BioChek
SMART VETERINARY DIAGNOSTICS

ELISA Technical Manual and Troubleshooting Guide

2014

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BioChek ELISA Test Kit Storage Guidelines

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General

The kit is composed of coated microtiter plates, sample diluent, substrate buffer, stop solution and positive and negative controls (aqueous solutions containing salts), conjugate reagent (aqueous solution with antibodies and enzymes), and freeze dried substrate tablets.

The most critical components of the kit are the microtiter plates. These are coated at precise concentrations with antigens that are immobilized to the surface. The coated microtiter plates are sensitive to moisture and temperature. Moisture will affect the stability of the binding of the antigen to the microtiter plates, therefore it is important to keep the kit box away from water, vapor or ice. High temperatures (> 45°C, 113°F) or low temperatures (< 2 °C, 35.6°F) may have a similar effect.

The other components are (due to the packing materials) only sensitive to temperature. The complete kit is most stable at a temperature between 4 – 8 °C (39.2-46.4°F).

Storage of a complete kit

The kit should be stored at 4 – 8 °C (39.2-46.4°F). Often there is condensation on the walls of the refrigerator, therefore make sure the kit doesn't come in contact with the walls of the refrigerator. Also, prior to storing the kit or components of a kit in the refrigerator, make sure there is no condensation on the kit components.

Using the kit

When planning to run an assay, remove from the refrigerator only the number of microtiter plate(s) that will be needed along with all the kit components and controls at least 2 hours prior to starting the assay.

Handling of partially used plates

In some instances, only a 'partial plate' may be required. When working with partial plates, cover the unused wells of the plate with adhesive tape (i.e. scotch) prior to and during the assay. After the assay, dry, mark, and cover the used wells with adhesive tape. Then REMOVE the tape from the unused wells and place the used plate with desiccant pouch in a re-sealable bag. Put the plate back in the original kit box and in the refrigerator.

Materials and Equipment Required for Running a BioChek ELISA Test

- Room with temperature between 22 and 27 °C (72-80°F)
- Refrigerator to store kits
- Freezer to store samples
- Personal computer (IBM compatible with minimum 4GB RAM) with BioChek software.
- Precision Pipette and disposable tips which are able to pipette 5 µl (i.e. single channel adjustable 1-10µl)
- 8 or 12 channel pipettes/repeater pipettes with disposable tips able to pipette volumes of 50, 90, 100, and 245µl (i.e. 5-50µl and 30-300µl)
- 96 well flat bottom dilution plates (min vol/well - 300µl)
- Distilled or de-ionized water: 250 – 300 ml is required for the full washing procedure of 1 test plate.
- 4- Clean Reagent reservoirs (Dedicate 1 for each specific reagent)
- Tissues or other absorbing paper
- 4- 10 ml Volumetric pipettes (Dedicate each for a certain reagent)
- Graduated cylinder – 1L
- Timer (portable and with alarm for 1 hour, 30 and 15 minutes)
- Microtiter plate reader with 405 nm filter
- Microtiter plate washer: manual or (semi)- automatic
- Vortex shaker
- Sealable tubes for sample collection

Performing the ELISA Assay

Before you start

Make sure that all required equipment, plastic ware, etc. is ready to use.

Critical factors for running an ELISA test are:

- Temperature of reagents (need to get to room temperature 22-27°C, 72-80°F)
- Temperature of laboratory (22-27°C, 72-80°F)
- Time for the various incubation steps

Other factors are:

- ELISA plates on an insulated surface
- No direct sunlight on ELISA plates/reagents
- Plates on a clean surface
- Use good quality and well maintained equipment

Standard Operating Procedure for BioChek ELISA test kits

- Preparation Running the assay

Preparation:

- **Take samples out of freezer/refrigerator to allow them to reach room temperature.**
  If samples were frozen, allow them to thaw. Prior to use, shake samples vigorously, either manually or with a vortex shaker.
- **Have all equipment ready to use on the bench (see previous chapter, pg. 4 for required equipment)**
- **Label reservoirs and graduated pipettes/cylinders**
- **Dedicate reagent reservoirs and graduated pipettes/cylinders to the same solution (DILUENT, CONJUGATE, SUBSTRATE AND STOP SOLUTION) if planning to reuse. Write on each item using a marker pen. Clean after use with distilled water.**
- **Prepare required conjugate volume (enough for each well)**
  Measure the required amount of conjugate into reservoir and **place the remaining conjugate back in the refrigerator immediately**. Note: 100µl of conjugate will be needed per well for all samples and controls being tested. For example, for a full plate, 9.6 ml will be needed. Therefore, it is recommended to dispense 11 ml into the reagent reservoir with a graduated pipette to easily cover require volume.
- **Let kit components warm to room temperature 2 hours prior to starting the test**
  Remove the required number of microtiter plates, all kit components (bottles), and the reference control from the box in the refrigerator in order to allow reagents to reach room temperature (22-27°C, 72-80°F).
  A minimum of 2 hours should pass before starting with the test. (see page 6 for preparation of wash buffer and substrate reagent).
- **Prepare dilutions of samples**
  Samples may be diluted during the 2 hour warm up time (see Making Sample Dilutions section).
- **Make a layout for sample location**

Make a layout of where samples will be dispensed on the microtiter plate. This can be done using the BioChek software (see software manual for instructions).

ATTENTION: When using only part of a plate, cover the unused wells of the plate with adhesive tape (scotch), prior and during the assay. The covered wells must remain dry while running the assay.

Making sample dilutions

In uncoated microtiter plate (recommended):

Preparation

Have layout for the samples ready.

For example, to prepare a 1:50 diluton, dispense 5 µl of sample in the bottom of the wells of an uncoated microtiter plate according to the plate layout. After all the samples have been added do a visual check to see that a drop of serum is present in each well. Add 245 µl of sample diluent to each well by **reverse** pipetting. This will give a 1: 50 dilution.

NOTE:

Whenever making a pipetting mistake while dispensing sample, NEVER try to remove wrongly added sample out of a well or tube and try to replace it with the correct sample! Just mark the well on the layout in order to skip it when entering the plate layout in the BioChek Software (see software manual).

COVERED DILUTED SAMPLES CAN BE STORED FOR 7 DAYS IN THE REFRIGERATOR.

Now everything is ready to start the assay. From now on, it will take about 2 hours to complete the test. However, some kits require more time to run. ***Don't start if time doesn't allow you to finish the test.***

Preparing the wash buffer

Dissolve one sachet of wash buffer in 1 liter of distilled water.

Pour the powder in a 1 liter bottle or flask and rinse the residual salt in the sachet once with a small volume of distilled water. Shake the bottle or flask very vigorously to be sure that all the salts have dissolved.

Preparing substrate reagent

Make the substrate reagent by calculating the number of tablets required (2 per plate) and the volume of substrate reagent (11 ml per plate). First add the tablets, then the reagent. Never touch the tablets with your hands***. It is recommended to prepare fresh substrate reagent during sample incubation. Mix substrate prior to use. Tablets must be fully dissolved.***

Running the Indirect ELISA Assay

1. Adding Samples and Pre-diluted Controls
   a. Fill the wells on the test plate, **except the wells which will contain the pre-diluted controls**, with either 90 µl (for assays requiring a 1:500 final dilution) or 50 µl (for assays requiring a 1:100 final dilution) of sample diluent. When adding diluted samples or pre-diluted controls, use clean tips for new samples.

b. Add 100 µl of pre-diluted controls (including reference controls) to the appropriate wells (see diagram A).
c. Use the multichannel pipette and dispense either 10 µl (for a final dilution of 1:500) or 50 µl (for a final dilution of 1:100) of the 1:50 diluted samples in the dilution plate in all the wells according to the layout.

2. Start a timer set at the required time for the assay after the addition of the last sample or control.
3. Cover the plate with a lid and let stand for the appropriate time according to the assay insert. Plate should not stand in direct sunlight and must be on a clean and insulated surface.

   NOTE: If the microtiter plate method was used, adjust the volume to 100 µl for pipetting after the washing steps.

4. **Washing the plate:** After the required incubation time has passed, dispose of the liquid in the plate and wash **4 times** with at least 300 µl of wash buffer (according to kit insert). Allow wash to soak for at least 10 sec. Between cycles dispose of the wash buffer in the plate. Gently dispense wash solution to the plate to avoid causing air bubbles. After the last washing step, tap the plate vigorously on absorbing tissue to remove any small trace of wash buffer present in the bottom of the wells. Then continue with the next step: the addition of conjugate reagent.
5. **Addition of conjugate reagent:** Add 100 µl of conjugate to each well using reverse pipetting. Start a timer set at the required time for the assay (see kit insert).
   ATTENTION: ONLY USE CONJUGATE BELONGING TO THE KIT BATCH IN USE. NEVER USE CONJUGATE FROM A DIFFERENT BATCH. DON'T POUR CONJUGATE BACK FROM REAGENT RESERVOIR INTO ORIGINAL BOTTLE.
6. After the conjugate incubation, repeat the ***Washing the plate*** step described above.
7. Add 100µl substrate reagent to all wells by reverse pipetting. **ATTENTION: the substrate reagent should be clear, not slightly yellow, prior to using**. One set of tips can be used for all wells. Start a timer set at the required time for the assay (see kit insert).
8. Aspirate the needed volume of stop solution with a pipette and dispense into a reservoir.
9. After the substrate incubation time has passed, add 100 µl of stop solution. ***Make sure to prevent bubbles.***

Reading the plate

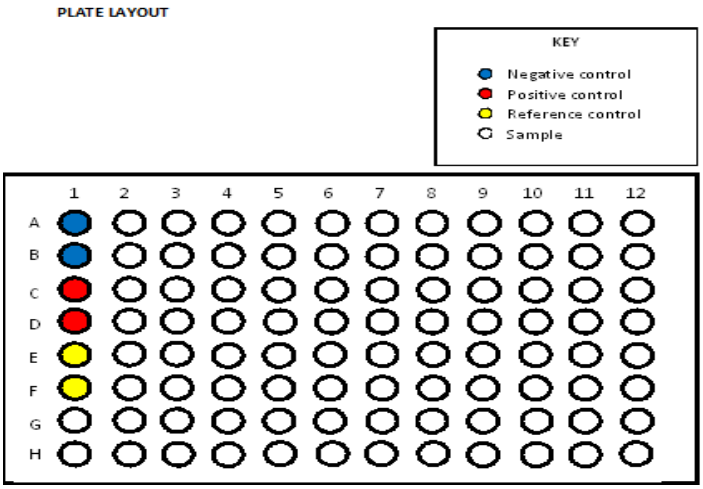
Read the Optical Densities (OD's) of the plate within 30 minutes of the finished assay, otherwise store the covered plate in the refrigerator for later reading. Plates can be stored cool and dark for 24 hours. Be careful to remove condensation after storage of the plate in the refrigerator.

ATTENTION

Partially used plate instructions*:* After running the assay, dry the used wells and mark, and cover them with adhesive tape. Then REMOVE the tape from the unused wells, place used plate with desiccant pouch

in a re-sealable bag. Put plate back in original kit box in refrigerator. *Partial plates can only be used when the unused wells remain dry.*

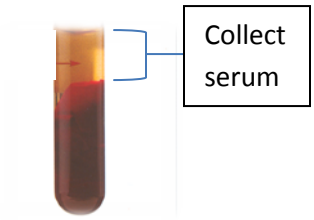
Diagram A.



Sample Handling and Storage

The following are guidelines for proper storage and handling of blood samples to assist in uniform quality samples:

- Collect 2-3 ml blood; this will yield 0.5 - 0.75 ml serum, which is more than sufficient for ELISAs.



- In general, light to moderate hemolysis and high lipid concentrations have little or no effect on an ELISA that has a high sample dilution.
- Allow blood to coagulate at room temperature for 1-2 hours.
- For clean sera it is advised to collect sera after centrifugation. Recommended protocol for poultry samples is 4000g for 10 min. For swine, the protocol is 2500g for 10 min.
- Store samples at 4-8^{O}C or 39-46^{O}F for short term storage (< 7 days).
- If storing samples long term, place them in plastic storage vials with airtight caps (micro centrifuge tubes 1.5 ml) and in labelled zip-lock bag to protect them from evaporation and store at -20° C. Evaporation may concentrate the protein (antibodies) content. Lyophilization of frozen samples is evidenced by crystallization and indicates evaporation.
- Samples that have been frozen and then thawed need to be mixed by vortexing or inverting (shaking) before making a dilution. This is because freezing serum can cause the protein fraction to settle out.
- Avoid many freeze/thaw cycles of serum samples as this may damage antibodies.
- Diluted serum samples (in sample diluent) can be stored in the refrigerator (4-8^{o}C or 39-46^{o}F) for 7 days.
- Do not use spoiled serum samples (bad smell). Spoiled samples contain proteolytic enzymes which may affect ELISA results.
- For international shipment of serum samples, samples should be heat treated for at least 30 min, at 56°C or 132.8°F to inactivate potential pathogens. Also make sure to use screw cap vials, to avoid leakage of serum during transport.
- ALWAYS LABEL SAMPLES PROPERLY to avoid mistakes. (Company ID, Flock ID, Age, Type Bird etc.)

TROUBLESHOOTING GUIDE

1. Low optical densities

- Check lab temperature must be 22-27°C (72-80°F)
- Check reagents temperature again must be 22-27°C (72-80°F)
- Check expiry date of kit is within range
- Check that incubation times were not cut short
- Check that pipettes are calibrated and correct volumes of reagents added in each step
- Check that you have correct filter installed on reader and the reader is functioning correctly

2. High optical densities

- Check lab temperature must be 22-27°C (72-80°F)
- Check reagents temperature again must be 22-27°C (72-80°F)
- Check that incubation times were not too long
- Check that pipettes are calibrated and correct volumes of reagents added in each step
- Check that you have correct filter installed on reader and the reader is functioning correctly

3. Poor replication of controls (CV's greater than 15%)

- Check that multichannel pipettes are calibrated and each channel dispensing correct volume
- Check washer for blockages and contamination. In addition, check number of washes and the quality of wash (volume =must be at least 300ul per wash).

4. No optical densities from test.

- Check visually if there color development on the plate.
  - If yes, check reader.
  - If no, the addition of one or more reagents has been performed incorrectly (such as failure to add tablets to substrate buffer; or washing after the substrate incubation) or the kit is malfunctioning. Repeat test to ensure operator has performed correctly and contact BioChek if result is the same.

5. All optical densities high, including negative control, and no difference between ODs.

- The substrate reagent has become contaminated –check the substrate reservoir. A strong yellow coloration will confirm contamination. Repeat the test.

Always keep conjugate reagent and substrate reagents separate and use separate equipment and reservoirs at all times; they are very sensitive to exposure of even the smallest amounts and this will invalidate the test.

6. Reference control out of range

- Check that you have the correct ranges for the relevant reference control.
- Check that the reference control temperature was 22-27°C (72-80°F) same as kit reagents

Note: With a properly run assay when all controls are within range, the reference

control should be within range. If it is not, contact BioChek immediately with Kit lot number and results for assistance.

PROBLEM: High background/excessive color development (high optical density readings).

POSSIBLE CAUSES	RECOMMENDED ACTIONS
Poor quality water used to wash plates, or to prepare wash solution.	Check water quality. If questionable, try substituting alternate water source, such as bottled demineralized water to prepare wash buffer solution.
Substrate solution prepared incorrectly, or deteriorated.	Make sure substrate tablets are diluted in substrate buffer in appropriate amounts; the solution will have little or no color when prepared properly. Prepare the substrate solution no further in advance then recommended. **When in doubt, throw it out.**
Insufficient washing or poor washer performance.	Make sure at least 300 µl wash solution is dispensed per well per wash and a soak time of 10 seconds per wash. Verify performance of washer system; have the washer repaired if any ports drip, dispenses or aspirates poorly.
Lab temperature is too high.	Maintain a temperature of 22-27°C (72-80°F). Install climate control to regulate temperature.
Washer system contaminated with microbials or an alternate wash formulation.	Eliminate microbials by flushing the system with a dilute solution of bleach, followed by copious amounts of distilled/deionized water; prime the system with the appropriate wash solution before use. Make sure that each unique wash solution is properly labelled to avoid mix-up and prime the system thoroughly when switching between the solutions.
Reader malfunctioning.	Verify reader performance, using a calibration plate; check lamp alignment.

Problem: Insufficient color development (Low optical density readings)

POSSIBLE CAUSES	RECOMMENDED ACTIONS
Lab temperature too low.	Maintain a room temperature of 22-27°C (72-80°F). Avoid running assays under air-conditioning vents or on metal or ceramic surfaces.
Substrate solution prepared incorrectly.	Make sure substrate tablets are diluted in substrate buffer in appropriate amounts.
Wash solution prepared incorrectly; wrong wash solution used, or washer system contaminated with an alternate wash formulation.	Make sure to use the BioChek wash buffer solution, prepared according to protocol. Make sure that each unique wash solution is properly labelled to avoid mix-up and prime the system thoroughly when switching between the solutions.
Too many wash cycles used.	Be sure to stay in the recommended range for the number of wash cycles (4 times for BioChek assays).
Incubation periods shorter than recommended.	Make sure to follow the guidelines for incubation times, described in the kit insert. Use a timer for each separate plate, to insure accurate incubation periods.
Wrong conjugate used, or conjugate is deteriorated.	Only use the original conjugate that came with the kit; all conjugates are kit-specific. Make sure to only measure out the amount required for immediate use, and do not return unused portions to stock bottle.
Reagents and plate are cold.	Bring reagents plates to room temperature (2 hrs prior to usage).
Assay plate read at wrong wavelength, or reader is malfunctioning.	Verify appropriate wavelength (405 nm) for the assay and reread the plate. Verify reader calibration and lamp alignment.
Positive control diluted.	Do not dilute controls.
Assay plates deteriorated or previously used.	Make sure to store plates in sealed bags with desiccant to maintain stability. Prevent condensation from forming on plates by allowing them to equilibrate to room temperature while in the packaging. If partial plates are used, be sure to mark the used wells to prevent reuse; use remaining wells as soon as possible.

PROBLEM: No color development.

POSSIBLE CAUSES	RECOMMENDED ACTIONS
Reagents used in wrong order.	Verify proper protocol and repeat assay.
Substrate solution prepared incorrectly, or deteriorated.	Make sure the substrate tablets are dissolved in the appropriate substrate buffer volume (1 tablet per 5.5 ml). Make sure that no metals come in contact with the substrate during preparation or degradation of the substrate will result

PROBLEM: Poor reproducibility of controls within a plate.

POSSIBLE CAUSES	RECOMMENDED ACTIONS
Too much time taken to add samples/ controls, or reagents to assay plate.	Make sure all materials are set up and ready for usage. Use multi- channel pipettes to add samples and reagents to multiple wells simultaneously.
Multi-pipette malfunctioning.	Verify pipette calibration and check that pipette tips fit on tightly. Make sure that all channels of the pipette draw/ dispense equal volumes.
Inconsequent washing, or washer system malfunctioning.	Verify performance of washing system: have the system repaired and cleaned if any ports drip, or dispense, or aspirate poorly.

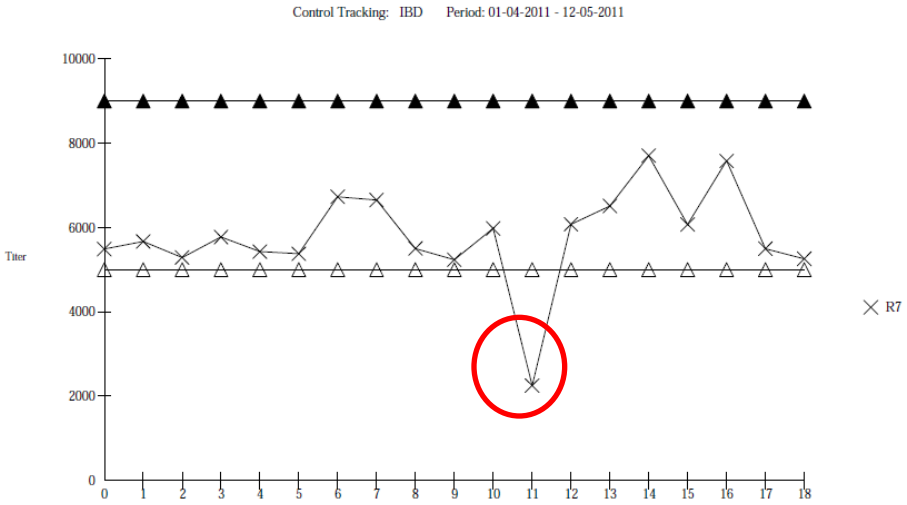
PROBLEM: Poor plate to plate reproducibility.

POSSIBLE CAUSES	RECOMMENDED ACTIONS
Inconsistent incubation times from plate to plate.	Time each plate separately to insure that plates have correct and consistent incubation times.
Inconsistent washing from plate to plate.	Verify the performance of washer system: have the system repaired and cleaned if any ports drip, or dispense, or aspirate poorly.
Pipette malfunctioning and/or inconsistent pipetting technique.	Verify pipette calibration and check that pipette tips fit on tightly. Make sure that all channels of the pipette draw/ dispense equal volumes. For sample dilution verify 1-10 µl pipette and dilution technique.
Kit controls and samples are at different temperatures.	Be sure to allow sufficient time for samples and kit components to come to room temperature.

TROUBLESHOOTING ACCORDING TO WESTGARD RULES

Random Error:

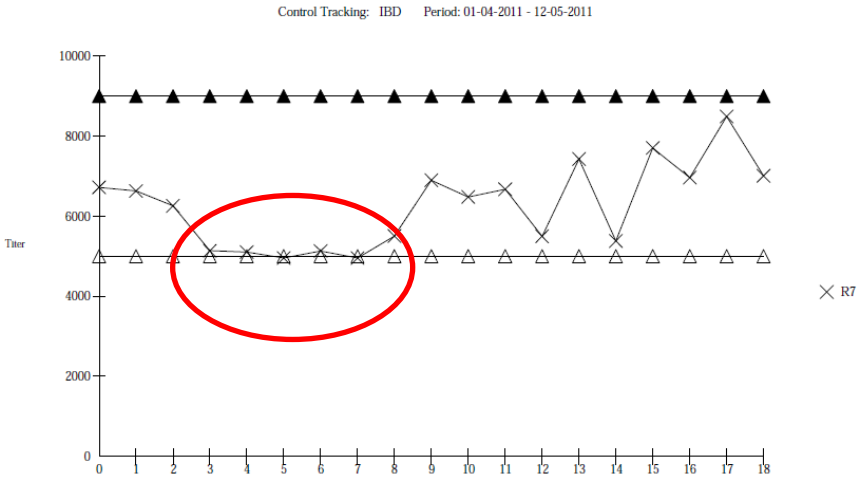
A single control measure is out of expected range



POSSIBLE CAUSES	RECOMMENDATIONS
Incorrect test procedure, incorrect washing procedure, equipment not calibrated, pipette not handled properly	Follow up all test procedures, washing procedures and ensure correct use of equipment and pipetting instructions. Repeat the reference control.

Shift:

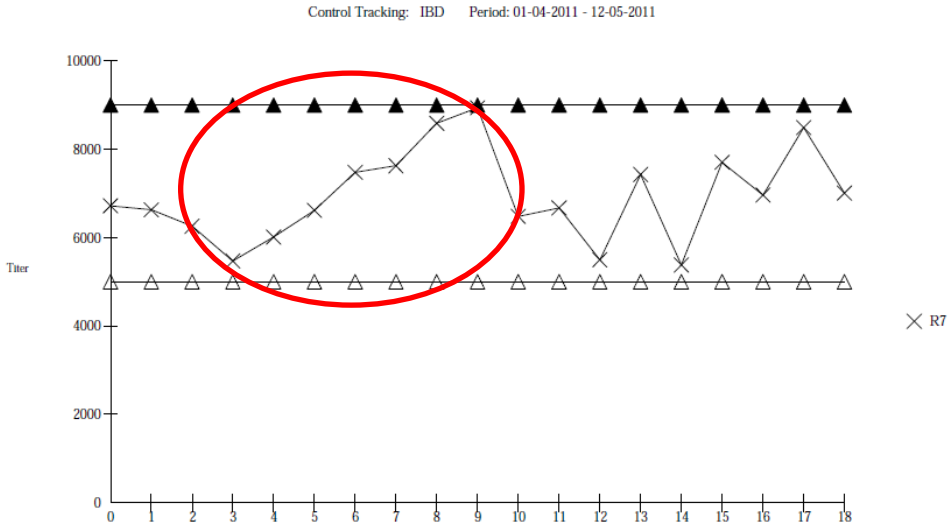
Consecutive control measures fall outside of the expected range



POSSIBLE CAUSES	RECOMMENDATIONS
A pH change in the distilled water, incorrect incubation temperature, incorrect incubation time, reagents not warmed up enough, reagents not constituted correctly	Check all details of requirements for water, temperature, time and reagent handling on the insert. Repeat the reference control.

Systemic Error:

A ‘trend’ in control measures outside of the expected range



POSSIBLE CAUSES	RECOMMENDATIONS
A change in distilled water pH, blocked washing equipment, problem with the lamp or filter in plate reader, reagent not stored properly	Check all details of water, equipment and reagent handling on the insert. Need to repair washing equipment. Calibrate plate reader.