IDEXX

ELISA Technical Guide

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Introduction

IDEXX manufactures diagnostic tests for the detection of diseases in ruminants, equines, swine, and poultry.

The enzyme-linked immunosorbent assay (ELISA) is one of the most sensitive and reproducible technologies available. These assays are rapid, simple to perform, and easily automated. IDEXX introduced the first commercial poultry ELISA for infectious bursal disease (IBD) in 1985 and the first commercial livestock ELISA for Aujeszky's disease/pseudorabies in 1986, enhancing the way laboratories test production animals.

As with any assay, the reproducibility and reliability of ELISAs depend upon proper technique and attention to detail. This ELISA technical guide will increase your awareness of ELISA techniques and help you maintain proficiency with this methodology.

Check your product insert for specific instructions for each assay you perform. Periodically, improvements and revisions are made to product inserts. Therefore, it is important to review the protocol on a regular basis.

If you have questions concerning any of the following information, please contact your local IDEXX representative.

ELISA technology

An ELISA is a set of standardized reagents and microwell plates manufactured for a specific test. An IDEXX ELISA may contain some or all of the following components: coated plates (solid and/or strip plates), sample diluent, controls, wash concentrate, conjugate, substrate, and stop solution. The tests are manufactured in batches or lots. Each component of each test lot is optimized and manufactured to work as a unit. The tests pass many quality-control procedures conducted by IDEXX, numerous worldwide reference laboratories and agencies, and/or the United States Department of Agriculture (USDA) before they are approved and released for sale.

The ELISA is a rapid test used for detecting and quantifying antibodies or antigens against viruses, bacteria, and other materials. This method can be used to detect many infectious agents in ruminants, equines, swine, and poultry.

In ELISA technology, the solid phase can consist of a 96-well polystyrene plate. The function of the solid phase is to immobilize either antigens or antibodies in the sample as they bind to the solid phase. After incubation, the plates are washed to remove any unbound material. In some assays, the conjugate is then added to the plate and allowed to incubate.

The conjugate consists of either an antigen or antibody that has been labeled with an enzyme. Depending upon the assay format, the immunologically reactive portion of the conjugate binds with either the solid phase or the sample. The enzyme portion of the conjugate enables detection.

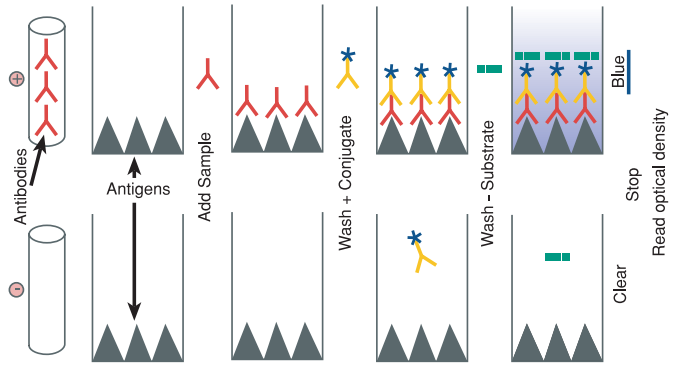
The plates are washed again and an enzyme substrate (hydrogen peroxide and a chromogen) is added and allowed to incubate. Color develops in the presence of bound enzyme, and the optical density is read with an ELISA plate reader.

Note: The steps and reagents used can vary in an ELISA. Refer to the product insert for specific information.

ELISA formats

Indirect format

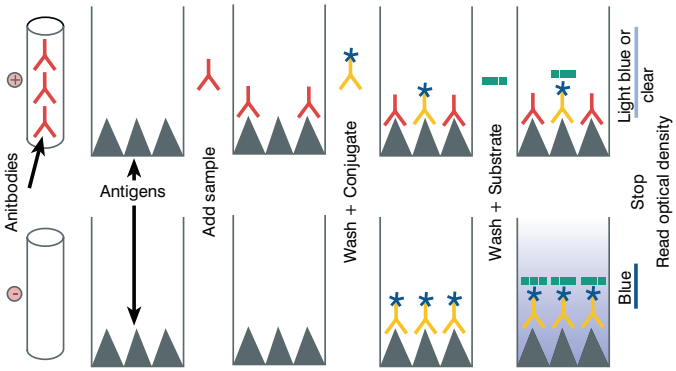
In the indirect format, antigens are coated on the plate. The sample antibody, if present, is sandwiched between the antigen coated on the plate and an enzyme-labeled, anti-species globulin conjugate. The addition of the substrate (enzyme substrate-chromogen reagent) causes color to develop in the wells where antibody is present. This color is directly proportional to the amount of bound sample antibody. The more antibody present in the sample, the stronger the color development in the test wells. This format is suitable for determining total antibody level in samples.



Steps in an indirect ELISA

Blocking format

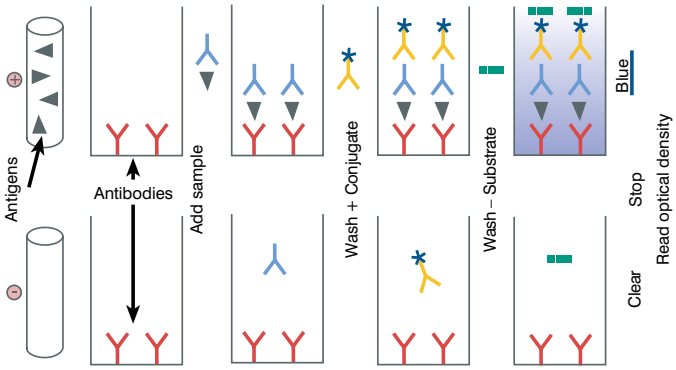
In this format, the specific sample antibodies block the enzyme-labeled, specific antibody in the conjugate. As shown in the graphic, the antibodies in the sample and in the conjugate both bind to the antigen coated on the plate. Adding an enzyme substrate-chromogen reagent causes color to develop. The color intensity is inversely proportional to the amount of bound sample antibody. The more antibodies present in the sample, the less intense the color development in the test wells.



Steps in a blocking ELISA

Indirect antigen-capture (sandwich) ELISA:

In the indirect antigen-capture ELISA, the antigen in the sample is bound by the antibody coated on the plate and by the detector antibody contained in the added detector solution. The detector antibodies are not enzyme-labeled. The conjugate added in the next step can bind to the antibody of the detector solution. If the conjugate binds to the detector solution, a color reaction takes place. The antigen is thus indirectly detected.



Steps in an indirect antigen-capture ELISA

ELISAs provide the ability to:

- Test a large number of samples at the same time.
- Automate the procedure using robotics or other types of automated equipment.
- Computerize the calculation and reporting of results.

ELISA components

Coated plates

The 96-well plates are made of polystyrene and coated with either inactivated antigen or antibody. This coating is the binding site for the antibodies or antigens in the sample. Unbound antibodies or antigens in the sample are washed away after incubation.

Sample diluent

Most assays require a specific dilution of the sample. Samples are added to the sample diluent and mixed prior to putting them onto the coated plates.

Controls

The positive control is a solution that contains antibody or antigen. The negative control is a solution without antibody or antigen. The controls help to normalize or standardize each plate. Controls are also used to validate the assay and to calculate sample results. In some tests the controls are prediluted and ready to use, and in other tests the controls must be diluted the same as the samples. Be sure to follow the instructions in the product insert.

Conjugate

ELISA conjugates are enzyme-labeled antibodies or antigens that react specifically to plate-bound sample analytes. Unbound conjugate is washed away after incubation and before the addition of substrate. The optical density of the colorimetric substrate is directly proportional to the quantity of bound enzyme present.

Substrate

For peroxidase conjugates, the substrate is a mixture of hydrogen peroxide and a chromogen that reacts with the enzyme portion of the conjugate to produce color.

Wash concentrate

The wash concentrate is a buffered solution containing detergent used to wash away unbound materials from the plates. Be sure to follow the instructions in the product insert for diluting the wash solution before use.

Stop solution

The stop solution stops the enzyme-substrate reaction and, thereby, the color development.

Note: Other reagents may be included depending on the test. These may include conjugate diluent, detector, etc.

IDEXX tests are manufactured in batches or lots according to strict quality standards. Each component or reagent in a test lot is optimized to work with the other reagents in the test. This includes measurements of sensitivity, specificity, and repeatability. Therefore, it is very important not to mix reagents from different lots. Typically, plates, conjugate, and test controls are kept together.

All components have an expiration date.

ELISA equipment

Equipment for ELISA testing is widely available. Readers, washers, and pipettes are available as manual or automated systems. Some of the factors affecting equipment selection are the number and types of tests and samples, technical training of staff, and financial considerations. Below is a brief outline of some equipment available for performing ELISA testing.

There is a large selection of equipment available. When purchasing a plate reader, call IDEXX Technical Services to make sure the reader can communicate with the current IDEXX software.

Pipettes

- Single-channel, fixed-volume, and adjustable-volume (1–20 µL, 10–100 µL, 20–200 µL, etc.)
- Multichannel, 8- and 12-channel
- Semiautomated dispensing units
- Fully automated systems that can process multiple plates

Dilutors

- Single-channel
- Multichannel
- Automated dispensing units

Washer systems

- Manual systems that wash one row or column at a time
- Semiautomated systems that handle one strip or plate at a time
- Fully automated systems that can process multiple plates

ELISA plate readers

- Manual readers that read one row or well at a time
- Semiautomated readers that read one plate at a time
- Fully automated systems that can process multiple plates simultaneously

Other

- Humidity chamber (not required for all ELISAs)
- Adhesive plate covers for assays that have long incubation times, to help prevent evaporation (not required for all ELISAs)
- Incubator or plate shaker incubator (not required for all ELISAs)

Multichannel pipette and single-channel pipette

Semiautomated wash system

Plate reader

Equipment maintenance and calibration

The maintenance and calibration of your laboratory equipment is extremely important in obtaining accurate and reproducible results.

The **Maintenance and Calibration Schedule (Appendix D)** can be used as a guideline. Adjust maintenance routines according to the amount of daily testing performed in your laboratory. Always refer to your equipment manufacturer's guide for their recommendations.

Be sure to label your pipettes with the calibration date and keep a log for the calibration and maintenance of all your equipment.

Calibration protocols

Equipment always needs to be in proper calibration. Equipment that is out of calibration can produce false or inaccurate results. As a guideline, refer to the **Maintenance and Calibration Schedule (Appendix D)** and your manufacturer's instructions for the proper calibration protocol and required frequency.

Options for calibrating pipettes

- Perform the gravimetric method outlined in **Appendix A**.
- Use a commercial automated calibration system in your laboratory.
- Send the pipette to the manufacturer; see your owner's manual for instructions.
- Send the pipette to a pipette calibration service.

Pipette with calibration label

Sending pipettes out for service is beneficial when repair or maintenance is necessary. However, this practice provides only a limited level of quality control, which can be increased with in-house calibration.

Operator technique and laboratory environment are two critical variables that determine how a pipette will perform when used on your bench top. A thorough quality-control program must include a quantitative account of these effects. It is beneficial to have a method in place that allows you to perform regular, routine performance verifications on your own pipettes. By doing so, you will be able to track pipettes that are drifting out of tolerance. When this happens, the failing pipette should be sent out for corrective maintenance or repair by a qualified service before it compromises your laboratory data and productivity.

It is recommended that the laboratory sets specifications about micropipettes performance based on their own internal or accreditation standards. As a guideline, micropipettes and multidispensing micropipettes with precision of ±10% for volumes <10 L and ±5% for larger volumes can be used.

Options for verifying readers

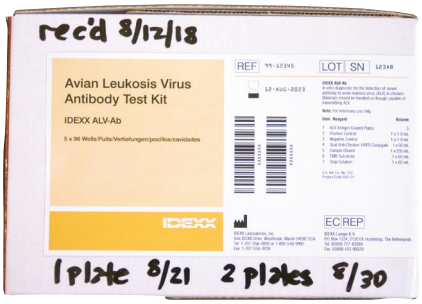
- Refer to the manufacturer's manual for the verification process.
- Contact IDEXX to inquire about using a calibration/verification plate.

Reagent handling and preparation

Receiving tests

When you receive your ELISA kit, we recommend recording the date on an Inventory Control Tracking Chart (see **Appendix B**) and on the boxes. Inspect them for damage and store them at the product insert temperatures. When using tests from your inventory, use the first-in-first-out (FIFO) method, i.e., use the tests that will expire first. Individual components may have longer expiration dates than the actual date on the outer box label. **However, abide by the expiration date on the outer box label.** If you do not use an entire test, mark the date it is opened and each time it is used thereafter.

Note: A test should not be used past its expiration date because the results would be invalid and not supported by IDEXX.



Label your kit with the date it was received.

General reagent handling

Be sure to check your product insert for guidelines on handling and preparing reagents. Some tests recommend that all reagents and plates be brought to room temperature (18°C–26°C) prior to use; others indicate that only specific reagents be brought to room temperature. When you need to bring a test to room temperature (18°C–26°C), take it out of the refrigerator and take the components out of the box at least 2–3 hours before beginning the assay.

After warming to room temperature, the reagent bottles should be mixed before use. Measure all reagents using sterile or clean labware. Be careful to measure only what is needed for the number of plates being run. This will help to maintain the integrity of the reagents. **Do not return reagents to the original reagent bottles.** We strongly recommend using disposable pipettes and reservoirs when handling reagents to minimize the risk of contamination. However, if you choose to reuse any disposable device, use a separate reservoir for each reagent and be sure to label them. Also, wash and thoroughly rinse the disposable reservoir with deionized or distilled water after each use. Change and discard the disposable reservoirs as frequently as possible. Never use the same reservoir for conjugate and substrate, even if it has been washed.

The contamination of reagents may compromise your test results. Labeling your reagent reservoirs and using a separate one for each reagent will help minimize the risk.

Labeled reservoirs

Test component handling and preparation

Most assay plates are packaged individually with a desiccant. If a partial, solid plate is used, aspirate all the liquid from the used wells and cover them with sealing tape. Store unused portion of plates with several desiccants in a new resealable bag. If a strip plate is used, warm up the full plate to room temperature, and then use only the strips needed. Store the remainder in a resealable bag with desiccant.

ELISAs can be sensitive to temperature extremes. Try to maintain a laboratory temperature of 18°C–26°C. Avoid running assays under or near air vents as this may cause excessive cooling, heating and/or evaporation. Also, do not run assays in direct sunlight as this may cause excessive heat and evaporation. Cold bench tops may affect your assay; avoid this issue by placing several layers of paper towels or some other insulating material under the assay plates during incubation.

Make sure the sample diluent and wash concentrate have come to room temperature (18–26°C) before use. These are usually the largest bottles in a test and require the most time to equilibrate. If the wash concentrate still shows crystal formation after reaching room temperature, mix it by inverting it several times.

Most tests are formulated with prediluted controls and are reconstituted. However, some require that you dilute them in the same manner as your sample. Controls should be added to the plate in the same method and at the same time as the samples.

If the test requires you to prepare a “working” conjugate solution, be sure to follow the instructions closely. Prepare only what you immediately need, and do not save leftover solution for future use. If conjugates are contaminated or improperly stored, they may lose enzymatic activity or may have an apparent increase in background color. Most tests supply a ready-to-use conjugate.

Our ELISAs include a ready-to-use substrate. The chemical activity of the substrate will be compromised if it is exposed to light or comes into contact with metal. Protect this solution by storing it in a dark container until ready for use.

Be sure to use the stop solution included with the test. Follow any safety precautions in the product insert. The stop solution should be at room temperature before use. If the stop solution shows crystal formation after reaching room temperature, mix it by inverting several times. The stop solution may crystalize at lower temperatures. Before use, make sure that it is completely dissolved and appears clear.

Refer to the product insert for specific details on the test you are using.

Do not exchange components between lot numbers, even if tests are of similar type. Test results may be severely and adversely affected.

Seal, label, and store partially used plates in a bag with desiccant.

Quality control

Internal controls

We recommend using internal assay controls to monitor your ELISA techniques and performance over time.

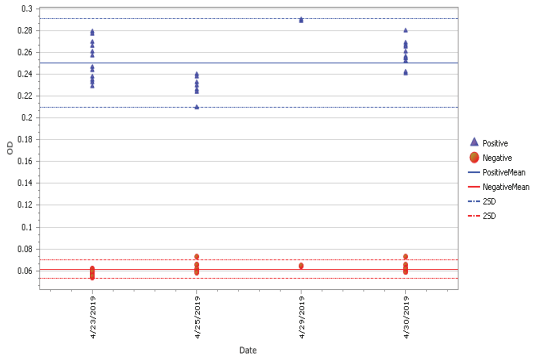
Because sera are generally received in small quantities, controls will need to be made by pooling samples.

Follow these general steps:

1. Collect negative and positive samples separately. When sufficient quantities of each have been collected, pool similar samples together, and mix the pooled samples thoroughly.
2. In small quantities, perform the serial dilution of positive sera in negative serum.
3. Assay each dilution according to the standard test protocol (the same sample dilution as described in the product insert). Select the dilution that is most comparable to the sample-to-positive (S/P) or sample-to-negative (S/N) values that you want to monitor. Make large quantities of that dilution.
4. Prefilter the prepared controls using a 0.45-micron filter membrane; you may then choose to filter with a 0.20-micron filter membrane (optional).
5. Aliquot the freshly made internal control into airtight vials, and then label, date, and store, frozen at -20°C to -80°C if possible.
6. Keep a record of all information.

To use this control, thaw, mix, and dilute it in the same manner as a routine sample. Run it on every plate next to the test controls. Do not refreeze your in-house control. You can store it for up to 5 days at 4°C.

Record your results in a tracking system, in the IDEXX software, or on the Laboratory Tracking Chart (**Appendix C**) and graph them or use the internal control feature within IDEXX software. Any variations or trends should alert you to review your technique and quality-control measures.

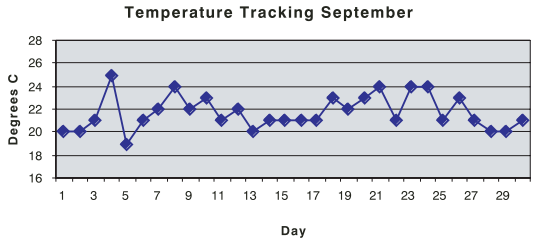


Tracking chart for controls

Monitoring temperature

Record and track the temperature during each assay. If your laboratory's temperature fluctuates from morning to afternoon, record this on your tracking chart. If you have conditions that are difficult to control, it is a good idea to use a temperature control chamber to incubate your plates. Using ELISA plate covers will help control evaporation and accidental spills.

To help troubleshoot questionable results, record and graph the laboratory temperature.



Laboratory temperature tracking chart

Quality control check

Use the Quality Control Quick Check (**Appendix E**) to troubleshoot any problems.

Sample handling

Incoming sample quality

Sample quality can have a significant impact on final assay results. Most labs have no choice regarding the quality of incoming samples. In many cases, the sample diluent formulation compensates for variations in sample quality.

Gross fungal or bacterial contamination can have adverse effects on the antibody or other protein components of a sample and may have an undesirable effect on test results. If sample quality is highly questionable, obtaining a fresh sample is strongly advised, when possible.

Serum/plasma samples

Serum samples with trace hemolysis (light-red color) and moderate lipemia (milky appearance) may have little or no effect on ELISA results. Avoid using samples that are heavily hemolyzed (dark-red color) or grossly lipemic. Check your product insert for information. When serum is on the clot, be careful not to aspirate any of the clotted material or blood cells.

Blood sample
Take sample from the area indicated.

Milk samples

Refer to the product insert to determine if skim and/or whole milk can be used as samples. In general, whole-milk samples can be used after centrifugation for 15 minutes at 2,000 x g, or after overnight refrigeration at 2°C–8°C. The sample should be drawn from below the cream layer. This sampling practice can vary throughout global regions. Some regions may thoroughly mix the whole milk and then draw the sample. Refer to the product insert to determine whether the test has any restrictions when using whole-milk samples.

Oral fluids

Avoid pipetting the sediment from these samples as this can be difficult to pipette and can cause unexpected results.

Ear notches

Refer to the product insert for soak solution and times. Avoid pipetting debris or hair as these can cause unexpected results.

Other sample types

Refer to your product insert for sample handling, preparation and storage of other sample types (e.g., albumin, cloacal swabs, whole blood, other tissues).

Note: For pooling or detection of a positive in a certain sample size (e.g., 1 in 250), see the specific product insert.

Sample handling (continued)

Storing samples

Be sure samples are properly stored. In general, serum samples should be refrigerated at 2°C–8°C for up to 5 days. If samples need to be stored for a longer period, they should be removed from the clot and frozen to a minimum of -20°C. Make sure all stored samples are properly labeled and sealed to prevent evaporation. Evidence of lyophilization (concentration of the sample) can be seen as crystallization and is common in self-defrosting freezers. This should be avoided because the integrity of the sample will most likely be compromised. It is recommended to avoid repeated freeze-thaw cycles so performance is not compromised. Milk samples are best stored refrigerated with a preservative such as bronopol. Ear notches or whole ears can be frozen for long-term use at -20°C and used again.

Using frozen samples

Frozen samples can be thawed at room temperature or in a refrigerator. All thawed samples need to be thoroughly mixed prior to dilution to ensure that the proteins are dispersed throughout the sample. Mix by gentle vortexing or inverting at least five times. Frothing or overmixing of samples will cause denaturation of serum proteins.

Avoid numerous freeze-thaw cycles, as this may damage the antibodies or antigens in the sample. We recommend no more than 3–5 cycles.

Light hemolysis

Dark hemolysis

Unmixed thawed sample; proteins settled on bottom of tube; mix prior to taking sample.

Pipetting methods

Two pipetting methods used for ELISA are standard (forward) and reverse. Not all pipettes are capable of reverse pipetting. Refer to the instructions included with your pipette for details.

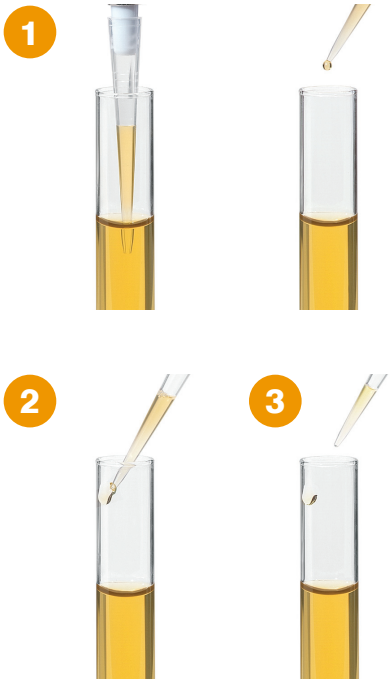
It is recommended to use standard (forward) pipetting for the preparation of sample dilutions and reverse pipetting for the addition of reagents.

Careful pipetting is crucial for accurate test results. Become familiar with the pipette and both methods before running actual tests. Be sure to use the correct pipette and tip (volume capacity) for the volume being transferred.

Pipetting technique

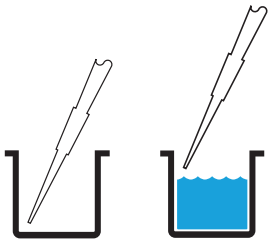
1. Draw the calibrated volume of sample into the tip. The drop on the tip needs to be removed.
2. Touch the side of the tube with the tip to remove the excess liquid.
3. Ensure that you have the proper volume of sample in the tip.

When using a multichannel pipette, if the wells on your plate are empty, position the tips into the lower corner of each well. If the wells on your plate contain liquid, position the tips above the liquid.



Proper pipetting

- Proper position to dispense reagents into empty wells using a multichannel pipette: in the lower corner of each well
- Proper position to dispense reagents into wells containing liquid using a multichannel pipette: above the liquid



Pipetting methods (continued)

Standard (forward) pipetting and sample preparation

1. Put a new tip on a single-channel pipette and make sure that it is on tight.
2. Press the plunger to the first stop.
3. Some manufacturers recommend that you prewet the tip by aspirating and expelling an amount of the sample. Check the instructions that came with your pipette.
4. Draw the calibrated volume of sample into the tip and pause for one second with the tip still in the sample. Be careful not to place the tip too deeply into the sample.
5. Touch the tip to the side of the sample container to remove any excess liquid on the outside of the tip.
6. Dispense the sample into the measured diluent by depressing the plunger past the first stop to the second stop. Be careful not to place the tip too deeply into the sample diluent.
   For samples less than or equal to 10 μL: After dispensing the sample into the diluent, rinse the pipette tip in the diluent by pushing the plunger down 2–3 times before ejecting the tip.
7. Mix samples with a multichannel pipette prior to dispensing samples onto the plate. You can do this by pushing the plunger down 3–6 times.
8. Eject the tip into a waste container.

Reverse pipetting using a multichannel pipette

1. Put new tips on the pipette. Make sure they are on tight and straight.
2. Press the plunger past the first stop and halfway to the second stop.
3. Draw the liquid slowly, being careful not to draw any air bubbles into the tips. Check for consistency of volume in the tips.
4. Touch the tips to the edge of the reagent reservoir to remove excess liquid on the outside of the tips.
   a. If the wells on your plate are empty, position the tips into the lower corner of each well.
   b. If the wells on your plate contain liquid, position the tips above the liquid.
5. Slowly dispense the liquid into the wells by depressing the plunger to the first stop. Be careful not to splash liquid out of the wells, and make sure there are no drops left on the tips.
6. To repeat, hold the plunger at the first stop and continue with step 3.
7. Eject the tips into a waste container.

Note: Reverse pipetting uses more reagent/volume (known as “dead volume”).

Automated dilution systems and competitive assays

For those systems and assays using neat samples or lower dilution factors, the sample can be put directly into the wells of the coated plates.

Follow the sequence below:

1. Add the diluent to the plate.
2. Add the sample into the diluent.
3. Mix by tapping the plate or repeating pipetting.

Automated equipment uses more reagent/volume than semiautomated. Check your manufacturer’s recommendations for purging and priming your system. The “dead volume” can be optimized with guidance from the manufacturer.

ELISA plate timing

Adding samples and controls

Incubations for assay plates should be timed as precisely as possible. Usually the process of adding samples to the plate requires the most time. When you dispense samples onto the plate, it is critical to keep the time difference between the first and the last sample to a minimum so results are not affected by differences in incubation times.

For tighter control over the time differentiation from when the controls and samples are added, you can put your controls in a tube that is racked in position with your samples. Then use a multichannel pipette and put the controls onto the plate at the same time you are adding the samples.

To minimize incubation time between controls and samples, rack the controls with the samples and add them to your plate using a multichannel pipette.

Multiple plate runs

When timing multiple plates, it is important to keep track of the time interval from the first plate to the last plate in the run. Keep your batch sizes small enough so your processes do not overlap. You do not want to be washing a plate while another needs to have conjugate added. It is recommended to use a timer for every plate or a single channel of the timer for each plate.

Use several timers when incubating multiple plates.

ELISA plate washing

Automated or semiautomated systems

In general, an automated or semiautomated wash system in proper working order will provide more consistent washing than manual methods. Check that all the dispensing needles are dispensing with a smooth, steady stream and that all aspiration ports aspirate uniformly.

Make sure your wash system is properly cleaned and maintained. Refer to the **Equipment maintenance and calibration** section in this guide **(page 8)** and your owner's manual for proper maintenance. The plate-washing technique should be consistent from plate to plate and from row to row within a plate. Avoid soak times unless specifically recommended in the product insert.

Prepare the wash solution according to the product insert. Use only the wash solution formulation included with for your test.

Aspirate reagents from the plate before dispensing the wash solution.

Follow the specific recommendations in your product insert for the number of washes to use at each step of an assay. Most assays require approximately 300–350 µL per well per wash. Be careful to fill the wells above the level of the reagents. Do not allow wells to overflow. If this occurs, the test results may be invalid.

Do not allow the plate to dry between plate washings and prior to the addition of the next reagent.

After the final aspiration, tap out any remaining liquid onto several layers of absorbent material.

When testing milk, albumin, or whole blood, take extra care to inspect the wells. Because of their protein or fat composition, these sample types are sometimes more difficult to wash from the wells and may require the maximum recommended number of wash cycles. If not washed well they could cause increased background and/or false positives.

After tapping out plates, check paper towels for any evidence of color. This may indicate that the plates were not washed properly and there are reagents remaining in or around the wells.

Microbial growth in wash system tube; could affect plate washing and increase background or generate unexpected results.

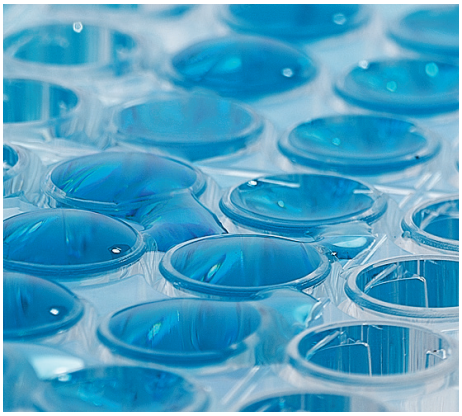
ELISA plate washing (continued)

Manual or semimanual systems

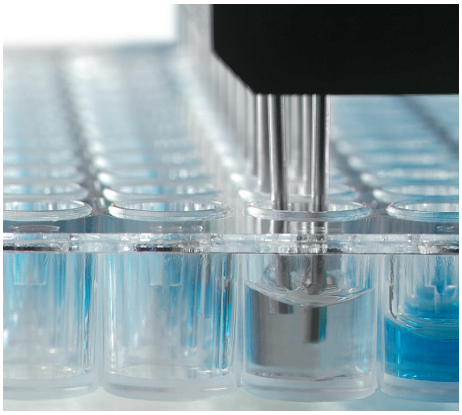
Work quickly so the time from washing the first well/row to the last is minimal. If the time is too long, the empty wells may dry out and the last wells will have a longer incubation than the first wells.

Make sure to aspirate all the liquid from the wells by placing the aspiration needles 1 mm above the bottom of the plate. Do not scrape the surface of the plate as this will remove the antigen/antibody bound to the surface and cause inconsistent or inaccurate results. After aspiration, wells should not dry before the addition of the next reagent.

After tapping out the plates, check the paper towels for any evidence of color. This may indicate that the plates were not washed properly and there are reagents remaining in or around the wells.



Overflowing plate; this can contaminate other wells.



Proper position of manual washer needles for dispensing wash solution.

Plate reading and data management

Reading plates

The last step in an ELISA is to read and interpret the results. For most assays, the optical density (amount of color) of the solution on the plate is read with a spectrophotometer, commonly known as a plate reader. There are many models and manufacturers of plate readers; refer to the manufacturer's instructions for details of operation.

The product insert specifies which wavelength is required for the assay. Most assays specify the absorbance reading at 450 nm or 650 nm. Most assays are optimized using a plate reader equipped with a 650 nm filter. The use of 630 nm or 620 nm filters will lower the optical density (OD) values of both the controls and samples but will do so equivalently across the entire plate. The use of these alternative filters will not affect the test results.

Plates should be read as soon as possible following the addition of stop solution. Absorbance readings may drift if excessive time elapses between stopping the reaction and reading the plates.

Data management

IDEXX provides software to assist you in the collection and management of the data from your ELISAs. The software interfaces with most common plate readers to read the plate, send the optical densities to the computer, and calculate the results. An IDEXX Technical Services representative can assist you in learning more about this software and validated readers.

ELISA troubleshooting

This information is intended to help you troubleshoot your ELISA procedure. If you need assistance, please contact your local IDEXX Technical Services representative.

Note: The conditions described here may not pertain to every ELISA because performance requirements vary for individual assays. Be sure to check your product insert for specifications.

High background or excessive color development (high optical density [OD] readings)

Possible causes	Recommended actions
Poor-quality water was used to wash plates or to prepare wash solution.	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.
Substrate solution has deteriorated.	Make sure the substrate is colorless prior to addition to the plate.
There was insufficient washing or poor washer performance.	Try using the highest number of washes recommended for the assay. Make sure that at least 350 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 or dispense/aspirate poorly.
Washer system had microbial contamination.	Clean out microbial contamination by flushing the system with a solution of tergezyme 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.
Wash system contained an alternate wash formulation.	Be sure each unique wash solution is properly labeled. Prime the system thoroughly when switching between solutions.
Reader was malfunctioning or not blanked properly; this is a possible cause if the OD readings were high and the color was not dark.	Verify the reader's performance using a calibration plate and check the lamp alignment. Verify the blanking procedure, if applicable, and reblank.
Laboratory temperature was too high.	Maintain the room temperature within 18°C–26°C. Avoid running assays near heat sources, in direct sunlight, or under air vents.
Reagents were intermixed with other tests, contaminated, or prepared incorrectly.	Ensure that the correct reagents were used, that working solutions were prepared correctly, and that contamination has not occurred.

ELISA troubleshooting (continued)

Insufficient color development (low optical density [OD] readings)

Possible causes	Recommended actions
Laboratory temperature was too low.	Maintain the room temperature within 18°C–26°C. Avoid running assays under air conditioning vents or near cold windows.
Wash solution was prepared incorrectly or the wrong wash solution was used.	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.
Washer system had microbial contamination or contained an alternate wash formulation.	Clean out microbial contamination by flushing the system with a solution of tergezyme 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.
Too many wash cycles were used.	Stay within the recommended range for the number of wash cycles. Try to use the lowest number of washes recommended for the assay.
Incubation periods were too short.	Follow protocol for incubation times. Time each plate separately to ensure accurate incubation periods.
Reagents and plates were too cold.	Make sure plates and reagents are at room temperature by taking them out of the refrigerator, and the test components out of the box, at least 2–3 hours before starting the assay.
Reagents were expired or intermixed from a different lot number.	Verify the expiration dates and lot numbers on the reagents.
Wrong conjugate was used, or conjugate was prepared incorrectly or has deteriorated.	Be sure that the conjugate used is the one that came with the test. All conjugates are test- 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.
Assay plate was read at wrong wavelength, or reader was malfunctioning.	Verify the correct wavelength for the assay and read the plate again. Verify reader calibration and lamp alignment.
Positive control was diluted (indirect format only).	Do not dilute controls unless specified in the product insert.
Excessive test stress occurred.	Check records to see how many times the test has cycled from the refrigerator. Check to see if the test was left out on a loading dock or other area for too long or at extreme temperatures.

ELISA troubleshooting (continued)

Replicates within a plate show poor reproducibility

Possible causes	Recommended actions
Excessive time was taken to add samples, controls, or reagents to the assay plate.	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.
Multichannel pipette was not functioning properly.	Verify pipette calibration and check that tips are on tight. Be sure all channels of the pipette draw and dispense equal volumes.
There was inconsistent washing or washer system malfunctioning.	Verify the performance of the washer system. Have the system repaired if any ports drip or dispense/aspirate poorly.
There was poor distribution of antibody in the sample.	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.

No color development

Possible causes	Recommended actions
Reagents were used in the wrong order or an assay step was omitted.	Check the product insert for the assay protocol and repeat the assay.
Samples were not added to diluent (indirect format only).	Verify that the samples were added to the diluent.
Wrong conjugate was used, or conjugate was prepared incorrectly or has deteriorated.	Be sure that the conjugate used is the one that came with the test. All conjugates are test- 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.

ELISA troubleshooting (continued)

Poor reproducibility plate to plate

Possible causes	Recommended actions
Inconsistent incubation times occurred from plate to plate.	Time each plate separately to ensure that plates have consistent incubation periods.
Inconsistent washing occurred from plate to plate.	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.
Pipette was working improperly.	Check the pipette calibration. Verify that pipette tips are on tight before use and that all channels draw and dispense equal volumes.
Controls and samples were at different temperatures.	Be sure to allow sufficient time for sample diluent, samples, and controls to come to room temperature by removing them from the 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.
Reagents were being used from different lots.	If running two different lots at the same time, make sure to label reagent trays, etc., so all reagents within a lot are used with the corresponding plates.
Sample identification error or mix-up.	Rerun the samples or have new samples taken for testing.

Appendix A: Gravimetric pipette calibration procedure

Materials

- Pipette
- Analytical balance
- Glass beaker
- Deionized water
- Weighing vessel
- Thermometer

Procedure

1. To avoid erroneous results due to evaporation, we recommend humidifying the analytical balance chamber at least 2 hours prior to calibration. This can be achieved by placing a small, half-filled beaker of water into the chamber with all doors closed.
2. Follow the manufacturer's directions for cleaning and lubrication of pipettes prior to calibration.
3. The room temperature should remain constant, preferably 18°C–26°C, ±0.5°C.
4. Allow a sufficient volume of deionized or distilled water to come to room temperature, and then take a temperature reading.
5. Record the beginning weight of the weighing vessel or zero the balance.
6. Using a new pipette tip with each delivery, pipette water into the weighing vessel and record the weight. Repeat this step 10 times.
7. Calculate the volume dispensed for each delivery.

Calculations

1. Calculate the actual volume delivered as follows:

$$Volume = \frac{Weight\ of\ water}{Density\ of\ water}$$

Density of water at 16°C–21°C = 0.998 mg/µL
Density of water at 22°C–25°C = 0.997 mg/µL

2. Calculate the mean (M), standard deviation (SD), and coefficient of variance (CV) of the 10 volumes to determine precision of the pipette.
3. Determine the accuracy of the pipette as follows:

(1-[Difference between stated and actual volume/stated volume]) × 100 = % accuracy

Recommended specifications

1. Precision: CV ≤5.0%
2. Accuracy: ≥95%

Labeling

Label the pipette with the calibration date, the technician's initials, the precision, and the accuracy. Also, record these data in a laboratory notebook or log for long-term storage.

Appendix B: Inventory control tracking chart

Test type	Lot number	Number of tests received	Date received	Expiration date	Comments

Appendix C: Laboratory tracking chart

Comments																					
Room temp.																					
Internal control																					
Internal control																					
Internal control																					
Internal control																					
Positive control																					
Negative control																					
Plate numbers																					
Test lot number																					
Technician																					
Date																					

Appendix D: Maintenance and calibration schedule

Procedure	Daily	Weekly	Monthly	Quarterly	Yearly
Pipettes					
Clean exterior	●				
Check calibration					●
Clean interior and O-rings				●	
Dilutor					
Flush system with DI water	●				
Purge system	●				
Check calibration†		●			
Soak syringes		●			
Check/change tubings				Check	Change
Washer					
Flush system with DI water when using wash solutions other than DI water and when changing between wash solution types					
Check traps, filters, and foaming	●				
Check aspiration and dispensing needles for drips and debris					
Check tubes and bottles for microbial growth	●	●			
Decontamination—flush system with with bleach, alcohol solution, or tergezyme†			●		
Calibration check—purge system			●		
Clean exterior	●				
Check/change tubing	●			Check	Change
Reader					
Calibration/verification plate‡					●
Lamp alignment	After bulb replacement				
Clean optics				●	
Clean exterior		●			

†Refer to your manufacturer's guide for specific instructions for your make and model.
‡Call IDEXX Technical Services or your reader manufacturer for recommendations on calibration/verification plates.

Appendix E: Quality control quick check

Monitoring and tracking assay performance on quality-control charts provides insight as to when it is necessary to troubleshoot problems. Below is a checklist to review if you are having problems with your ELISA. If you have followed all the steps below and are still having problems with your assay, contact your local IDEXX Technical Services representative.

Equipment

- ☐ Keep preventive maintenance up to date.
- ☐ Calibrate and clean pipettes.
- ☐ Calibrate reader.
- ☐ Clean and maintain washer system.

Reagents

- ☐ Maintain inventory control—FIFO.
- ☐ Inspect components.
- ☐ Bring reagents to room temperature.
- ☐ Avoid contamination.
- ☐ Verify proper storage.

Technique

- ☐ Monitor sample quality and handling.
- ☐ Verify reagent preparation.
- ☐ Verify appropriate sample mixing.
- ☐ Verify proper pipetting.
- ☐ Check timing—multiple plate runs.
- ☐ Check washing of assay plates.
- ☐ Use internal controls—track results.

Other

- ☐ Monitor laboratory temperature.
- ☐ Use sterile disposables and reservoirs.
- ☐ Monitor assay performance.

Notes

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