoid stacking.
Pipetting is inconsistent	Ensure pipettes are working correctly and are calibrated. Ensure pipette tips are pushed on far enough to create a good seal. Take particular care when diluting down the plate and watch to make sure the pipette tips are all picking up and releasing the correct amount of liquid. This will greatly affect consistency of results between duplicates.
Antibody dilutions/reagents are not mixed well	To ensure a consistent concentration across all wells, make sure that all the reagents and the samples are mixed before pipetting onto the plate.
The wells are allowed to dry out	Ensure the lids are left on the plates at all times when incubating. Place a humidifying water tray (bottled clean/sterile water) in the bottom of the incubator.
Inadequate washing	This will lead to some wells not being washed as well as others, leaving different amounts of unbound antibody behind which will give inconsistent results.
Bottom of the plate is dirty which affects absorbance readings	Clean the bottom of the plate carefully before re-reading the plate.

Problem: Color developing slowly

Plates are not at the correct temperature	Ensure all plates are at room temperature and that the reagents are at room temperature before use.
The conjugate is 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.
Solutions are contaminated	Presence of contaminants, such as sodium azide and peroxidise can affect the substrate reaction. Avoid using reagents containing these preservatives.

Helpful links / references http://www.protocol-online.org/