ELISA – a simple experiment ?

ELISA experiments follow a simple work routine and thus it is tempting to believe that this type of experiments needs no special expertise. Especially workers who are dealing with large samples for years sometimes often neglect the accompanying instructions due to their working routine. Thus it can happen that an experiment fails or does not provide clear results. This short introduction will draw your attention to some important facts and will assist you to solve potential problems.

Sampling

Correct sampling is in fact the key to successful ELISA testing. Thus, by designing the experiment, please reconsider the following questions:

- Did you choose the right season and temperature for sampling?
- Did you choose the right parts of the plants (leaves, blossoms, roots, stems, seeds, tubers)
- What about the age of the plants?
- Did you take mixed samples or individual samples form different parts of the plant?
- Are enrichment steps necessary (culture of bacteria or fungi on selective or enrichment media)?
- Is transportation and storage well organized (short time, cool environment, refrigerator, no storage or transport during weekend or holidays)

Guidelines for sampling are often stated in official diagnostic protocols published by plant health organisations such as EPPO (*European Plant Protection Organisation*) or ISTA (*International Seed Testing Association*) for a particular pathogen.

Sample preparation for ELISA

Leaves and juicy samples can be squeezed in simple plastic bags using commercial homogenizers, a hammer, Pollähne juice presses etc. Some fibrous tissues (mycelia of fungi, woody plant parts), however, need grinding with sand in mortars to assure proper crushing of the cell walls.
It is very important to dilute the samples sufficiently, i.e. min. 1:20 to 1:30. Exceptions are indicated in our product specification sheet. Positive samples - when used too concentrated - sometimes give no or too low signals. At the same time, negative samples can exhibit high background reactions.

Never leave a sample or solubilized control standing at room temperature for several hours, but prepare immediately before use if possible and store it in the refrigerator.

Choice of the ELISA plate

The quality of the ELISA plate has **a really dramatic effect** on the sensitivity of the ELISA assay in terms of O.D. value, detection limit, and background behaviour. LOEWE is using exclusively NUNC Maxisorp™ and Greiner Microlon High-Binding plates for standardizing its ELISA reagents.
To obtain high-quality results it is absolutely necessary to use a high-affinity plates.

Washing

Careful washing is also a crucial point. Best and reproducible results are obtained by using automated ELISA washers. Washing by hand represents the most gentle method but often leads to irreproducible results. Automated washers on the other hand have to be optimized because the pressure with which the washing buffer is injected into the wells is often too high. Too high pressure and too extensive washing can lead to weak results. Also, daily cleaning of the washer is important for reproducible function.
Only use the wash buffer formulation described in our protocol for plate washing!

Storage of plates between individual ELISA steps:

This is important in case many plates have to be handled simultaneously. We recommend to store plates covered with a tape in the refrigerator until use.

ELISA Buffers

Do not use other buffer formulations besides the ones given in our protocol because our ELISA reagents are standardized using these buffers.

It is important to adjust and control the pH value of the buffers. The pH of the substrate buffer is especially important, because the enzymatic activity of the alkaline phosphatase has a very small optimum at pH 9.8. Deviations lead to a considerable reduction of the O.D. values. Especially albumin-containing sample and conjugate buffers are prone to microbial growth if stored in the refrigerator, which can lead to odd ELISA results. **Use ready-to-use buffer solutions as fresh as possible. Store aliquots in the freezer until use!** Alkaline buffers should be stored in glass bottles.

Dilution of IgG and IgG-AP-conjugate

Our reagents are pre-diluted to minimize deviations from pipetting errors. Please follow our pipetting scheme:

Dilution Table for DAS ELISA
(based on a working dilution of 1:200)

IgG or AP-Conjugate stock solution from vial	Buffer
10 µl	2 ml (1x8 wells)
17 µl	3.4 ml (2x8 wells)
34 µl	6.8 ml (4x8 wells)
100 µl	20 ml (1 plate)
500 µl	100 ml (5 plates)

Dilution of Controls

The controls represent lyophilized samples from healthy (negative controls) or infected plants (positive controls). Bacterial and fungal positive controls are routinely made from inactivated extracts of bacterial or mycelial suspensions. Lyophilized controls can be stored in the refrigerator, for long-term storage they can be frozen.

Dilute content of the vial with 1ml of sample buffer. Solubilized positive controls should be stored frozen in suitable aliquots until use. Please note, that after solubilisation, losses in activity occur with many pathogens upon freezing and thawing.

Sample Volume

Our ELISA reagents are standardized for a sample volume of 0.2 ml/well, if not indicated otherwise in the product manual. We do not recommend smaller volumes and cannot guarantee the OD valued stated in our product specifications if other volumes are used.

Incubation Temperatures and - Times

We refer you to our standard protocol. In case there are practical reasons against this scheme, it is in the responsibility of the user to test whether modified incubation temperatures and times lead to the same reliable result as the standard protocol.

Sensitivity of the Assay / Establishing the + / - Threshold

To determine the sensitivity of your assay we recommend to prepare a dilution series from our positive control in steps of ten. Thereby you can estimate whether the detection limit you obtain is comparable to the one we state in our certificate.

Determining the +/- threshold in plant disease diagnostics is still a crucial problem and needs not only personal expertise but also comparison with a second independent method of pathogen detection. One frequently used method is to calculate the mean value + 3 x standard deviation. For calculating this threshold it is absolutely necessary to have several healthy plants of the same species at the same plate. Run all samples in duplicate.

Last but not least: always cover your plate with sealing tape!

TROUBLESHOOTING

ERROR	CAUSE	SOLUTION
No colour development	IgG and/or AP-Conjugate omitted	repeat tests
	wrong positive control, wrong reagents	replace reagents
	water used for washing	repeat with wash buffer
	positive control lost activity	use fresh positive control
Weak colour development	low quality ELISA plate	use high-affinity plate
	wrong dilution	repeat test with correct dilutions
	wrong storage of reactants	repeat test with fresh material
	water used for washing step	repeat with wash buffer
Irregular colour	incomplete washing or spillage	check washing procedure
	insufficient mixing of the reagents	mix all reagents (especially IgG and AP-Conjugate) thoroughly
All wells turn yellow	AP-Conjugate used for coating step	repeat test
High background	wrong dilution of IgG and/or AP-Conjugate	repeat test with correct dilutions
	plate has not been covered	cover with sealing tape
	wrong sample/extraction buffer	repeat test with our buffer formulations
	old ELISA buffers	use buffers as fresh as possible freeze aliquots until use!
	IgG contaminated with AP-Conjugate by contamination of pipettes or buffer reservoir with AP-Conjugate	repeat test using clean labware Never use the same reservoir or pipettes for IgG and AP-Conjugate **Use filtered tips and one-way labware only!**
	Insufficient washing of plate	Check washing procedure