QUALPRO DIAGNOSTICS

TECHNICAL SERIES

ELISA

Troubleshooting Aspects

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Foreword

Qualpro Diagnostics is a part of the innovative **TULIP** Group of companies based at Goa, India.

The group's commitment in building products of international standards, through indigenous R&D has accorded the company virtual leadership in most product segments in the Indian marketplace. Its state-of-art manufacturing facility conforms to the strictest FDA (India) and GMP regulations. In its efforts to build world-class Quality products, the group has recently received the ISO 9001(2000) certification from TUV. It is this commitment to Quality, which has given the group international acclaim

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The products are now exported to over 45 countries globally with an ever-increasing user base. With decades of experience in *in-vitro* diagnostics (IVD), **TULIP** has created a strong knowledge base. **TULIP** believes that in the knowledge-based society of the 21st century, regular upgradation of knowledge is essential not only for better diagnosis and patient care, but also to improve the overall quality of life

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Publishing of **Technical Series** is one such initiative to make available to the Laboratory professionals and clinicians updated knowledge that is vital for them to set trends in their day-to-day practice.

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INTRODUCTION

ELISAs have emerged as the mainstay for diagnosis of various human diseases in a modern clinical laboratory. Despite being an extremely sensitive & specific assay format, several pre-analytical and analytical factors may affect the performance of ELISAs. Thus it is imperative that laboratory professionals be aware of the problems so that he/she has more control over the final assay results. Many errors can be avoided if the protocol is read and fully understood before starting the assay.

On identifying assay failure, check the expiration dates of the individual reagents and ensure that all the reagents have been stored as indicated on the product label.

Once this has been established, check for signs of instability or deterioration in reagent solutions, (e.g., precipitation or discoloration).

All substrate solutions should be colorless.

Use clean plastic disposable pipettes, tips, and containers for reagent preparation and storage.

Avoid cross-contamination of kit reagents by changing pipette tips between addition of each calibrator, sample and reagent.

Ensure that specified incubation times and temperatures have been adhered to and that no substitution of kit reagents has occurred.

To improve accuracy, it is recommended that samples and standards be run in duplicate.

In this technical series we present various problems commonly encountered in ELISAs and the necessary corrective action.

Problem: High negative control value or high background

Possible Causes	Corrective Action
• Contamination of negative control wells by positive control.	• When washing, do not allow wells to overflow.
• Contamination of negative control vial.	• Check pipette barrel for residual fluid of dried material. Remove if present. • Always format negative control wells before positive control. • Use new pipette tip for each sample. • Check that pipette tips are long enough to provide air space between top of tip and the barrel. • Re-run with fresh reagents.
• Insufficient washing or contamination of negative control by conjugate.	• Make sure wells are completely filled. While washing ensure residual conjugate is removed from well. • Pipette all specimen and reagents in the center bottom of the micro well. Avoid contact with inner wall and rim. • Re-wash
• Non specific attachment of antibodies.	• Unsuitable blocking buffer or omission of blocking buffer. • Wells not pre-processed to prevent non specific attachment of antibodies.
• Antispecies conjugate reacts with reagent coated on plate.	• Set-up controls to assess weather any reagent binds unexpectedly to any reagent.

Problem: Low positive control value or low absorbance

Possible Causes	Corrective Action
Reagent not at room temperature.	Make certain all kit components are at RT (22-28°C).
Test volume low.	Ensure pipette tips are fitted correctly / tightly. Check pipette barrels for obstructions. Check calibration of pipettes.
Substrate A & B not freshly combined or incorrectly prepared (in case of 2-reagent substrate system)	Prepare substrate immediately before use. Follow working reagent preparation.
Contamination of substrate with or bacterial contamination of positive control	Re-run the assay with fresh reagents.
Incubation time too short,	Check calibration of timers. Record time of incubation.
Moisture in pouches.	Check whether desiccant in pouch is in working condition. Seal unused wells in pouches. Date pouches when first opened
Improper incubation temperature.	Check incubator temperature/ Room Temperature (22-28°C).
Room temperature too low for substrate incubation	Check temperature of the working area.
Washing step too vigorous.	Reduce pressure in wash system.
Reagent not mixed before using.	Mix the reagents before use.
Wells allowed to dry after assay has started.	Complete all assay steps without interruption.
Failure to add stop solution.	Addition of stop solution increases intensity of colour reaction and stabilizes final colour reaction.
Insufficient conjugate concentrate added in preparing working stock.	Prepare conjugate accurately. Follow working reagent preparation as described by the manufacture.

Problem: Entire plate gives positive OD or colour all over plate

Possible Causes	Corrective Action
• Inadequate wash volume or contamination of substrate by residual conjugate left in well.	When washing, fill the wells to the rim and ensure no overflow
Too strong conjugate.	Check dilution.
Antispecies antibodies react with absorbed antigen.	Check suitable controls.
Serum factors in heated sera.	Do not heat sera.
Substrate solution contaminated by conjugate.	Check pipette barrels for residual fluids or dried material, remove if present. Pipette tips should be long enough to provide air space between top of tip and pipette barrel. For automated system, make sure regent lines are in proper position. Do not switch lines.
Substrate solution is not fresh.	Do not hold substrate solution longer than Manufacturer claims.
Failure to stop reaction. Acid not added.	Check bottle before use. Check assay procedure.
Plate sat idle too long before reading.	Read within 30 minutes of adding stop solution.
Chromogen may not be working.	Use fresh chromogen.
Substrate solution container is dirty.	Do not add fresh substrate to reagent bottle containing old substrate. Clean old solution bottle with acid and thoroughly rinse with distilled water.
Plate exposed to light during substrate incubation	Place plates in dark immediately after addition of substrate solution (Check Product Insert)

Problem: False positive reactions

Possible Causes	Corrective Action
Inadequate washing.	Check washer before use to determine they are working properly. Perform routine maintenance.
Clogged cannulas in washer.	
Contamination of wells by conjugate.	Carefully add conjugate to wells. Pipette reagent to center bottom of microwell. Avoid contact with sides and rims of wells. Check alignment and delivery of automated systems. Check pipette barrels for residual fluid or dried material. Remove if present. Check pipette tips are long enough to provide air space between top of tip & pipette barrel.
Splashing of conjugate on rims of wells during conjugate addition.	
Contamination of substrate solution by conjugate.	
RBCs in test sample.	Centrifuge before use.
Evaporation of sample of conjugate during the 37°C incubation (if 37°C is a must, not applicable in RT incubation).	Place the covered test plate in a pre-warmed (37°C) moist incubation box inside the incubator (dry or humidified).
Mold in incubation box and / or wash buffer bottle.	Visually check incubation boxes & wash buffer bottles. Clean any moldy containers. Be sure all containers are free of cleaning agents before using. Set up routine cleaning schedule.
Too much conjugate concentrate used in preparing working stock.	Prepare fresh working conjugate. Follow conjugate preparation.
Incubation temperature too high.	Check room temperature, whether at 22-28°C / Check AC.

Problem: Poor reproducibility or bad duplication

Possible Causes	Corrective Action
Bubbles in wells.	Use pin or needle to burst. Use separate pin for each well.
Dispensing error.	Check dispensing instrument.
Finger tips on plates.	Clean bottom surface of plate with wash buffer, blot to dry.
Mis-aligned wells in plate.	Re-align wells.
Improper washing technique.	Be certain to wash the specified no. of times. Fill each well to the rim with wash buffer. Do not allow well to overflow. Blot plate dry at end of wash.

Problem: Poor sensitivity

Possible Causes	Corrective Action
Usage of non human serum based calibrators(for quantitative assays)	Re-run with human serum based calibrators (Primary Standards)
Insufficient conjugate concentration added in preparing working stock	Prepare fresh working conjugate, follow working procedure.
Error in pipetting working conjugate.	Check calibration of pipettes.
First incubation time insufficient.	Repeat run using proper incubation time.
Temperature more than suggested by manufacturer. Plates being held too long after first incubation before further processing.	Process plate continuously throughout entire assay procedure.

Problem: High absorbance of calibrator

Possible Causes	Corrective Action
Plates being held too long after first incubation at a higher temperature than what is recommended by the manufacturer before further processing.	Process plate continuously throughout entire assay procedure
Insufficient sample volume added.	Check calibration of pipettes.

Problem: Specimen absorbance out of range of calibrators

Possible Causes	Corrective Action
Concentration in specimen is too high.	Dilute with '0' calibrator & re-assay.

Problem: Overall low absorbance

Possible Causes	Corrective Action
Temperature of room < 20°C.	Increase time of reaction between enzyme/ substrate (Check with manufacturer). It is recommended to maintain 22-28°C ambient temperature in the laboratory.

Problem: Controls out of range

Possible Causes	Corrective Action
Contamination of controls.	Re-run assay with new controls.
Contamination of calibrators.	Re-run assay with new calibrators.

Problem: Strips slip from holder

Possible Causes	Corrective Action
Improper handling.	Grasp holder on grip marks when tapping.

Problem: Strips do not fit in holder

Possible Causes	Corrective Action
Improper alignment or incorrect holder.	Rotate strip 180° & re-insert or use correct holder.

Problem: Substrate A is blue

Possible Causes	Corrective Action
Contaminated.	Obtain fresh Substrate A.

Problem: Substrates A and B turn blue when mixed

Possible Causes	Corrective Action
Contaminated.	Obtain Fresh substrate A & B.

Problem: Stop solution yellow

Possible Causes	Corrective Action
Contamination.	Obtain fresh stop solution.

Problem: Waited over 30 minutes before measuring plate

Possible Causes	Corrective Action
End product of enzyme reaction may precipitate and cause error.	Re-run the essay.

Problem: No colour even after 30 minutes incubation with substrate

Possible Causes	Corrective Action
Improper mixing of Substrate A & B.	Re-mix the substrates.
Substrate not working.	Contact manufacturer.

Problem: Colour develops very quickly

Possible Causes	Corrective Action
Contaminated enzymes.	Common in wells, pre-treatment may be necessary. Make sure all reservoirs are clean.

Problem: Colour develops too slowly

Possible Causes	Corrective Action
Sample not at room temperature.	Bring samples to room temperature before assay run
Conjugate too weak.	Check dilutions & time when diluted.
Contamination inhibits activity of enzyme e.g. sodium azide on peroxidase.	Avoid wrong preservatives.
Low temperature of laboratory or substrate solution.	Makes sure temperature of substrate is correct.

TECHNICAL TIPS

Washing

The purpose of washing is to separate bound and unbound (free / unwanted) reagents/serum components. This involves the emptying of microwells of reagents followed by the addition of liquid into the wells. Such a process is performed at least 3-6 times for every well. The liquid used to wash wells is usually buffered (PBS) in order to maintain isotonicity, since most Ag-Ab reactions are optimal under such conditions. Tap water is not recommended, since tap water varies greatly in composition (pH, molarity, and so on). Generally, the mechanical action of flooding wells with a solution is enough to wash wells of unbound reagents. Some workers leave washing solution for a short time (soak time) after each addition (1-5 minutes). Sometimes detergents, notably Tween-20 (0.05%) are added to washing buffers. This can cause problems where excessive frothing takes place producing poor washing conditions, since air is trapped and prevents the washing solution from contacting the well surface. For most cases, this addition does not contribute significantly to the washing procedure. When using detergents, care has to be taken that they do not affect reagents adversely (denature Ag), and greater care is needed to prevent frothing in the wells.

Normal Washing

In washing plate manually, the most important factor is that each well receives the washing solution so that, no air bubbles are trapped in the well or a thumb is not placed over corner wells.

Strip / Plate Washers

- Various washing cycles can be programmed.
- Careful maintenance is essential, since they are prone to machine errors, such as having a particular nozzle being blocked.

Washing Tips

- Follow procedure for preparation of wash buffer.
- Check washer alignment daily as part of routine instrument start-up procedures.
- Ensure that the plate is leveled.
- Make certain will is completely filled, when washing, to ensure residual conjugate is removed.

- Examine the fill volume (a slight dome should be observed at the top of the well).
- When washing do not allow wells to overflow.
- Reduce pressure in wash system.
- Check washers before use to determine they are working properly. Perform routine maintenance.
- Be certain to wash the specified number of times.
- Allow approximately 20 seconds soak-time between the addition of wash solution and subsequent aspiration (if soak-time is not indicated in the assay pack insert).
- Examine the wells for complete aspiration of contents.
- Upon completion of wash cycle, blot to remove residual fluid.

Pipetting Tips

- Calibrate pipettes regularly according to manufacture's instructions.
- Avoid touching side wall of well with tips.
- Avoid splashing of sample and reagents.
- Avoid blowing out tip contents.
- Use a new tip for each sample/control/reagent addition.
- New tips should be used on the multichannel pipettes for each reagent to be added.
- Reverse pipette when using the multichannel pipettes to add conjugate and substrate solution.
- Forward pipette when using the multichannel pipettes to add stop solution.
- Check pipette tips are long enough to provide air space between top of tip and pipette barrel.
- Check pipette barrel for residual fluid of dried material, remove if present.
- Ensure pipettes tips are fitted tightly.

Microplates

- Bring microplate pouches to room temperature before opening.
- Level microwells evenly in microplate frame as the individual breakaway wells have very flexible plate frames leading to bowing off wells and yield poor washes.
- Place plates in dark immediately after addition of substrate solution, provided the substrate is sensitive to light.
- Grasp holder on grip marks when tapping to avoid strips slipping from holder.
- Rotate strips 180° and re-insert or use correct holder if strips do not fit in holder.

- Seal unused wells in purchase along with the desiccant.
- Date the pouches when first opened.
- Clean bottom surface of plates with wash buffer to remove fingerprints.
- Make sure microwells are at level during washing, reagent addition and plate/strip reading.
- Wipe the bottom the plate with a lint-free cloth/ towel before reading.
- Do not allow microwells to become dry once the assay has begun.

Substrate Preparation

- Use freshly prepared substrate A and substrate B (in 2-reagent substrate systems)
- Do not hold substrate solution longer then 1 hour.
- Follow procedure of working substrate solution.
- The temperature of solution is important because it effect s rate of colour reaction.
- Do not add fresh substrate to reagent bottle containing old substrate.
- Clean old substrate solution bottle with H_2SO_4 and thoroughly rinse with distilled water.

Conjugates

- Store at recommended temperature.
- Never store excessively diluted conjugate for use at some later time.
- Always make up the working dilution of conjugate just before you need it.
- Never leave conjugate on the bench for excessive time.

Addition of Samples

Problems caused by :

- Failure to put sample into buffer in well, leaving it on the side of the plate.

Stopping Reagents

Stopping reagents are added to prevent further enzyme reaction in ELISA. The stopping is usually made at a time when the relationship among the enzyme-substrate product is in the linear phase. Molar concentration of strong acids or strong bases stops enzyme activity by quickly denaturing enzymes. Some stopping reagents are enzyme-specific. Sodium azide is a potent inhibitor of HRPO, whereas EDTA inhibits Alkaline phosphatase by the chelation of metal ion cofactors. Since addition of stopping agents may alter the absorption spectrum of the product, the absorption peak must be known.

Temperature

- Bring test reagents to room temperature (22-28°C) approximately 30 minutes prior to use.
- Maintain proper incubation temperature :
  - Lower temperature can decrease OD values.
  - Higher temperatures can increase OD values.
  - Evaporation in wells can cause edging effect.
- The optimal temperature for incubation is 22-28°C
- Check temperature against calibrated thermometer.
- Strict adherence to time must be maintained :
- Check calibration of timers.
- Record time of incubation.
- Read plate with specified time limits of adding stop solution

Rotation of plates while incubating reagents

In certain ELISA systems, the plates are rotated during incubation for better antigen-antibody reaction. The effect of rotating plates is to mix the reactants completely during the incubation step. Since the solid-phase limits the surface area of the absorbed reactant, the mixing ensures that, potentially reactive molecules are continuously coming into contact with the solid-phase.

During stationary incubation, mixing only takes place because of diffusion of reagents. Thus, to allow maximum reaction from reagents in stationary conditions, greater times of incubation may be required, than if they are rotated.

Rotation also allow ELISA to be performed independent of temperature conditions. The interaction of antigen & antibodies relies on their closeness, and the kinetic energy provided to the system, which is encouraged with the mixing during rotation. Stationary incubation relies on the diffusion of molecules & thus is dependent on temperature.

Laboratory conditions

The laboratory should be devoid of any acid fumes.

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NOTES

For the use of Registered Medical Practioners and Laboratories only

QUALPRO DIAGNOSTICS

Gitanjali, Tulip Block,Dr.Rego Bagh, Alto Santacruz,Bambolim Complex
Post office, Goa - 403 202, India Te.l:+91-832-2458546-51 Fax:+91-832-2458544
E- mail : tulip@sancharnet.in, Website:www.tulipgroup.com