BioChek

BETTER DIAGNOSTICS FOR BETTER RESULTS

Kit handling & Troubleshooting Manual

WITH BIOCHEK ELISA KITS

BIOCHEK technical manual

Contents:

BioChek ELISA test kits storage guidelines.

- General
- Storage of a complete kit
- Using the kit
- Handling of partially used plates

General
The kits are 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 micro titer plates. These are coated at precise concentrations, with antigens that are immobilized to the surface of the microtiter plate.

The coated microtiter plates are sensitive to moisture and temperature. Moisture will effect 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 temperature (> 45°C.) or low temperature (< 2 °C) 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.

Storage of a complete kit:
Store at 4 – 8 °C. As there often is condensation on the walls of the refrigerator, 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 one microtiter plate and all kit components from refrigerator at least 2 hours prior to moment of starting the assay.

Handling of partially used plates:
Also important is how to work with partial plates. In that case we recommend to cover the unused wells of the plate with adhesive tape (scotch), prior and during the assay. After the assay, dry the used wells, also mark the used wells and cover the used wells with adhesive tape. Then REMOVE the tape from the unused wells, place used plate with desiccant pouch in a resealable bag. Then put plate back in original kit box in refrigerator.

Materials/equipment required for running a BioChek ELISA test:

Room with temperature between 22 and 27 °C

Refrigerator to store kits

Freezer to store samples

Precision Pipette and disposable tips able to pipette 1 µl

8 or 12 channel pipette / repeater pipette with disposable tips able to pipette volumes of 100 and 500 µl (500 µl is can be done bydispensing 2 x 250 µl)

96 plastic tubes for sample dilution in a rack:
Minimal volume 1.0 ml
An empty rack for diluted samples

Distilled or de-ionized water: you need about 250 – 300 ML for the full washing procedure of 1 test plate.

4 clean Reagent reservoirs*

Kleenex tissues or other absorbing paper

4 Volumetric pipettes 10 ml*

Timer (portable and with alarm for 30 and 15 minutes)

Microtitre plate Reader with 405 nm filter

Microtitre plate Washer: manual or (semi)- automatic

Vortex shaker

Sealable tubes for sample collection

Personal computer (IBM compatible) with BioChek software.

* Dedicate each for a certain reagent.

Running the ELISA assay:

Before you start:
Before starting, make sure that all required equipment, plastic ware etc is ready to use.
The most important feature is the temperature of the laboratory and the temperature of the reagents.

Critical general factors for running an ELISA test are:
- Temperature of reagents (need to get to room temperature 22 – 27 °C)
- Temperature of laboratory (22 – 27 °C)
- Time for the various incubation steps

Other factors are:
- ELISA plates on insulated surface
- No direct sunlight on ELISA plates/reagents
- Plates on clean surface
- Use good quality and well maintained equipment

Standard Operating Procedure Biochek ELISA test kits

- Preparation (Start preparation 2 hours prior to running the test)
- Running the assay

Preparation:
- **Let kit components warm to room temperature 2 hours prior to starting the test**
- **Take samples out of freezer/refrigerator**
- **Prepare required conjugate volume and put conjugate flask back in refrigerator immediately.**
- **Have equipment ready on the bench**
- **Make a layout for sample location**

Have all equipment ready to use on the bench (see chapter equipment).
Allow the kit components (and reference control) to warm to room temperature (22 – 27 °C) by removing one microtiter plate and all kit components out of the refrigerator and placing the kit components (bottles) out of the kit box on the bench.. Do the same for the reference control. (Measure amount of conjugate required and place conjugate bottle back in refrigerator)
A minimum of 2 hours should have passed before starting with the test. *One may start preparing dilution of the samples during these 2 hours provided the samples are thawed and mixed by using the vortex shaker.*

Take the frozen samples out of the freezer and allow them to thaw. Once thawed shake vigorously, either manually or with a vortex shaker. Now the samples are ready for use.

Make a layout for where you plan to dispense the samples on the microtiter plate. This can be done using the BioChek software.
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.

Turn on the computer and start up the Biochek software program. Click on "READ" button and select a kit name by double clicking on it. Then go to READ window, fill in the lot number (on the side of the kit box FSxxxx number) under the plate layout and make a print by clicking on the "Print" button. You now have a plate layout printed.

Fill in the flock sample codes manually on the respective wells on the paper. You can choose between a horizontal and a vertical orientation. Whenever you are not going to use a whole plate and you have either an 8 or 12 – multichannel pipette, it might be wise to use the vertical orientation when you have an 8 multichannel pipette and a horizontal layout when you have a 12 multichannel.

Be as clear and consistent as possible in your coding, i.e. use same names for same flocks and enter all other information such as type of chicken, age etc in the code fields.

Dedicate reagent reservoirs and GRAD pipettes to the same solution if you want to reuse them. Using a marker pen write DILUENT, CONJUGATE, SUBSTRATE AND STOP SOLUTION on each item. Clean after use with distilled water.

- Whenever making a pipetting mistake while dispensing sample onto the ELISA plate, never try to remove wrongly added sample out of a well and try to replace it by the correct sample! Just mark the well on your paper layout, so you can skip it from your results in the software by using "delete cupcode". *(right mouse button click and select delete cupcode)*

Making sample dilutions and dispensing diluted sample to the microtiter plate:

There are two ways of making dilutions of the samples.
In dilution tubes:
Preparation:
Have 2 racks for dilution tubes on the bench. One filled with dilution tubes, the other one empty.
Have layout for the samples ready, take the dilution tubes in the position of a prediluted control out of the rack with tubes.

Pipette 1 ul of sample to the bottom of each dilution tube, according to the plate layout. Remove the dilution tube containing the sample to the same position in the empty rack.

After you have added all the samples do a visual check. You should see a drop of serum in the bottom of each tube. Then add 500 ul of sample diluent (green liquid), when possible with a multichannel pipette. (*This can be done by adding 2 x 250ul to the tubes)*

In uncoated microtiter plate (recommended):
Preparation
Have layout for the samples ready.

Dispense 5 ul of sample in the bottom of the wells of an uncoated microtiter plate according to the plate layout. After you have added all the samples do a visual check if you see a drop of serum in each well. Add 245 ul of sample diluent by **reverse** pipetting. You now have a 1: 50 dilution.

DILUTED SAMPLES CAN BE STORED FOR A FEW DAYS IN THE REFRIGERATOR.

Now everything is ready to start the assay. From now on it will take about 1 hour and 45 minutes to complete the test. Don't start if time doesn't allow you to finish the test. Attention some kits require more time to run.

First:
Prepare the wash solution
Preparation of 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.

Prepare substrate solution
Make the substrate reagent by calculating the number of tablets required (you will need 2 for 1 plate) and the volume of substrate reagent (you will need 11 ml for 1 plate. First add the tablets, then the reagent. Never touch the tablets with your hands. Pour them out of the bottle or use tweezers.

Then start the assay:
Dilution tube Method
Start filling the ELISA test plate, preferably using the reverse pipetting method with **100 ul** for one of the negative, positive control and one of the reference control samples. Mix shortly by aspirating and dispensing with new tips on the multichannel pipette or with a vortex and add 100 ul of this dilution to the coated plate (ELISA test plate) of the kit. Use the **forward** pipetting technique. After that, end with the other negative, positive and reference control. The advantage of dilution tubes is that you can throw away an individual tube when you have made mistakes and replace it by another unused one.
Microtitre Plate Method
Fill the wells, **except the wells for prediluted controls**, on the test plate with 90 ul of sample diluent.
When adding diluted samples or prediluted controls use clean tips for new samples.

Add 100 ul of prediluted control (neg and pos and optional R4) to the appropriate wells.
Then use the multichannel pipette for dispensing 10 ul of the (1:50) diluted samples in the dilution plate in all the wells according to the layout. Use the **forward** pipetting technique a fresh tips for each sample!
When finished, start timer and don't forget to adjust the volume to 100 ul for the pipetting after the washing step.
Start a timer set at 30 minutes after addition of the last sample (control) to the plate. Cover the plate with a lid and let it stand for 30 minutes. Plate may not stand in direct sunlight, and must been on a clean and insulated surface.

Washing the plate
After 30 minutes have passed, dispose of the liquid on the plate into the sink and wash **4 times** with at least 300 ul of wash buffer. Between cycles dispose of the wash buffer on the plate into the sink.
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.

Addition of conjugate reagent
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

Calculate the amount of conjugate reagent you need for the number of samples you're testing. For a full plate you will need 9.6 ml, so to cover requirements easily, dispense 11mls into the reagent reservoir with a grad pipette. You will need 100 ul of conjugate reagent per well. Use reverse pipetting for filling the plate.
Start a timer set at 30 minutes before starting the second incubation.

After this second period of incubation repeat the **Washing the plate** step described above.

After tapping the plate again vigorously add 100 ul 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.

Set a timer at 15 minutes.

Aspirate the needed volume of stop solution with a pipette and dispense it in a reservoir.

After the 15 minutes have passed, add 100 ul of stop solution by reverse pipetting.

Reading the plate:
Read the OD's of the plate within 30 minutes, otherwise store 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.

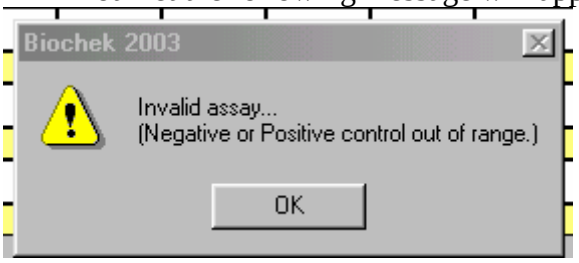
After pushing the READ button and retrieving OD results, continue by making a hardcopy of the results by clicking on the PRINT button. Immediately after that, click on the "SAVE RESULTS" button. From now on your results will be saved in your database.

Attention partially used plate instruction:
After the assay, dry the used wells, also mark the used wells and cover the used wells with adhesive tape. Then REMOVE the tape from the unused wells, place used plate with desiccant pouch in a re-sealable bag. Then put plate back in original kit box in refrigerator. *The better the unused wells remain dry, the less the quality of the partially used plate will be impaired.*

INVALID ASSAY

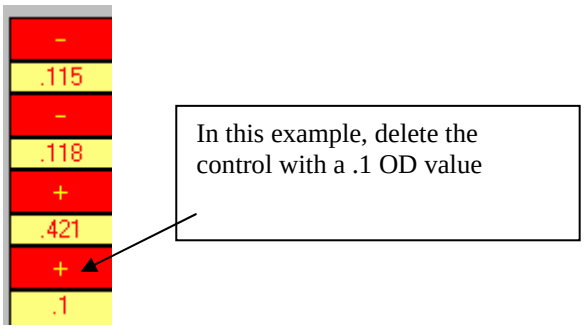
Message from software:
"Invalid assay…. (Negative or positive control out of range.)

The difference in OD between the mean positive and the mean negative control (=P-N) of the assay needs to be at least 0.15 (most assays) or 0.3 (Mg,Ms,Mm,AI,Se,St) for our software to save the data, otherwise the data will not be saved and the test is considered as "invalid". Furthermore, the maximal value for the mean negative control should not exceed 0.3. If these criteria are not met the following message will appear:



What's the consequence of this error message and what are the possible causes of this error message?

The consequence of this message is that the data cannot be saved. However there is one escape, if the problem is caused by just one of the controls, this control can be deleted. Put the mouse on the faulty control, then use the right mouse click and delete the code.



The most common cause of this error message is that somehow the temperature of the reagents were to low when running the test. This is either caused by a to low temperature of the environment (bench) or by a to low temperature of the ELISA reagents when running the test.

Other possible causes / What to check?

1. Check the expiry date of the kit. *When expired, this is the most likely cause.*
2. Check the color of the desiccant in the kit (shouldn't be pale pink). *When pink, contact BioChek representative. This might have caused the problem*
3. Check the temperature of the lab; temperature should be at least 22 °C with maximum of 27 °C. *When too low, it is the most likely cause of the problem. Some*

kits will be more sensitive than others to low temperature. Increase lab temperature to at least 22 – 23 °C.

4. Check that the warming up time for the reagents is sufficient for the kit taken from the refrigerator (should normally be at least 2 hours)
5. Ensure the plate is not positioned on a cold surface (stone, metal etc.)
6. Check if the batch numbers of the kit components plate, conjugate and positive control corresponds with the numbers on the side of the box or on the certificate of analysis
7. Is the substrate tablet completely dissolved prior to use? It takes 10 minutes to dissolve completely.
8. Is the right filter selected for the reader, or is the filter not functioning properly?
9. To determine if the problem is caused by one of above reasons visually check the color of the negative and positive control on the plate. Put the plate on a white background and check if the negative control is colorless and the positive control should be light yellow.
10. FILTER SELECTION: It should be 405 nm. If the intensities of the yellow color for the different wells do not correlate with the OD values, it is likely that a wrong filter has been selected. Try to read the plate manually and print and compare OD's with OD's obtained through the BioChek software.(note: For Labsystems and Dynatech readers **not** the wavelength (405) should be selected in the software, but the position of the filter in the filter wheel (which is typically "1").

For further help with trouble shooting , please make a print of the plate with OD's and send to BioChek. (fax +31 182 599 360)

Sample Handling and Storage

During and after sampling , the next important step is to make sure that the blood samples are correctly handled and stored, to ensure a uniform and good quality of the samples. The following are some guidelines for the proper handling and storage of your samples:

- Collect 2-3 ml blood , this will yield 0.5 - 0.75 ml serum, which is more then sufficient for ELISA's

- In General, light to moderate hemolysis and high lipid concentrations have little or no effect on an ELISA that has a high sample dilution.

- Separate serum from blood by centrifugation or coagulation (1-2 hrs at room temp)

- Short term storage samples (< 48 hrs): 4- 8 ^{o}C

- Long term storage : -20 ^{o}C in plastic storage vials with airtight caps (microcentrifuge tubes 1.5 ml) and in labeled zip-lock bag, to protect them from evaporation, which may concentrate the protein (antibodies) content. Lyophilisation of frozen samples is evidenced by crystallization and indicates evaporation.

- Since freezing serum, causes the protein fraction to settle out, samples that have been frozen and then thawed, need to be mixed by vortexing or inverting (shaking) before making a dilution.

- Avoid many freeze/thaw cycles of serum samples as this may damage antibodies.

- Diluted serum samples (in sample diluent) more stable than non-diluted samples. In the refrigerator these can be stored for at least 3 months. The ideal temperature is + 4 ^{o}C in sealable tubes.

- Do not use spoiled serum samples (bad smell). Spoiled samples contain proteolytic enzymes which may affect ELISA results.

- For international shipment of serum samples, it is advisable to heat treat the samples (30 min, at 56 ^{o}C) 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 from test when compared to certificate of analysis.
Check lab temperature must be 22-27'C
Check reagents temperature again must be 22-27'C
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 from test when compared to certificate of analysis.
Check lab temperature must be 22-27'C
Check reagents temperature again must be 22-27'C
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 >350ul per wash).**N.B. This is most likely cause.**

4. No optical densities from test when compared to certificate of analysis.
An addition has been performed wrong 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 and no difference between ODS from test when compared to certificate of analysis.
The substrate reagent has become contaminated –check visually in substrate reservoir for strong yellow colouration this will confirm. Repeat the test.
Always keep conjugate reagent and substrate reagent separately 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 was warmed to 22-27'C same as kit reagents
Check the CV of the control if higher than 15% retest if lower rely on the result
N.B. 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.

SUMMARY
Do's And Don'ts For Good ELISA results

The following technique tips should be considered general guidelines;

ALWAYS REFER TO THE PACKAGE INSERT FOR SPECIFIC INSTRUCTIONS.

1. **DO** allow reagents to equilibrate to room temperature before use, if so designated by the package insert. (If reagents are needed for use right away in the morning let them equilibrate to room temperature overnight.)
2. **DO** monitor your laboratory temperature and try to maintain it at approximately 22-27 ^{o}C.
3. **DO** prepare reagents and samples according to package insert recommendations. Use specified diluents when designated; use distilled or deionised water when designated.
4. **DO** regularly check calibration on all pipettes to verify performance.
5. **DO** pipette samples and reagents accurately and pipette to the bottom corner of test wells to avoid splashing.
6. **DO** use a new pipette tip for each sample or control.
7. **DO** time incubations accurately; use a separate timer for each plate if possible.
8. **DO** prime washer apparatus prior to washing assay plates to remove air bubbles and insure even dispensing/aspiration from all ports.
9. **DO** use the number of washes recommended in the package insert. After washing plates, invert and tap gently on an absorbent surface to remove excess liquid from wells.
10. **DO** flush the washer apparatus daily with distilled/deionised water to prevent clogs and microbial contamination in the ports and tubes.
11. **DO** check reader calibration regularly to verify performance.
12. **DO** use reference controls to verify accuracy and reproducibility of results.
13. **DO** calibrate pipettes at least 1x per year.
14. **DON'T** touch any optical surfaces in the reader (lamp, lens, detector, prism) with your fingers. If optical surfaces require cleaning; use a fibre-free cloth.
15. **DON'T** leave kits at room temperature unnecessarily; store them at refrigerated temperature to maintain kit stability.
16. **DON'T** exchange components from different serial lots.
17. **DON'T** deviate from the recommended assay protocol.

TROUBLE SHOOTING GUIDE FOR BIOCHEK ELISA

PROBLEM: High background; excessive colour development

(high optical density readings).

POSSIBLE CAUSES	RECOMMENDED ACTIONS
1. Poor quality water used to wash plates, or to prepare wash solution.	**1.** Check water quality. If questionable, try substituting alternate water source, such as bottled demineralised water to prepare wash buffer solution.
2. Substrate solution prepared incorrectly, or deteriorated.	**2.** Make sure if the substrate tablets are diluted in substrate buffer in appropriate amounts; the solution will have little or no colour when prepared properly. Prepare the substrate solution no further in advance then recommended.
3. Insufficient washing, or poor washer performance.	**3.** Try using the highest number of washes recommended for the assay. Make sure at least 300-350 ul wash solution is dispensed per well per wash. Verify performance of washer system; have the washer repaired if any ports drip, or dispense or aspirate poorly.
4. Lab temperature is too high.	**4.** Maintain a temperature of 20-27 ^{0}C. Optimal is 23 ^{0}C. During warm weather conditions run assay early in the morning and avoid running assay during afternoon at peak heat. Install climate control to regulate temperature.
5. Washer system contaminated with microbials or an alternate wash formulation.	**5.** Eliminate microbials by flushing the system with a dilute solution of bleach, followed by copious amounts of distilled/deionised 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.
6. Reader malfunctioning.	**6.** Verify reader performance, using a calibration plate; check lamp alignment.

TROUBLE SHOOTING GUIDE FOR BIOCHEK ELISA

PROBLEM: Insufficient colour development (Low optical density readings).

POSSIBLE CAUSES	RECOMMENDED ACTIONS
1. Lab temperature too low.	**1.** Maintain a room temperature of 20-26 ^{0}C. Optimal is 23 ^{0}C. Avoid running assays under air-conditioning vents and on metal or ceramic surfaces.
2. Substrate solution prepared incorrectly.	**2.** Make sure if the substrate tablets are diluted in substrate buffer in appropriate amounts.
3. Wash solution prepared incorrectly; wrong wash solution used, or washer system contaminated with an alternate wash formulation.	**3.** 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.
4. Too many wash cycles used.	**4.** Be sure to stay in the recommended range for the number of wash cycles (4-5 for BioChek assays). Try using the lowest number of washes recommended for the assay.
5. Incubation periods shorter than recommended.	**5.** Make sure to follow the guidelines for incubation times. Use a timer for each separate plate, to insure accurate incubation periods.
6. Wrong conjugate used, or conjugate is deteriorated.	**6.** Only use the original conjugate that came with the kit.; all conjugates are kit-specific. Do not prepare a working conjugate too far in advance. Make sure to only measure out the amount required for immediate use, and do not return unused portion to stock bottle. Return stock conjugate immediately to refrigerator, after removing the required amount for immediate use.
7. Reagents and plate are cold.	**7.** Bring reagents plates to room temperature (1 to 2 hrs) before usage.

TROUBLE SHOOTING GUIDE FOR BIOCHEK ELISA

PROBLEM: Insufficient colour development (Low optical density readings), *Continued.*

POSSIBLE CAUSES	RECOMMENDED ACTIONS
8. Assay plate read at wrong wavelength, or reader is malfunctioning.	**8.** Verify appropriate wavelength (405 nm) for the assay and reread the plate. Verify reader calibration and lamp alignment.
9. Positive control diluted.	**9.** Do not dilute controls, unless designated by package insert.
10. Assay plates deteriorated or previously used.	**10.** 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 off used wells to prevent reuse; use remaining wells as soon as possible.

PROBLEM: No colour development.

1. Reagents used in wrong order.	**1.** Verify proper protocol and repeat assay.
2. Substrate solution prepared incorrectly, or deteriorated.	**2.** Make sure if the substrate tablets are diluted in substrate buffer in appropriate amounts. Make sure that no metals come in contact with the substrate during preparation or degradation of the substrate will result.

TROUBLE SHOOTING GUIDE FOR BIOCHEK ELISA

PROBLEM: Reference controls within a plate show poor reproducibility.

POSSIBLE CAUSES	RECOMMENDED ACTIONS
1. Too much time taken to add samples/ controls , or reagents to assay plate.	**1.** Make sure to have all materials set up and ready to use Quickly. Use multi- channel pipettes to add samples to multiple wells simultaneously. Use multi-channel pipettes for reagents.
2. Multi-pipette malfunctioning.	**2.** Verify pipette calibration and check that pipette tips fit on tightly. Make sure that all channels of the pipette draw/ dispense equal volumes.
3. Inconsequent washing, or washer system malfunctioning.	**3.** Verify performance of washing system: have the system repaired if any ports drip, or dispense, or aspirate poorly.

PROBLEM: Poor plate to plate reproducibility.

1. Inconsistent incubation times from plate to plate.	**1.** Time each plate separately to insure that plates have correct and consistent incubation times.
2. Inconsistent washing from plate to plate.	**2.** Use the same number of washes for each plate. Verify the performance of washer system : have the system repaired if any ports drip, or dispense, or aspirate poorly.
3. Pipette malfunctioning and/or inconsistent pipetting technique.	**3.** 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 ul pipette and dilution technique.
4. Kit controls and samples are at different temperatures.	**4.** Be sure to allow sufficient time for sample diluent, samples, and kit controls to come to room temperature: larger volumes will require longer equilibration time.