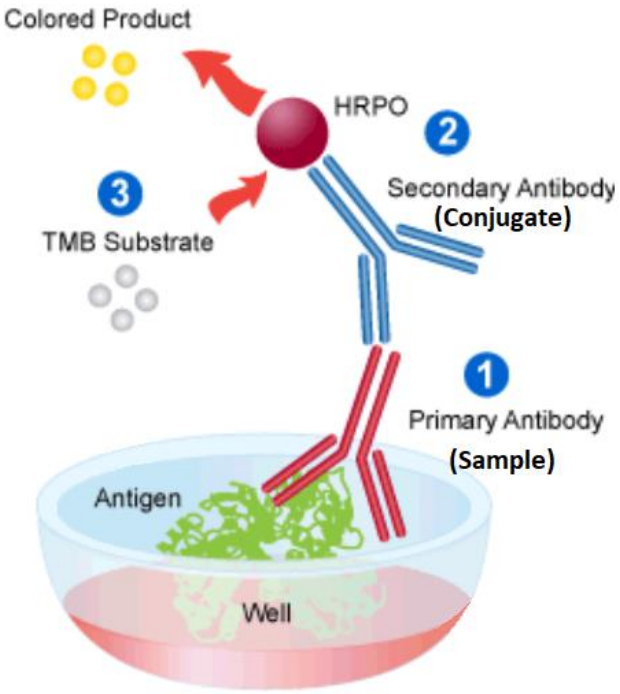
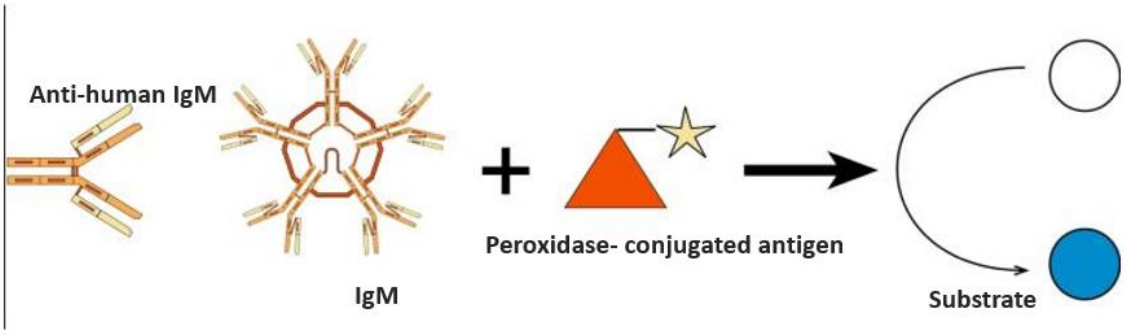
ELISA Laboratory Practice

Principle of the test:

The ELISA method is based upon the reaction of antibodies in the sample tested with the antigen absorbed on the polystyrene surface. Unbound immunoglobulins are washed off. An enzyme-labelled anti-human globulin binds the antigen-antibody complex in a second step. After a new washing step, bound conjugate is developed with the aid of a substrate solution (TMB) to render a blue coloured soluble product which turns into yellow after adding the acid stopping solution.



Note: our Capture ELISA tests have a slightly different principle; this ELISA method is based upon the capture of IgM in the sample with anti-IgM antibodies adsorbed on the polystyrene surface. Unbound immunoglobulins are washed off. Then, the antigen labelled with peroxidase react with the IgM captured, and the unbound is eliminated by washing; bound antigen is developed with the aid of a substrate solution (TMB) to render a blue coloured soluble product which turns into yellow after adding the acid stopping solution.



Vircell supplies different ELISA presentations to detect immunoglobulins in human serum/plasma:

- IgA
- IgG
- IgM
- IgG/IgM
- IgG+IgM

We also provide different Capture ELISA:

- Dengue IgM (1/100; needs predilution)
- Toxoplasma IgM
- Rubella IgM
- Cytomegalovirus IgM (1/100; needs predilution)
- Parvovirus IgM (extra step)

As well as kits that allow you to semiquantificate.

Demonstration kit: indirect immunoenzyme assay

The kit contains:

- Vircell plate: 1 96-well plate coated with antigens.
- Vircell serum diluent: 25 ml of serum dilution solution: a blue coloured phosphate buffer containing protein stabilizers and Neolone and Bronidox.
- Positive control: 500 ul
- Negative control: 500 ul
- Cut off control: 500 ul
- Conjugate: 15 ml (in two bottles) of anti-human Ig peroxidase conjugate dilution in an orange-coloured buffer.
- TMB substrate solution: 15 ml of substrate solution containing tetramethlylbenzidine (TMB).
- Stop reagent: 15 ml of stopping solution: 0.5 M sulphuric acid.
- Wash buffer: 50 ml of 20x washing solution.

Note: before starting the assay the kit, as well as the wash buffer, should be left to reach room temperature.

Wash buffer preparation:

The wash buffer bottle 20x supplied with the kit has to be reconstituted in order to obtain a 1/20 dilution. To do so, add **950 ml of DI water** to a test tube, and transfer it to a dedicated wash bottle. Then, add the whole **wash buffer bottle 20x (50 ml)** into the same bottle and gently shake it to obtain a homogeneous mixture. In this way, you will obtain **one litter** of reconstituted wash buffer at 1/20 (**50 ml wash buffer 20x + 950 ml DI water**). The DI water used to reconstitute the wash 20x has to be at room temperature.

It is also recommended to leave the wash buffer 20x to reach room temperature before reconstituting it, as there could be precipitates when it is cold.

STORAGE OF REAGENTS ONCE OPENED:

Reagent	Stability
1x washing solution	4 months at 2-8ºC
Rest of reagents	Refer to package label for expiration date (at 2-8ºC)

Although the reconstituted wash buffer is stable for an extended period of time when stored at 2-8ºC, we recommend to finish it as soon as possible, which is quite feasible in a lab with a minimum workload (this will avoid potential contamination by user mishandling). In a lab with a normal routine, they will likely finish 1 L of wash buffer within a week. The downside of storing the wash buffer in the fridge is that 1L can take around 3:30 hours to reach room temperature.

Note: using cold wash buffer could lead to lower Optical Densities (OD). For the wash buffer preparation only use good quality DI water.

Samples/controls dispense and First Incubation:

Material required:

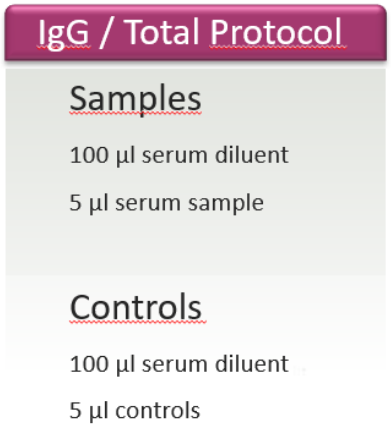
- Pipette 5 ul
- Pipette 100 ul
- Multichannel pipette 100 ul
- Pipette tips
- For IgM or IgA testing, human IgG sorbent (not provided with the kit)
- Shaker or predilution plate/tubes
- Thermostatized incubator/water bath

Note: Clean pipette tips must be used for every assay step. Use only clean, preferably disposable material.

Determine the numbers of wells to be employed counting in **four wells for the controls**: two for the cut off and one each for the negative and positive controls.

It is recommended to tap the control vials as well as the samples to homogenized them before proceeding to dispense them.

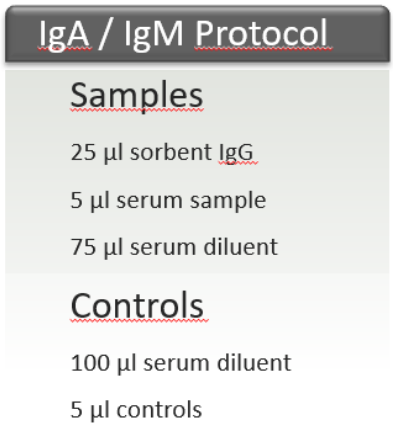
For IgG test (and total tests; IgG+IgM), add **100 ul of serum diluent** to all wells. Add **5 ul of each sample**, **5 ul of positive control**, **5 ul of cutoff** control (in duplicate) and **5 ul of negative control** into the corresponding wells. If the assay is performed manually, shake the plate in a plate shaker (**2 min**) in order to achieve a homogeneous mixture of the reagents.



If for some reason correct shaking cannot be guaranteed, a pre-dilution of the sample/controls in a separate tube or plate should be made, using extra volume of diluent and sample (**120 ul of diluent** + **6 ul of sample**). Mix homogeneously with the pipette and dispense **105 ul** of each diluted sample (and controls) to the wells.

The final dilution for samples/controls will always be **1/21**, no matter if you dispense directly to the wells or perform a predilution step.

Alternatively, for IgM tests (and IgA), as the samples have to be treated with sorbent, instead of adding 100 ul of diluent, add **75 ul of diluent** + **25 ul of sorbent**. Then, add **5 ul** of sample. The same dilution will be reached: 1/21. **Do not treat the controls with sorbent**.



Again, if there is no shaker available, perform a predilution step of the sample; **90 ul of diluent** + **30 ul of sorbent** + **6 ul** of sample. Mix homogeneously with the pipette and dispense **105 ul** of each diluted sample (and controls) to the wells.

The purpose of using sorbent is to block the IgG's present in the sample. Failing to use sorbent could lead to FN reactions; the IgG's will bound the antigens present in the well surface, leaving less antigens available to be bound by IgA/IgM, which will result in a weaker signal.

Another advantage of prediluting the samples/controls is that you minimize the incubation at room temperature; if you have many samples to dispense, the first samples will incubate at room temperature for a considerable amount of time. If you do a predilution step and then transfer the samples with a multichannel pipette, the incubation at room temperature will be considerably reduced.

Lastly, cover the wells with the sticker provided with the kit, and **incubate 45 minutes at 37ºC**. Failing to cover the wells will lead to evaporation and the content of the wells will get concentrated, which will likely result in higher OD's. Also, it helps to retain the temperature, as it will not dissipate as easy.

- *Tip 1:* when aspirating small volumes (like the 5 ul of sample/control), place the pipette in the vial vertically, and just dip 1 mm the tip inside the liquid, and aspirate slowly. With small volumes the error tends to be higher, and immersing the tip too much will drag some drops that will remain attach to the exterior of the tip, and eventually will end up into the reaction well. As a result, the OD will be higher.
- *Tip 2*: as general rule of thumb, aspirate slowly and dispense fast.
- *Tip 3*: at the end of the run, if the results are lower/higher than expected, the incubator should be reviewed. There are dedicated plates for this purpose although a contact probe could also be used; the temperature on the surface of the incubator should be around 38-40ºC. Alternatively, you can measure the temperature of the liquid inside the wells with a dedicated temperature probe.
- *Tip 4*: if you use a multichannel pipette, secure each of the tips before aspirating. When you aspirate make sure that all the tips are even and no air bubble is aspirated.
- *Tip 5*: close the control vials as soon as possible in order to avoid evaporation and eventual concentration. This will lead to higher OD's.

Note 1: the serum diluent is a generic reagent, and therefore compatible with all our ELISA tests.

*Note 2: Vircell ELISA captures do not require control predilution; **100 ul** of each control go directly to the well. The incubation time is **60 minutes**.*

First Washing Step:

Material required:

- ELISA plate washer (alternatively a multichannel pipette)

The washing step consists **of 5 cycles of 0,3 ml of wash buffer per well**. If you use an automated washer, configure the wash program. If no washer is available, use a multichannel pipette for this purpose. The manual wash is more prone to errors and we recommend to use it as a last resource.

Before starting, make sure that the wash buffer is at room temperature, and that you have enough volume in the wash bottle.

Place the plate in the washer and start the washing program. If you have an 8-tips washer, you might need to add some empty wells if the strips are not complete.

Once the washing has finished, drain off any remaining liquid by hitting the plate on an absorbing paper.

Proceed with the conjugate dispense immediately.

- *Tip 1*: draining off any remaining liquid after the washing will avoid that the conjugate is partially diluted.
- *Tip 2*: if you obtain non-repetitive or erratic results at the end of the run, the washer might need to be checked. If there is any entrance of air, some tips could be dripping, and this will have an impact on the results. Check it by being in front of the washer while the washing takes place; no dripping should be observed, the wells should get filled with wash buffer correctly, and afterwards the wash buffer should be completely removed from the well every cycle.
- *Tip 3*: in order to avoid any washer malfunction, you should follow the maintenance procedure recommended by the manufacturer.

Conjugate dispense and Second Incubation:

Material required:

- Multichannel/single channel pipette 100 ul
- Pipette tips
- Thermostatized incubator/water bath

Dispense **100 ul of conjugate per well**. If you have many wells to dispense, pour approximately the volume of conjugate that you are going to use into a Petri dish, and use a multichannel pipette to dispense faster. Do no return leftovers to the conjugate bottle.

Vircell provides the conjugate in two separate bottles; in the unlikely scenario that one bottle gets contaminated, there is a second one available. In the majority of cases it is due to a serum contamination by a user mishandling; the serum will react with the conjugate, causing the OD's to be lower over time.

Once the conjugate has been dispensed, seal the plate and incubate it for **30 minutes at 37ºC**.

*Note: the conjugate incubation of Vircell ELISA captures is **60 minutes**. Some of the antigen/conjugates come **lyophilised** and have to be reconstituted (reconstitute it one hour before use). Our recommendation is that once they are reconstituted, are kept stored at 2-8ºC no longer than one week. If it has not been finished within a week, freeze it for further use. They can only be thawed once.*

Second Washing Step:

Follow the same protocol as in the first washing step.

Substrate dispense and Third Incubation:

Dispense **100 ul of substrate** (TMB) to each well. If there are many wells to be dispense, transfer a part of the bottle to a Petri dish and use a multichannel pipette. Do not return leftovers to the bottle.

Incubate the plate **20 minutes in darkness at room temperature**.

- *Tip 1*: if using a multichannel pipette, secure the tips before aspirating, making sure that the aspiration is even and no air bubbles are taken.
- *Tip 2*: the incubation can take place inside a drawer, as it will be dark inside.
- *Tip 3*: the substrate reagent has to be completely transparent. If it is blueish, it is a symptom that it has been contaminated, and higher OD's could be obtained. Direct light contact can also spoil it.

Note: the substrate (TMB) is a generic reagent, and therefore compatible with all our ELISA tests.

Stopping solution dispense:

After the substrate incubation, directly dispense **50 ul of stop solution** to each well. If there are many wells to be dispense, transfer a part of the bottle to a Petri dish and use a multichannel pipette.

If there is reaction the colour will turn blue; the bluer, the more positive the sample is. As soon as the stop solution is added, the reaction is stopped and the colour turns yellow.

Reading:

Read with a spectrophotometer at 450/620 (630 nm is also fine) nm within 1 hour of stopping the reaction.

The purpose to read with the reference at 620 nm is to eliminate interferences; at 620 nm the specific yellow colour of a positive does not interfere, whereas any opacity present in the well will be absorbed. In this way, there is no need to use a blank well.

- *Tip 1*: if anomalous OD's are obtained, it might be due to an issue with the reader. In order to check it, read the plate in parallel with a second reader and compare the results, or use a calibrated plate to check the reader performance.

Note: the stop solution is a generic reagent, and therefore compatible with all our ELISA tests.

Validation criteria:

Positive, negative and cut off (in duplicate) controls must be run with each test run. It allows the validation of the assay and kit. Optical densities (OD) must fall in the following ranges. Otherwise, the test is invalid and must be repeated.

Control	O.D.
Positive control	>0.9
Negative control	<0.5
Cut off control	>0.55
	<1.5

Note: there could be a slight variation in OD values from run to run. This variation can be caused by many factors (such as temperature in the lab, operator, length of time that kits have been opened, etc.) and is normal; this is one of the reasons why the validation criteria for the controls has a range. What is more important for most ELISA is not the OD values (although they should always be within specifications) but the concentration values; this means that if a given OD value for a sample is higher in one assay, the cutoff will proportionally be higher as well, and therefore the index (which determines the result) will not vary.

Results interpretation:

Calculate the mean O.D. for cut off serum.

Antibody index=(sample O.D./ cut off serum mean O.D.) x 10

Index	Interpretation
< 9	Negative
9-11	Equivocal
>11	Positive

- Samples with equivocal results must be retested and/or a new sample obtained for confirmation.
- Samples with indexes below **9** are considered as not having specific antibodies against the pathogen.
- Samples with indexes above **11** are considered as having specific antibodies against the pathogen.

Note: it is important to run the cutoff control in duplicate because it is used to calculate the results; if we use the average of the two replicas, pipetting imprecisions will be balanced.

Information about Vircell ELISA tests in automated platforms:

Our kits are adjusted manually because they are intended to be used either manually, or in any automated platform with the required technical specifications that allow the correct performance of the assay.

Vircell already has the testfiles to run our ELISA assays in Thunderbolt (GSD) and DS2 (Dynex).

If you want to run our ELISA tests in other open automated platforms (i.e. Triturus (Grifols), DSX (Dynex)) you should first program the test with the dedicated method editor software, and **validate** it.

Before validating the assay, we recommend to make sure that the timing and synchronization of the assay are correct. In order to not to waste reagents, this first assays are normally water tests (all reagents are water). Afterwards, the logfiles generated by the instrument, which contain all the information of the assay, should be analyzed in order to make sure that the incubations are fulfilled for all the wells.

Once the timing is correct you can run the controls. It might be convenient to add the controls at the beginning and end of the plate, to see if there could be drift affecting the assay. If everything is correct and all the assays steps are programmed properly, the results of the controls should correlate with those indicated by the manufacturer.

If the results obtained are not correct, we recommend to check the program step by step, in order to identify where the problem is. To do so, you would start the assay in the instrument, and test only the first step, for example, the dispense of controls and samples. Then, you would take the plate out and finish the assay manually. If the results are fine, you can conclude that this step in the instrument is correct. Now, you would test in the instrument the first step + second step and follow the same procedure; take the plate out and finish it manually. Following this procedure, eventually, you will isolate the faulty step. Once you isolate it you should work to improve it, until you get correct results.

Please, note that to validate a kit for a specific instrument can be a long procedure where the instrument as well as the kit performance have to be well known.

Validating a kit in an automated platform implies testing that kit in standard lab conditions with **repeatability, reproducibility** and **correlation assays**, which implies the testing of samples too.

These results should also be reproduced at the customer site.

Note 1: whenever you have anomalous results in an automated platform also perform the assay manually in order to replicate the results, as the manual assay is the reference method. In this way, we will have more information as to decide if it is a lot or an instrument-related issue. Also, share results from other parameters, so we can have a general overview of the instrument performance.

Note 2: OD values in automated platforms might be slightly higher or lower than the manual assay. Nevertheless, the indexes will not vary, because all the samples/controls will be lower/higher proportionally.

Note 3: Vircell provides training for the Test Designer Software for the Thunderbolt system during the VirClia training.

Details to open a complaint:

General information:

- Reference or product name
- Lot number
- When was the kit opened/used for the first time?
- Anomalous results were found in the first use of the kit?
- How have the kit components been stored after the first opening? (2-8ºC, R.T, -20ºC, -80)
- Does the final customer use this kit in routine?
- Does the customer use other Vircell ELISA with correct results?

Specific information:

- Controls values: positive control, negative control, cut-of control, others
- Results of the discrepant samples in the same assay
- Was the assay performed in an automated platform or manually? Specify platform and share results
- If the assay was performed manually, specify kind of washer and incubator
- If the assay was carried out in an automated platform, did you have the opportunity to run the affected kits manually, in order to see if the results were replicated?
- If the assay was carried out in an automated platform, does the customer run other Vircell ELISA kits? Share the results with us

Troubleshooting – Tips:

Instruments:

- Incubator: verify that the temperature of the incubator is correct. Review page 5, tip 3.
- Washer: check that the washing step is correct. Review page 6, tip 2. Also, verify that the user did not run out of wash buffer during the assay.
- Reader: check that the reader status is correct. Review page 8, tip 1.

Reagents:

- Verify that the wash buffer was prepared adequately (950 ml of DI water + 50 ml of wash 20x → 1/20 dilution) with quality DI water, and that it was at room temperature. If you doubt, prepare fresh wash buffer.
- Before opening the controls for the first time, shake the vial off (or do a centrifuge pulse) to make sure that any drop that could be in the vial cap returns to the body of the vial.

- The control vial caps should be placed facing upwards in order to avoid contamination. It is recommended to open and close each vial sequentially, in order to avoid cross contamination.
- The controls could be contaminated by accident by mixing up the vial caps.
- The conjugate could be contaminated by serum if no attention is paid while dispensing, the pipette tips are dirty, or they are not changed after dispenses. If there is a suspicion of conjugate contamination, use the second bottle the kit supplies.
- In IgG/IgM kits, the customer might use the wrong conjugate bottle.
- Avoiding to pipette directly from the reagent bottles will minimize the chance of reagent contamination. To do so, transfer the volume to a clean container (like a Petri dish) and do not return leftovers to the original reagent bottle.
- Verify that the substrate reagent is completely transparent. If it is blueish it means that it could have been contaminated, or that it was spoiled by the light.
- Some of the reagents have big volumes, which means that it might take more than 1 hour to reach room temperature. You can use a warm bath or in an incubator at 37ºC for this purpose.

Environmental:

- The lab temperature might be higher than usual. Remember that the substrate incubation should be at room temperature; a high temperature could result in a higher peroxidase activity. If the lab temperature is too high and cannot be changed, see if shortening a little bit the incubation time of the substrate improves the results.
- Too much light during the incubation of the substrate. Remember that it should take place in darkness.
- Evaporation of the reagents could lead to an increase of the OD values over time. Close the vials (specially the controls) after its use. This is especially important in automated platforms, where the temperature can be high.
- Environmental microbial contamination; verify that there is no turbidity in the controls nor reagents. Nevertheless, they contain preservatives that should avoid this. If the wash buffer has been stored for a long time at room temperature, it could also get contaminated.

Material:

- Make sure that the pipettes are calibrated and dispense correctly. The important volumes are 100 ul and 5 ul.
- Always clean and disposable pipette tips should be used. Always store them protected from dust or dirtiness.
- Load the pipette tips into the boxes using gloves.

Automated platforms:

- See suggestions in page 10.
- Verify that the daily, weekly, monthly and preventive maintenance are up to date.
- Verify that there are no hardware issues.
- Analyze the logfiles generated after the run is completed in order to find what could have caused the anomalous results (overincubations, running out of reagents, insufficient volume, etc.).