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GOOD TECHNIQUE FOR THE ELISA ASSAY

Background

The ELISA (Enzyme Linked Immunosorbent Assay) is a very sensitive assay when properly performed. Often the difference between a good assay and a poor assay is the attention to detail in multiple steps. The following suggestions describe in detail how to achieve the best results using the GTI Diagnostics ELISA assays.

Pipetting

Always purchase high-quality pipette tips that have a uniform bore size and exhibit reliable sealing to the pipettor. Always warm all reagents to room temperature before pipetting. Pipetting a cold solution into a warm pipette tip can cause fluctuations in the volume aspirated and the volume dispensed. Pipetting errors can cause the total failure of an assay. The following precautions were taken from the operating instructions of some commonly used pipettes:

Adjustable pipettes

- Make sure the pipettes are calibrated.
- The minimum recommended frequency is yearly, but this should be dependent on usage.
- Develop pipetting precision before you perform the assay. If the instrument is an adjustable pipette, read the instructions that came with it. When setting the volume, some pipettes suggest that you turn the ring past the desired volume and then back to the desired setting when changing from a lower to a higher setting.
- Use the proper tips. Make sure the tips are tightly attached and fit properly.
- Wet the tip with the solution to be measured. Press the control button down to the first stop. Holding the pipet vertically, immerse the tip 3mm into the solution. Do not rest the tip on the bottom of the container. This could cause a vacuum and then a sudden splash when the solution fills the tip. Let the control button slide back gently and slowly. The anti-IgG or anti IgG/A/M are quite viscous and require a gently release of the control button to fill properly.
- Remove the pipette from the liquid and wipe the tip with a lint-free tissue. Hold the tip at an angle against the inside of the receiving vessel. Slowly press the control button down to the first stop and wait until no more liquid is emptied.
- If using a “to contain” (tc) pipette, press the button down to the second stop (blow out) to empty the tip completely. Hold the control button down. Slide the tip out along the inside of the vessel. Then let the control button slide back. Eject the tip. Never lay the pipette down with liquid in the tip as it can flow into the pipette and subsequently contaminate the next solution. Avoid using the blowout step when manually pipetting. This function can introduce a bubble into the fluid in the well. If the bubble remains during the reading on the ELISA reader, it can cause as much as a 0.100 OD variance. If bubbles are inadvertently introduced, puncture them with a wooden stick or needle but be careful not to cross-contaminate the wells.

- Always follow the manufacturer's directions for the pipette that you are using.
- Pre-rinse the tip in the liquid before pipetting the first volume of solution. Liquids such as serum form a thin film on the inside of the pipette tip. Without rinsing, the first volume dispensed would be too small due to the retained fluid. When pipetting fluids with high viscosity such as serum or anti-IgG (or G/A/M) wait a few more seconds when aspirating and dispensing. Pipette tips are typically hydrophobic and will immobilize biomolecules. By pre-wetting the inner and outer surfaces the pipette tips become saturated and little to no biomolecule loss is detected with subsequent pipetting steps. If pre-wetting is not employed the first well(s) may receive a less concentrated solution than the following wells.
- For volumes near 10µl, the values stated for accuracy and precision can only be achieved when the sample is rinsed into another liquid. In this case the tip must not be pre-wet.
- Differences between the temperature of the pipette and solution can result in incorrect dispensing volumes.
- Make sure that the pipettes used for the ELISA assay are clean and are designed to deliver the amount desired. The operator instructions that accompany the pipette usually include instruction for calibration.
- Always pipette below the fluid line or against the sidewall of the well to remove the last drop that adheres to the pipette tip. The volume in this last drop can vary from tip to tip and with each pipetting maneuver.

Multichannel Pipettes

- Multichannel pipettes are of special concern. Be sure that the tips are appropriate for the pipette. They should fit properly and be firmly attached.
- Do not set the volume below the minimum volume or above the maximum volume printed on the pipette.
- ALWAYS hold the pipette upright. The same procedure for filling and dispensing applies to multichannel pipettes.
- When ejecting the tips, remove tips by hand that are jammed on tightly. Never use force when operating the ejector.
- Check the pipette daily by filling it to the maximum volume. Hold it upright for one minute. If a drip forms at one of the tips, troubleshooting is in order.
- When calibrating the pipette, check each channel separately according the operating instructions. Follow the cleaning and maintenance instructions provided by the manufacturer.

Repeating Pipettes

Be sure to use the proper pipette tips. It is important to use tips for delivery of small volumes. Before the first volume is dispensed into the assay, one volume should be dispensed into a discard.

General

If a sample has aggregated proteins, those proteins can bind non-specifically to the wells and cause false positive results. Samples that contain particulates should be centrifuged or filtered before pipetting into the assay.

Washing

Most ELISA assays require at least one wash step. The GTI Diagnostics assays require two wash steps. The first removes any unbound antibody and serum proteins. The second removes unbound anti-IgG or anti-IgG/A/M. Good washing is critical to good precision. The following suggestions will ensure improved results:

- The volume of wash should be adequate to rinse down the sides of the wells and remove any serum or anti-IgG that might have adhered during the addition step. We suggest filling the wells nearly to the top. The wash solution contains a detergent to help loosen proteins from the wells.
- Studies have shown that three well-performed washes are adequate. Four washes are also effective but five washes generally start to remove more than just the unbound proteins.
- Add an equal volume of wash solution with equal force to all wells. If a jet pipette is used, direct the fluid at the side of the wells rather than at the bottom. Make sure the manifold has no air by turning it upside down during the priming and slowly expressing the wash solution until all needles are dispensing.
- The plates should be completely decanted between washes and the plate firmly rapped on a blotting towel after the last wash. Do not over dry the plate by pounding after all wash solution has been removed.
- All manufacturers of the microwell plates suggest that hand washing is best for the ELISA assay.

Automated Plate Washers

If an automated plate washer is used, the pressure of the dispensing wash solution must be carefully checked. Wash solution that is added too forcefully can wash the coating off of the well. Aspiration of the wash solution is also critical. If the vacuum is too strong or too weak, it can adversely affect the assay in unpredictable ways. A vacuum that is too strong can cause drying of the wells. Drying of the wells containing enzyme can seriously weaken the enzyme activity in that well.

The following example shows the effect of vacuum aspiration on assay results:

Vacuum mmHg	**No. of plates per run**	**No. of plates out of spec.**	**CV**
400	112	0	2.0
550	10	2	3.3
250	10	3	2.4

The best method of aspiration is from the top down. The needles should begin aspirating as soon as they enter the liquid so that aspiration occurs as the needles descent to the bottom of the well. This type of aspiration reduces shear action and prevents air currents from drying out the surface bound protein. It is crucial that the needles stop aspirating as soon as the liquid is removed or they will draw air over the protein-coated surface and cause unnecessary drying. Needles that reach their lowest position before they start suction will aspirate for a given amount of time, regardless of the amount of fluid in the well. This can result in “dry aspiration” once the fluid is removed. Even minimal drying can result in a loss of protein activity, especially enzyme activity.

If using a plate washer that washes one strip at a time, there may be a significant delay between washing the first strip to the last. To minimize the risk of drying it is best to have the first strip washed three (3) times and leave the wells filled with the wash solution from the 4th wash, move to the second strip and repeat. When all strips have been washed three (3) times and remain full of wash solution the washer should then aspirate the liquid from each strip to complete the 4th wash.

Optimization of vacuum strength is crucial to maintaining low CV’s in an assay. If vacuum force is too high, shear forces and air currents will denature bound protein and inactivate enzymes. If vacuum strength is too low, excessive residual wash solution will remain in the wells and depress enzyme activity.

Even with optimal settings on a preferred plate washer, Costar has suggested that at least 5% of their assays had to be repeated.

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Multiple Assays
Do not allow the plates to dry between steps. When running multiple plates or if delays are unavoidable keep all wells filled with wash solution until ready to proceed with the next step or leave plates upside down on a moist towel after removal of the last wash if delay is unavoidable.

The following example shows the loss of enzyme activity as protein dries:

Summary Data N=6 plates/time group

Time delay	Avg. OD	CV	Low well	High well
0 min.	1.080	1.77	3.5%	4.8%
15 min.	0.995	2.14	5.9%	4.8%
30 min.	0.711	9.96	43.6%	9.2%
60 min.	0.317	40.0	59.9%	86.1%

The following example demonstrates edge effects from a 20-minute delay:

Average OD = 0.669 Minimum = 0.224 Maximum = 0.759
Wells > 10% of the average:

0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	.759	0	0	0
0	0	0	0	0	.744	0	0	.751	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0

Wells < 10% of the average:

0	.233	.225	.224	0	.330	.534	.326	.589	.452	.585	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	.504	0	0	0	0	0	0	0

Final Decant
A delay between the final wash and the addition of substrate can cause the loss of enzyme activity because drying is accelerated in the outer wells. One study showed that with a delay of 30 minutes in adding the substrate, the average OD dropped 30%. The Coefficient of Variation was nearly 10% and the lowest sample was 44% away from the mean. If multiple plates are being tested at once, a delay of 15 minutes could easily take place in the processing of the plates. Do not decant the final wash until the next reagent is ready to be added. Prepare the

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substrate 5-10 minutes before the end of the previous incubation. Store it in the dark. It will then be ready to dispense immediately after the wash step.

After the last wash, rap the plate on a dry blotter or paper towel until no more fluid can be seen. If wetness is observed, move the plate to a new spot and rap it again on the dry towel. If the towel is dry, turn the plate upright and promptly add the next reagent.

Incubation
Improper incubation can lead to uneven results. The most obvious sign of improper incubation is called the “edge effect”. The wells on the outside of the plate, especially the four corners have higher or lower than expected values. The edge effect is caused by uneven temperature, loss of enzyme activity due to drying, and exposure to light.

Stray light can also affect color development. Be sure the plate is kept entirely dark for even color development.

Polystyrene is a poor conductor of heat so outer wells will reach the desired incubation temperature much more quickly than inner wells. The increased temperature increases the kinetics within the wells. For this reason, a dry heat incubator is less effective than a water bath or heat block. If using a dry incubator place the plate on a paper towel in the middle of the incubator. If using a heat block, a small gap must exist between the block and the bottom of the wells.

Air drafts can also affect temperature. If a room temperature incubator is used for the final incubation, a plate sealer should be used to cover the wells to prevent evaporation and cooling in the wells. If a room temperature incubator is not being used, the plate should be placed in a draft-free area on a surface that is not cooler than the air temperature. The GTI Diagnostics outer box can be used as a room temperature incubator.

The following example shows the effect of temperature gradients at 37°C on assay results:

	Avg. OD	CV	Low well	High well	# out of spec.
Incubator	1.20	5.5%	7.1%	28%	4
Heat block	1.30	3.0%	6.3%	7.3%	0

A technical bulletin by NUNC describes the edge effect in more detail.

Conclusion
Often problems develop as cumulative errors. Accurate pipetting, careful and thorough washing and proper incubation will result in excellent ELISA assays. Each step if not controlled may contribute only a small amount of variation. When pipetting is poor, washing is not adequate, and incubation temperatures are not controlled; the results will be inconsistent and the cause will not be evident. With knowledge of how each step must be controlled, the technologist can achieve satisfactory results from the very beginning.

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