KRISHGEN BioSystems

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KRISHGEN BioSystems

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TEST GUIDE

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Optimize your laboratory's ELISA performance and eliminate error.
Tips, hints and troubleshooting Q &A from KRISHGEN's Technical Services team

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Introduction

KRISHGEN Biosystems is a leading manufacturer of life sciences reagents and kits. Our commitment to marketing products of international standards has accorded it increasing market share worldwide.

With man-hours put in for building and understanding in-vitro diagnostics (IVD), KRISHGEN has created a strong knowledge base. KRISHGEN believes that in the knowledge-based society of today, regular upgradation of knowledge is essential not only for better diagnosis and patient care, but also to improve the overall quality of life.

KRISHGEN BioSystems strives to provide quality immunoassays that guarantee high performance. Our Quality programs are designed to guarantee the highest quality immunoassays. Each kit is put through rigorous validation and stability testing to ensure precision, accuracy, sensitivity, specificity and the results obtained are reproducible for the life of the kit and from lot to lot.

The Quality Control programs ascertain that:
- intra- and inter-assay precision must have a CV that is less than 15%.
- controls, calibrators and plates must meet established specifications.
- minimum one (1) year of accelerated or real-time shelf life for all kit components.

We have designed this troubleshooting booklet as an accessory to help guide you through any questions you may have. Immunoassay failure can be attributed to many factors. Most technical errors can be avoided if the kit insert is read carefully before starting the assay.

Variables to Consider

In general, most problems encountered with ELISA's can be solved upon careful reflection about the assay conditions and techniques and by reviewing the product insert. When troubleshooting, pay close attention to factors such as conditions of kit components, timing, temperature, pipetting/loading technique, washing, and location of the plate during incubation. Variations in any of these conditions may greatly affect results. Tips to help control variations in lab conditions follow:

Conditions of Kit Components and Materials

• Do NOT substitute kit reagents or mix reagents from different kit lot numbers.
• Ensure that plate pouches are sealed correctly and undamaged. Return unused portion of plate along with desiccant and reseal tightly after using strips from pouches. An unsealed pouch reduces shelf life.
• Adhere to storage conditions as indicated in the product insert.
• Inspect reagents for signs of instability or contamination. The presence of precipitate in the solution or discoloration are indicators.
• Avoid frothing of reagents. This will have a deleterious effect on the stability of proteins in solution.
• Reseal plate pouch completely after each use and return to 4℃ storage. Be sure that the desiccant packet is replaced in the pouch with the plate. For most plates, prolonged exposure to outside air will cause the solid phase to decay much more rapidly.
• Use distilled or deionized water when diluting wash buffer powdered concentrate.
• Check the expiration date. Make certain it has not lapsed.
• When using an automated plate washer or manifold dispenser, be sure all pins on the wash head are clear.

Timing

Quick Points:
• Always use times indicated in the product insert.
• Load plates quickly (within 2 minutes).
• Be prepared before starting assay.

Adhere to the timing indicated in the product insert as closely as possible. Variations in timing may cause results to vary greatly. This is especially true of short incubation times. Binding will continue if the incubation time is allowed to lapse, yielding high results while shorter incubation times may cause low results. It is prudent to have all reagents ready so that plates can be loaded as quickly as possible, not to exceed 5 minutes.

These assays were developed according to the incubation times listed in the product insert and variation will effect results negatively.

To remedy timing problems, always be ready with required equipment (pipettes, tips, test tubes, reservoirs, etc.). Also warm all reagents to room temperature prior to starting the assay so they can be used immediately (except when special conditions indicated in the product insert demand cold reagents be used at the start of 4℃ incubation period). If multiple assays are run, time each plate individually so incubation times do not lapse. Allow for washing and pipetting time by spacing assays 3-5 minute apart.

Timing is also important when loading plates. The reagents should be loaded in the same order and with the same timing as previous reagents. This is especially true of the substrate and stop solutions. This ensures that each well is exposed to substrate for the same amount of time before the reaction is stopped.

Temperature
Quick Points:
• Allow time for reagents to equilibrate to room temperature.
• Be certain that the laboratory and work surfaces are within the temperature range recommended in the insert.

Attention to temperature is essential for accurate, reproducible results. Bring all reagents to room temperature (except when special conditions indicated in the product insert demand cold reagents be used at the start of 4℃ incubation period). Cold reagents will cause lower results and uneven warming of the wells may yield an “edge effect.” If this is the case, samples located in wells toward the edge of the plate will warm faster and therefore falsely yield higher readings than those located in the center.

Ensure that the room and/or incubator temperature are within the required temperature range, preferably in the middle of that range. If the laboratory is especially warm, it is highly recommended that a controlled room temperature incubator be used for room temperature assays. These assays have been developed based upon the given temperature range and results differ when conditions vary.

A frequently overlooked factor to consider is the temperature of the benchtop on which the assay is being performed. For example, if the laboratory has heat turned down or off overnight, the temperature of the benchtop will remain lower than the air temperature for some time after the heat is turned on. A cold benchtop will cause the signal to be much lower and may contribute to the edge effect mentioned above. Also, locations near heat sources, in direct sunlight or close to warm equipment will cause the benchtop to warm and may affect results.

Pipetting/Loading Technique

Quick Points:

• Ensure that instruments are calibrated.
• Do not cross contaminate wells.
• Wipe outside of tips and visually confirm that no droplets are present.
• Always use clean tips.
• Read the product insert carefully.

It is important to follow a few guidelines when pipetting. These techniques can be important during the processes of loading plates with calibrators, samples and reagents and making sample dilutions. Many changes can be made during the pipetting process that greatly increase the accuracy and precision of the ELISA assay.

· Do not use pipettes below or above the recommended volume range of the manufacturer. Pipettes are not designed to be accurate outside of this range.

· While making sample dilutions, adhere to the methods indicated in the product insert. The assays were developed using these specific methods for dilution and volumes indicated, so variation from this may change results.

· Residual serum often remains on the outside of the pipette tip when transferring to the serum diluent. Avoid transferring extra serum to the diluent by wiping the outside of the pipette tip free of liquid on the rim of the sample container before transferring. Also, avoid immersing the pipette tip far into the liquid while retrieving or dispensing the sample by touching only the surface of the liquid with the pipette tip.

· When dispensing a reagent or serum out of the pipette tip, some liquid adheres to the inside of the tip and the full volume is not dispensed, especially with viscous solutions. To avoid this, draw the reagent or serum up into the pipette tip and, keeping the tip immersed, successively eject to the first stop of the pipette, then proceed to draw the reagent back up. This effectively rinses the pipette tip.

The liquid can then be dispensed accurately by ejecting only to the first stop of the pipette. Successive uses of this pipette tip(s) for the same reagent or sample do not require additional rinsing.
· Do not scratch the bottom of wells when dispensing reagents onto the microtiter plate. Place the pipette tip on the side of the well to prevent droplets from remaining on the end of the tip, while avoiding contact with the bottom of the well.
· To avoid cross-contamination, do not drag pipette tips over into other wells.
· Pipette slowly to prevent bubbles in the pipette tip due to turbulence. These bubbles will prevent liquid from being dispensed completely when the pipette is ejected to its first stop.
· Gently agitate plates briefly after loading is complete to be certain that all of the liquid in each well is at the bottom. An air pocket may form at the bottom of a well due to the surface tension of the liquid. Brief agitation will eliminate this.
· Always use separate reservoirs and change pipette tips with each new reagent. It is important to avoid mixing reagents.
· Take care to press pipette tips firmly onto the pipette. Check tips visually to be sure that liquids are being dispensed and aspirated completely.
· Ensure that any pipettes being used are calibrated properly. The precision of each pipette should have a 2-3% CV at the minimum volume of the pipette as well as at the maximum volume. Accuracy should also be within 2-3% of the volume indicated on the pipette. Accuracy can be determined by weighing the liquid dispensed and calculating its volume using the density of the liquid. Distilled water is the easiest liquid to use for this test. Water has a density of 1g/ml, so 100 µl (0.1 ml) will weigh 0.1 g.

Location of Plates
Quick Points:
• Do not move or agitate plates during incubation.
• Avoid areas of high light intensity.
• Stay away from air currents or machines that radiate heat.

It is important to choose the location of your plate carefully. An incubator set to the middle of the given temperature range is an ideal location for ELISA plates.

Do not incubate plates in areas of high light intensity. Direct exposure to sunlight will increase the temperature of the plate or the reagent.

Enzyme substrate is also light sensitive and backgrounds will increase if the plate is exposed to light during the substrate incubation period. Be sure to avoid areas where other workers will bump or move plates. Repeated agitation of plates may increase development rates or cause reagents to spill out of the wells.

Also, avoid placing plates near air vents or other objects such as refrigeration units that will emit warm or cool air.

Washing

Quick Points:

- Use an automated plate washer if possible.
- Check for clogged pins in wash head, rinse with deionized water if necessary.
- Decontaminate weekly / monthly.
- For manual washing, ensure all wells are filled completely.
- Firmly tap plates before adding new reagents.

It is recommended to use an automated plate washer for the wash steps to ensure that complete and even amounts of wash buffer are administered. If an automated plate washer is not available, manual washing may be done. It is important though, to avoid creating bubbles which limit contact of wash buffer with the well surface. Always fill each well completely with wash buffer. After filling wells, decant plate by inverting and “flicking” contents into a sink. Repeat this process 4 times. After washing with either an automated plate washer or manual washing, invert plate and tap on an absorbent paper towel. Tapping technique is a critical step in the washing process.

The number of repetitions necessarily depend on how firmly or gently the plates are tapped. For example, more repetitions are necessary when gentle tapping is required. Check the product insert for information about which method is appropriate for your particular kit. Always visually check the plate for remaining liquid. More tapping may be necessary if liquid is remaining in the wells. After washing, proceed to the next step immediately to avoid drying out the plate.

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Frequently Asked Questions

Q: Why do I have poor precision in my results?

A: Many things can cause a lack of precision, including the following:

· Wavelength correction was not used. Read plates at 405nm with a correction wavelength of 630nm.
· Too much time taken while dispensing samples, calibrators and reagents. Perform loading procedures as quickly as possible, preferably in less than 5 minutes.
· Incomplete filling of the wells during washing.
· Extra wash buffer remaining in wells. Be sure to remove all liquid from wells by tapping plates after each set of washes. Check wells for remaining liquid.
· Evaporation of liquid from wells during incubation. If long incubation times (>1 hour) or circulating air in the incubation area are encountered, a plate sealer should be used (do not use during enzyme substrate incubation).
· Problems with pipetting volume. Ensure that correct quantities are being dispensed while making dilutions and loading wells. Check pipette volumes often to ensure correct reading on the dial. Also, check that pipette tips are firmly pressed onto fittings.
· Samples may have been diluted in serum diluent used for another kit.
· Liquid from wells has been transferred to adjacent wells. Incubate plate on a flat, immobile surface. Do not move the plate during the incubation time unless absolutely necessary.
· Sera are hemolyzed, lipemic or contaminated with bacterial growth. Remove any precipitants by centrifugation before making dilutions.
· Internal standards have been exposed to freeze-thaw cycles. If internal standards are used as a reference for precision, be sure that they are stored in a set temperature -20oC freezer. Frost-free freezers frequently warm up to remove frost. Avoid freeze-thaw cycles that will negatively effect the stability of the standards.
· Pipettes need calibration.

Q: Why do I get high backgrounds?

A: Here are a few possibilities:

· Temperature is above the recommended range. In warm laboratories, it is highly recommended that controlled room temperature incubators be used.
· Incubation is extended beyond the recommended time.
· Substrate has been contaminated, stored improperly or exposed to light prior to use. This will be indicated by a yellow color in the substrate when removed from the bottle.
· Exposure of plate to light while color is developing. Enzyme substrate is light sensitive.
· Liquid from adjacent wells has been transferred. Take care to prevent mixing of solutions in adjacent wells while pipetting and manipulating plates

Q: What should be done in the event that wavelength correction is not available?

A: Read the plate at both wavelengths.

Subtract out readings at the correction wavelength from the primary wavelength readings. This will correct for optical imperfections inherent to 96 well plates.

Q: Can I substitute the primary wavelength filter for another?

A: No.

The recommended primary reading wavelength is based upon the peak absorbance of the substrate's development color. The signal will vary significantly if another wavelength is used.

Q: Can another correction wavelength filter be substituted?

A: Yes.

As long as the filter is close to the recommended one.

Q: There is low or no signal after performing the assay. Why would this happen?

A: Here are some possible explanations:

· On the plate reader, a 450nm or other primary wavelength filter was used instead of the 405nm filter.
· Reagents added in a different order than the protocol calls for. Check the product insert carefully for the order of steps to be performed.
· Sample dilutions may not have been mixed. Thorough mixing of samples is necessary before loading the plate.
· Plate was washed after addition of substrate. Stop solution should be added directly to the substrate without washing the plate first.
· Assay was performed below the recommended temperature range and/or the reagents and samples were not brought up to temperature before starting the assay (for room temperature assays).
· Standards were not added or standards from another kit were added for the assay being performed.
· Storage conditions were less than optimal and one or more of the components of the kit were damaged or decayed.

Q: What is the recommended method of storage for samples that cannot be tested at the time of collection?

A: It is recommended to store samples at –20°C or less.

Avoid freeze-thaw cycles that may be damaging to samples. Use a fixed temperature freezer. Frost-free freezers warm up periodically and may degrade samples.

Q: Are plate sealers necessary?

A: Plate sealers will be beneficial for incubation times of more than 1 hour.

They may also be used to prevent evaporation when plates are exposed to circulating air.

Q: Can a plate sealer be reused?

A: No.

Over each well, the plate sealer may contain residue from the previous step. Contact with this residue will cause contamination and will reduce the level of precision in the assay.

Q: Why am I getting false positive results?

A: The following are possible explanations:

· Standard curve was calculated using calibrator values from another kit, or calibrators from another kit have been run.
· Incubation times or temperatures were not followed as per product insert protocol.
· Screen assays and single point determination assays sometimes give false positive results. Run the assay using an entire standard curve to obtain more accurate results.
· Values assigned to calibrators may not be appropriate for the representative sample population being tested. It may be necessary, and it is highly recommended, to use normal human sera from the sample population at a Mean + 3 Standard Deviations cutoff to reassign values to calibrators.

Q: Is it necessary to use a multichannel pipette?

A: In general, yes.

It is recommended to use a multichannel pipette whenever possible to reduce the time needed to load plates. Precision will increase by reducing the loading time.

Q: May the wash buffers from other kits be used for this one?

A: Not all of the wash buffers included in the kits are the same.

Check the product Code Number located on the label to ensure that the two wash buffers are of the same type.

Q: Can I change the method of washing, for example, using less wash buffer or cutting down on the number of washes?

A: No.

We recommend that the number of washes and the volume of wash buffer indicated in the product insert be strictly adhered to. Lowering the volume of wash buffer or number of washes will affect the accuracy and precision of the assay.

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Q: My standard curve does not follow a linear pattern or all of the calibrators have nearly the same optical density. Why would this occur?

A: Here are some possible explanations:

· Wavelength used for reading the plate was not at the peak absorbance of the development color. For example, the commonly used 405nm and 450nm wavelength filters are often confused with one another.
· Washing was not complete. This may arise from clogged tips in the washer or incorrect washing technique. If two reagents from successive steps become mixed in the wells due to incomplete washing, unspecific binding will occur and the plate will have a uniformly high optical density.
· Conjugate or enzyme substrate is not performing properly. To test, mix equal volumes of each. If strong color does not develop immediately, there may be a problem with the storage conditions of the kit.
· Pipette tips that were used for one reagent were used to load another reagent, or an unclean reservoir was used to pipette from. This may cause unspecific binding, and give an artificially strong positive signal.
· Calibrators from another kit were used.
· Plate was loaded too slowly. Do not exceed 5 minutes during this step, since the incubation times must be adhered to within a +/- 5-minute range of the suggested incubation time.
· Plate was not read within the acceptable time limit (see product insert).
· Incubation times and/or temperatures deviate outside the range recommended in the product insert.

Q: Can a standard curve from one plate or from the product insert be used to calculate the values of samples on multiple plates?

A: No.

It is necessary to run a set of calibrators and obtain a standard curve for each plate. Variations in assay conditions for each plate prevent the use of one standard curve for several plates.

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Q: What is the difference between a standard curve and a single cutoff calibrator? Is one better than the other ?

A: A standard curve is better at predicting the value of a patient sample

because it is more accurate to calculate value from a 4 point curve than from a single cutoff calibrator. Cutoff calibrators are beneficial in screen assays where you are only interested in determining whether a patient is positive or negative. Fewer wells are used for a cutoff calibrator and more wells are available for patient samples. To determine the absolute value of a screen positive sample one would use the appropriate antibody specific kits that have 4 point standard curves. With the exception of screen assays all KRISHGEN kits are supplied with a standard curve, and the calibrator 4 can be used separately as a cutoff calibrator.