Recommendations for ELISA Testing

ELISA – a simple experiment?

This short introduction will draw your attention to some important facts, that might be neglected and will assist you to solve potential problems.

Sampling

Correct sampling is in fact the key to successful ELISA testing. Thus, 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 from different parts of the plant?
- Are enrichment steps necessary (culture of bacteria or fungi on selective or enrichment media)?

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 value stated in our product specifications if other volumes are used.

Choice of the ELISA plate

The quality of the ELISA plate has a dramatic effect on the sensitivity of the ELISA assay in terms of OD value, detection limit, and background behaviour. LOEWE® Kits contain high-quality plates as NUNC Maxisorp™ or Greiner Microlon® High-Binding 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 of washing buffer injection into the wells is often too high. Wrong pressure and too extensive washing can lead to weak results. Also, daily cleaning of the washer is important for reproducible function.

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, as the enzymatic activity of the alkaline phosphatase has a very small optimum at pH 9.8. Deviations lead to a considerable reduction of the OD values. Especially 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.

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 results 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 an unsolved issue. One frequently used method is to calculate the mean value + 3 x standard deviation. For calculating this threshold it is absolutely necessary to have samples of several healthy plants of the same species on the same plate. Run all samples in duplicate. Comparison with a second independent method of pathogen detection is recommended for borderline results.

LOEWE®

Product Manual: Complete Kit Standard DAS - ELISA

Assay Principle - Double Antibody Sandwich (DAS) ELISA

The Double Antibody Sandwich (DAS) ELISA is one of the most common methods for serological plant pathogen detection, which consists of four basic steps:

During the first step, the surface of a microtiter plate is coated with a coating-antibody (IgG), which is directed against a specific antigen. When an antigen-containing sample is added during the second step, it will bind to the immobilized IgG, forming an IgG-antigen complex. During the third step, a complementary IgG, which is labelled with alkaline phosphatase (AP-conjugate) is added, binding to the antigen-IgG-complex and forming a double-antibody sandwich. During the fourth step the substrate 4-nitrophenyl-phosphate is applied and the alkaline phosphatase (AP) enzymatically forms yellow coloured 4-nitrophenol. The colour development can be evaluated visually or measured in a spectrophotometer at 405 nm.

Content of the Kit

Components	96 tests	480 tests	960 tests	4800 tests
Antibody (IgG)	0.1 ml	0.5 ml	1.0 ml	5 x 1.0 ml
Antibody-AP-conjugate	0.1 ml	0.5 ml	1.0 ml	5 x 1.0 ml
Positive Control	1 vial	1 vial	2 vials	5 vials
Negative Control	1 vial	1 vial	2 vials	5 vials
Coating Buffer	1 liter	1 liter	1 liter	1 liter
Wash Buffer	1 x 5 liter	1 x 5 liter	2 x 5 liter	5 x 5 liter
Conjugate/Sample Buffer	1 x 1 liter	2 x 1 liter	4 x 1 liter	10 x 1 liter
Substrate Buffer (5x)	1 x 25 ml	1 x 25 ml	2 x 25 ml	200 ml
Substrate Tablets	4 x 5 mg	5 x 20 mg	10 x 20 mg	5 g (powder)
Tween® 20	5 ml	5 ml	10 ml	50 ml
High-binding ELISA plates	strips à 8 wells	5 plates	10 plates	50 plates
Sealing Cover	1 sheet	5 sheets	10 sheets	50 sheets

Handling and Storage of Antibodies and Controls

Our reagents are shipped at room temperature without ice packs.

The stability at ambient temperature has been evaluated without observing a significant loss in activity. However, after receipt the antibodies must be kept refrigerated (ca. 4°C). Once opened, we recommend using the reagents within 5 months. Our DAS-ELISA reagents are standardized for use at a dilution of 1:200 and a test volume of 200 µl/well.

Positive and Negative Controls must be kept refrigerated. Once dissolved, it is advised to aliquot the controls and store them frozen until use. However, depending on the stability of the particular pathogen, prolonged storage might result in reduced activity. Repeated freezing and thawing should be avoided as it can result in loss of activity.

Analytical data and other product specifications can be derived from the Product Specification sheet that is included with each reagent set.

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Preparing the Assay

Buffer Preparation

Coating Buffer	Dissolve the content of the sachet in approx. 900 ml distilled water, adjust pH to 9.6 and fill up to 1 l. Store refrigerated until use. Keep frozen in glass bottles for long-term storage.
Wash Buffer	Dissolve content of the sachet in 5 l of distilled water. Add 2.5 ml of Tween ™ 20 (0.05% final concentration), adjust pH to 7.4. Store refrigerated. Keep frozen for long-term storage.
Conjugate/ Sample Buffer	Dissolve content of the sachet in approx. 900 ml water and adjust pH to pH 7.4 with sodium hydroxide. Add 0.5 ml Tween® 20 and fill up to 1 l. If desired 0.01% sodium azide can be added. Store refrigerated for not longer than 1 week. We recommend freezing aliquots and use the buffer as fresh as possible.
Substrate Buffer	Dilute 25 ml of 5x concentrate with approx. 100 ml of water. Adjust pH to 9.8 before filling up to 125 ml. Store refrigerated. Keep frozen in glass bottles for long-term storage.
Substrate Solution	Dissolve substrate, equivalent to 1 mg/ml in diluted substrate buffer (1x) **directly before use.**

Please note: Our buffer formulations **do not contain sodium azide.**

Buffer formulations are according to Clark, M.F. & Adams, A.N. 1977. Characteristics of the micro-plate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol., 34: 475-483.

Preparation of Antibody Working Solution

Our antibodies are pre-diluted to minimize deviations from pipetting errors. Please follow our pipetting scheme to obtain the working dilution:

Nr. of wells to be filled	IgG or Conjugate Stock Solution from original vial	Coating Buffer (for IgG Dilution) Conjugate/Sample Buffer (for Conjugate Dilution)
1x8 (1 strip)	8.5 µl	1.7 ml
4x8	34 µl	6.8 ml
96 (1 plate)	100 µl	20 ml
480 (5 plates)	500 µl	100 ml

Preparation of Samples

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) need to be grinded 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. Positive samples - when used too concentrated - sometimes give no or too low signals. At the same time negative samples can exhibit high background reactions.

Please also consult official diagnostic protocols published by plant health organisations such as EPPO (European Plant Protection Organisation) or ISTA (International Seed Testing Association) for sampling and sample preparation recommendations.

Performing the Assay

Coating Plates with Antigen-specific Antibodies (IgG)

1

- Dilute IgG from original vial **1:200*** in Coating Buffer, mix gently but thoroughly.
- Add **0.2 ml***of the IgG working solution to one well of the ELISA plate.
- Tightly cover plate with sealing tape.
- Incubate at 37°C for 4 hours or overnight in the refrigerator.
- Remove IgG with Wash Buffer by four washing cycles using an automated washer or by manual washing.

Sample Preparation and Application

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- Homogenize samples with ratio of **1:20 w/v** in Conjugate/Sample Buffer.
- Dissolve LOEWE® Positive or Negative Controls in ca. 2 ml Conjugate/Sample Buffer.
- Add **0.2 ml***of sample extract or Control solution to one well of the ELISA plate.
- Tightly cover plate with sealing tape.
- Incubate overnight in the refrigerator.
- Remove sample with Wash Buffer by four washing cycles using an automated washer or by manual washing.

Application of Antibody-AP-conjugate

3

- Dilute Conjugate **1:200*** from original vial in Conjugate/Sample Buffer, mix gently but thoroughly.
- Add **0.2 ml*** of the conjugate working solution to one well of the ELISA plate
- Tightly cover plate with sealing tape.
- Incubate at 37°C for 4 hours or overnight in the refrigerator.
- Remove conjugate with Wash Buffer by four washing cycles using an automated washer or by manual washing.

**unless stated otherwise in the product specification sheet*

Enzymatic Assay

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- Dissolve substrate equivalent to 1 mg/ml in 1x substrate buffer directly before use.
- Add **0.2 ml** of the solution to one well of the ELISA plate.
- Incubate at ca. 25°C in the dark.
- After 1 and 2 hours of substrate incubation, evaluate the reaction visually or read photometrically at 405 nm.

Evaluation

We strongly advise to add the positive and negative controls to each plate for verification of a strong positive and a low negative reaction. To determine potential background of healthy plants, add fresh non-infected extracts of the tested species and tissue at the same dilution to each plate. However, the positive/negative threshold needs to be determined by the user, as it depends on many factors, such as plant species and its physiological conditions (e.g. tissue type, age).