Product no AS25 5114
SA | Salicylic acid ELISA quantitation kit

Background

This ELISA assay utilizes the principle of competitive binding to measure the concentration of SA in plant extracts. The SA (salicylic acid) has been pre-coated on the surface of the reaction wells. The plant extract sample, containing unknown amount of SA, or standards, are mixed in the appropriate reaction wells with a known amount of the biotin-conjugated anti-SA antibody. During incubation, the competitive inhibition reaction occurs between pre-coated SA and hormone in the samples with the SA-specific antibody.

Unbound hormone and plant extract are washed out of the reaction wells. Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated followed by the addition of TMB substrate solution for the reaction development. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution, and the colour change is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The colour develops in opposite to the amount of SA in the sample or standards. The concentration of SA in the samples is then determined by comparing the OD of the samples to the standard curve.

The SA ELISA kit reagents:

Pre-Coated Microplate	6 strips x 8 wells	4°C/-20°C
Standard (Lyophilized)	1 vial	4°C/-20°C
Biotinylated-Conjugate (100×)	30 µL	4°C/-20°C
Streptavidin-HRP (100×)	60 µL	4°C/-20°C
Standard/Sample Diluent Buffer	10 mL	4°C/-20°C
Biotinylated-Conjugate Diluent	5 mL	4°C/-20°C
HRP Diluent	6 mL	4°C/-20°C
Wash Buffer (25×)	10 mL	4°C/-20°C
TMB Substrate Solution	6 mL	4°C/-20°C (Store in dark)
Stop Reagent	3 mL	4°C/-20°C
Plate Covers	1 piece	RT

Assay development time: 2.5 hours
Sensitivity: 0.6 ng/mL
Detection range: 1.57-100 ng/mL
Intra-assay Precision (Precision within an assay): CV% < 8%
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.
Inter-assay Precision (Precision between assays): CV% < 10%
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.
Plant extract volume: 50 µL/well
Detection wavelength: 450 nm
Cross reactivity to other plant hormones: No significant cross-reactivity or interference between Plant Salicylic Acid and analogues was observed.
Sample type: plant leaf tissue

This kit should not be used beyond the expiration date. Please refer to the outer packaging label of the kit for the specific shelf life.

Storage

1. If the kit is opened, Store the whole kit at 4°C. If the kit is not used up in 1 week. Store the Pre-Coated Microplate, Standard, Biotinylated-Conjugate and Streptavidin-HRP at -20°C, the rest reagents at 4°C, please used up within 6 months.
   *If the kit is not opened, store the whole kit: 4°C (short time storage, valid for 6 months); -20°C (long-term storage, valid for 1 year). Avoid repeated freeze-thaw cycles.
2. If the whole kit is stored at -20°C, place the kit at 4°C the day before the experiment.
3. After opening the package, please check that all components are complete.
4. The cap must be tightened to prevent evaporation and microbial contamination. The reagents volume is slightly more than the volume marked on labels, please use accurate measuring equipment and do not pour directly into the vial.

All kit components have been formulated and quality control tested to function successfully. Do not mix or substitute reagents or materials from other kits, detection effect of the kit will not be guaranteed if utilized separately or substituted.

Not provided with the kit but required to conduct the test

Microplate reader capable of measuring absorbance at 450 ± 10 nm | High-speed centrifuge | Incubator with stable incubation temperature up to 37°C | Squirt bottle, manifold dispenser or automated microplate washer | Absorbent paper for blotting the microtiter plate | Double distilled water or deionized water | Single or multi-channel pipettes with high precision and disposable tips | Precision pipettes to deliver 2 µL to 1 mL volumes.

Important notes

This kit is only used for lab research and development and should not be used for human or animals. Reagents from other lots should not be mixed. In case analyzed samples are generating values which are higher than the highest standard, samples should be diluted, and the assay repeated. Variation in the assay can occur due to operator, pipetting technique, washing technique, incubation time and temperature as well as kit expiry date. Reagents should be regarded as hazardous substances and should be handled carefully and correctly.

Safety measures

Gloves, lab coats, and goggles should always be worn to avoid skin and eyes in contact with Stop Reagent and TMB. In case of contact, wash thoroughly with water.

Procedure instructions

Please, read the whole manual carefully before proceeding with your experiment.

Sample collection Preparation and Storage

Plant Xylem Sap - The xylem sap of wild plants can be collected by cutting the stem 10-15 cm above the ground (preferably in the morning to make full use of root pressure). Xylem sap accumulates in silicone tubing due to root pressure. If there is a risk of excessive exposure to light, the silicone tubing should be wrapped in aluminum foil. Depending on the plant and treatment, approximately 0.5 mL can be obtained within 1-2 hours. Use a pipette to collect into a centrifuge tube, freeze immediately, and store at -80°C until testing.

Crude Extract - (Homogenizer Method)

1. Prepare 0.9% sodium chloride in pure water, homogenizer, and other equipment necessary for protein extraction.
2. Always use plants which are clean, without any surface dust on the plant parts. Make sure that the material is dry before it is weighted. To reduce weighing errors, the tissue weight should not be less than 50 mg.
3. Add 0.9% sodium chloride in pure water at a mass-to-volume ratio of 1:9. For example, weigh 100 mg of tissue sample and add 900 µL of normal saline.
4. Homogenize the tissue to obtain a 10 % homogenate suspension.
5. Centrifuge the homogenate at 4000xg in a 4°C centrifuge for 5 min, then collect the supernatant and aliquot for testing.
6. If not tested for a long time, it is recommended to store samples at -80°C.

For sample testing, dilute the sample appropriately during the testing process according to the detection range.

Crude Extract - (Liquid Nitrogen Method)

1. Prepare 0.9% sodium chloride in pure water, liquid nitrogen, etc.
2. First, clean the plant sample with pure water to remove surface dust, wipe dry, and grind with liquid nitrogen. Place the crushed sample into a tube for later use.
3. Add 0.9% sodium chloride in pure water at a mass-to-volume ratio of 1:9. For example, weigh 100 mg of tissue sample and add 900 µL of normal saline.
4. Place the plant tissue with 0.9% sodium chloride in pure water in -80°C freezer and perform repeated freeze-thaw cycles more than 3 times to obtain a 10% homogenate suspension.
5. Centrifuge the homogenate at 4000xg in a 4°C centrifuge for 5 min, then collect the supernatant and aliquot for testing.
6. If not tested for a long time, it is recommended to store extracted material at -80°C.

For sample testing, dilute the sample appropriately during the testing process according to the detection range.

Notes

1. Samples which are to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤ 1 month) or -80°C (≤ 2 months) to avoid loss of bioactivity and contamination. Avoid repeated freeze-thaw cycles.
2. Shortly before performing the assay, bring samples to room temperature.
3. If the concentration of the test material in your sample is higher than that of the Standard product, please make the appropriate multiple dilutions according to the actual situation (it is recommended to do preliminary experiment to determine the dilution ratio)

Summary

1. After the kit is equilibrated at room temperature, add 50 µL of Standard Working Solution (gradually dilution refers to Reagent Preparation) or 50 µL of sample to each well, immediately add 50 µL of 1× Biotinylated-Conjugate Working Solution to each well, mix well, incubate at 37°C for 60 min.
2. Discard the liquid in the plate, add 200 µL1× Wash Buffer to each well, and wash the plate 3 times. After patting it dry against clean absorbent paper, add 100 µl 1× Streptavidin-HRP Working Solution to each well, incubate at 37°C for 60 min.
3. Discard the liquid in the plate, add 200 µL1× Wash Buffer to each well, and wash the plate 5 times. After patting it dry against clean absorbent paper, add 90 µL TMB Substrate Solution to each well, incubate at 37°C for 20 min in the dark.
4. Add 50 µL Stop Reagent to each well, shake plate on a plate shaker for 1 min to mix. Record the OD at 450nm immediately, calculation of the results.

Reagent Preparation

1. Bring all kit components and samples to room temperature (18-25°C) before use. Make sure all components are dissolved and mixed well before using the kit.
2. If the kit will not be used up in the planned experiment, use only strips and reagents for present experiment, and save the remaining strips and reagents as specified.
3. Dilute the 25× Wash Buffer into 1× Wash Buffer with double distilled water.
4. **Standard Working Solution** - Centrifuge the Standard at 1000 × g for 1 min. Reconstitute the Standard with 1.0 mL of Standard Diluent Buffer, kept for about 10 min at room temperature, shake gently (not to foam). The concentration of the standard in the stock solution is 100 ng/mL. Please prepare 7 tubes containing 0.5 mL Standard Diluent Buffer and use the Diluted Standard to produce a double dilution series according to the picture shown below. To mix each tube thoroughly before the next transfer, pipette the solution up and down several times. Set up 7 points of Diluted Standard such as 100 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.13 ng/mL, 1.57 ng/mL, and the last EP tubes with Standard Diluent is the **Blank** as 0 ng/mL. In order to guarantee the experimental results validity, please use the new Standard Solution for each experiment. When diluting the Standard from high concentration to low concentration, replace the pipette tip for each dilution. Note: the last tube is regarded as the **Blank** and **do not** pipette solution into it from the former tube.
5. 1× **Biotinylated-Conjugate** and 1× **Streptavidin-HRP Working Solution** - Briefly spin or centrifuge the stock Biotinylated-Conjugate and Streptavidin-HRP before use. Dilute them to the working concentration 100-fold with Biotinylated-Conjugate Diluent and HRP Diluent, respectively. For example, 10µl of Streptavidin-HRP with 990µl of HRP Diluent
6. **TMB Substrate Solution** - Aspirate the needed dosage of the solution with sterilized tips and <u>do not</u> dump the residual solution into the vial again.

Notes

1. After receiving the kit, please store the reagents according to the instructions. The plates can be disassembled to single strips. Please use it in batches on demands.
2. The test tubes, pipette tips and reagents used in the experiment are all disposable and are strictly prohibited from being reused; otherwise, the experiment results will be affected. Kit reagents of different batches cannot be mixed (except TMB, Washing Buffer and Stop Reagent).
3. Lyophilized Standards, Biotinylated-Conjugate, and Streptavidin-HRP are small in volume and may be scattered in various parts of the tube during transportation. Please centrifuge at 1000 × g for 1 min before use. Then, carefully pipette 4-5 times to mix the Solution. Please configure the Standard, 1× Biotinylated-Conjugate and 1× Streptavidin-HRP Working Solution according to the required amount, and use the corresponding Dilution Solution, cannot be mixed used.

4. Bring all reagents to room temperature (18-25°C) before use. If crystals form in the concentrate (25×), it is a normal phenomenon. Heat it to room temperature (the heating temperature should not exceed 40°C), gently Mix until crystals are completely dissolved.
5. Prepare to dissolve Standard within 15 min before the test. This Standard Working Solution can only be used once. If the dissolved Standard is not used up, please discard it. The sample addition needs to be rapid. Each sample addition should preferably be controlled within 10 min. To ensure experimental accuracy, it is recommended to test duplicate wells, and when pipetting reagents, keep a consistent order of additions from 1 well to another, this will ensure the same incubation time for all wells.
6. During the washing process, the residual washing liquid in the reaction well should be patted dry on absorbent paper. Do not put the paper directly into the reaction well to absorb water. Before reading, pay attention to remove the residual liquid and fingerprints at the bottom, so as not to affect the microplate reader reading.
7. TMB Substrate Solution is light-sensitive, avoid prolonged exposure to light. Dispense the TMB Substrate Solution within 15 min following the washing of the microtiter plate. In addition, avoid contact between TMB Substrate Solution and metal to prevent colour development. TMB is contaminated if it turns blue colour before use and should be discarded. TMB is toxic, avoid direct contact with hands.
8. Bacterial or fungal contamination of either samples or reagents or cross-contamination, between reagents may cause erroneous results.

Samples Preparation

1. Equilibrate all materials and prepared reagents to room temperature prior to use. Prior to use, mix all reagents thoroughly taking care not to create any foam within the vials.
2. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
3. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their experiments.

Manual

1. Determine wells for Diluted Standard, Blank and Sample. Prepare 7 wells for Standard, 1 well for Blank. Add 50 µL of Standard Working Solution (please refer to **Reagent Preparation**) or Sample into each well (**Blank** is Standard Diluent). Then, add 50 µL of Biotinylated-Conjugate (1×) to each well immediately. Mix well, cover with the Plate Cover. Incubate for 1 h at 37°C. Note: solutions should be added to the bottom of the micro-ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Pour out the liquid of each well. Aspirate the solution and wash with 200 µL of 1× Wash Solution to each well and let it sit for 1-2 minutes. After the liquid has been decanted, completely remove the remaining liquid from all wells by snapping the plate onto absorbent paper. Repeat washing procedure 3 times. Complete removal of liquid at each step is

essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid. Notes: (a) When adding Washing Solution, the pipette tip should not touch the wall or bottom of the wells to a void contamination.
(b) Pay attention to pouring the washing liquid directly to ensure that the washing liquid does not contaminate other wells.

3. Add 100 µL of Streptavidin-HRP Woking Solution (1×) to each well, cover the wells with the Plate Cover and incubate at 37°C for 60 min.
4. Repeat the aspiration, wash process for total 5 times as conducted in step 2.
5. Preheat the Microplate Reader for about 15 min before OD measurement. Avoid placing the plate in direct light.
6. Add 90 µL of TMB Substrate Solution to each well. Cover with a new Plate Cover. Incubate for exactly 20 min at 37°C (do not exceed 30 minutes) in the dark. The liquid will turn blue by the addition of TMB Substrate Solution.
7. Add 50 µL of Stop Reagent to each well. The liquid will turn yellow by the addition of Stop Reagent. Mix the liquid by tapping the side of the plate. The insertion order of the Stop Reagent should be the same as that of the TMB Substrate Solution.
8. Remove any water drops or fingerprints from the the bottom of the plate and make sure that there is no bubble on the surface of the liquid. Immediately make the reading of the plate on the microplate reader at 450 nm.

Note: Depending upon analysed materia, some samples may require dilution (please refer to Sample Preparation section).

Calculation of Results

This assay employs the competitive inhibition enzyme immunoassay technique, so there is an inverse correlation between Plant Salicylic Acid concentration in the sample and the assay signal intensity. Average the duplicate readings for each standard, control, and samples. Create a standard curve with the Plant Salicylic Acid concentration on the y-axis and absorbance on the x-axis. Draw the best fit straight line through the standard points and it can be determined by regression analysis. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor. Using some plot software, for instance, curve expert.

Concentration (ng/mL)	OD
100	0.232
50	0.336
25	0.557
12.5	0.897
6.25	1.119
3.13	1.596
1.57	1.861

0	2.101

Recovery

Matrices listed below were spiked with certain level of Plant Salicylic Acid and the recovery rates were calculated by comparing the measured value to the expected amount of Plant Salicylic Acid in samples.

Matrix	**Recovery range**	**Average**
Serum (*n*=5)	78-92%	85%
EDTA plasma (*n*=5)	92-106%	99%
Heparin plasma (*n*=5)	87-95%	98%

Linearity

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of Plant Salicylic Acid and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	**1:2**	**1:4**	**1:8**	**1:16**
Serum (*n*=5)	86-93%	87-101%	82-97%	89-97%
EDTA plasma (*n*=5)	95-106%	87-101%	82-97%	87-102%
Heparin plasma (*n*=5)	92-105%	82-91%	86-93%	81-94%

Declaration

1. For research use only. Not intended for diagnostic use.
2. The kit may not be suitable for analysis of gene knockouts.

Common Problems while working with ELISA assays

High background/Non-specific staining

Description of results	Possible reason	Recommendations and precautions
After termination, the whole plate results show a uniform yellow or light colour, or the Standard curve is linear, but the background is too high	The yellowing of the whole plate may be caused by wrong addition of other reagents	Check the components and lot numbers of the reagents before the experiment and confirm that all components belong to the corresponding kit. Reagents from different kits or different lot numbers cannot be mixed.
	ELISA plate was not washed sufficiently	Make sure that the same amount of Washing Solution is added to each microwell during the washing process. After washing, press the ELISA plate firmly on the fresh absorbent paper to remove the residual buffer.
	Incubation time too long	Please strictly follow the steps of the manual
	Streptavidin-HRP contaminates the tip and TMB container or positive control contaminates the Pre-coated Microplate	When absorbing different reagents, the tips should be replaced. When configuring different reagent components, different storage vessels should be used. Please use a pipette during operation.
	Biotinylated Antibody or Streptavidin-HRP concentration too high	Check whether the concentration calculation is correct or use after further dilution.
	Substrate exposure or contamination prior to use	Always store in the dark before adding substrate.
	Colour development time is too long	Please strictly follow the steps of the manual.
	The wrong filter was used to measure absorbance	When TMB is used as the substrate, the absorbance should be read at 450 nm

No colour development

Description of results	Possible reason	Recommendations and precautions
After the colour development step, all wells of the ELISA plate are colourless; the positive control is not conclusive	Mixed use of component reagents	Please read labels clearly when preparing or using
	In the process of plate washing and sample addition, the enzyme marker is contaminated and inactivated, and loses its ability to catalyse the colour development	Confirm that the container holding the ELISA plate does not contain enzyme inhibitors (such as sodium azide, etc.) and confirm that the container for preparing the Wash Solution was cleaned.
	Mixing up a reagents with each other or a step of the protocol	Review the manual in detail and strictly follow the operating steps

Weak colour development

Description of results	Possible reason	Recommendations and precautions
The Standard is developed correctly but there is no reaction in the samples	The sample contains sodium azide or other preservative, which inhibits the reaction of the enzyme	Samples cannot contain inhibitors of enzymatic reaction
	Level of analysed compound is too low for the kit to detect it	Decrease sample dilution, use other material
The visual result is correct, but the reading value of the microplate reader is low	Wrong filter was used for absorbance reading	When TMB is used as the substrate, the absorbance should be set up at 450 nm.
All wells, including Standard and Samples, are not developing any colour	Insufficient incubation time	Keep accurate timing
	Too low concentration of anylased compound and too extensive washes	Reduce the impact of washing, dilute the concentrated lotion and wash time according to the manual, and accurately record the washing times and dosage.
	Distilled water	Check the pH value which should be neutral
	In the process of plate washing and sample addition, the enzyme marker is contaminated and inactivated, and loses its ability to catalyse the colour developing agent	Confirm that the container holding the ELISA plate does not contain enzyme inhibitors (such as sodium azide etc.), confirm that the container for preparing the Washing Solution has been washed, and confirm that the purified water for preparing the Washing Solution meets the requirements and is not contaminated.
	The kit has expired or has been improperly stored	Do not use the kit beyond expiration date.
	Reagents and samples are not equilibrated before use	All reagents and samples should be equilibrated at room temperature for about 30 minutes
	Insufficient suction volume of the pipette, too fast discharge of pipetting suction, too much liquid hanging on the inner wall of the tip or the inner wall is not clean.	To calibrate the pipette, the tips should be matched, each time the tips should fit tightly, the pipetting should not be too fast, and the discharge should be complete. The inner wall of the tips should be clean, and it is best to use it once.
Poor reproducibility	Varied incubation temperature	Keep the temperature constant to avoid the local temperature being too high or too low

	Wells of the plate not dried completely before adding liquid in the next step of the protocol	Dry the plate completely before each step, immediately apply reagents following the protocol
	Reuse of consumables	Do not re-use tips, bottler or containers
	The bottom of the microwell is scratched or the well is not completely clean	Be careful when operating, be careful not to touch the bottom and wipe the bottom of the microplate to remove dirt or fingerprints.
		Technical repetition of the same sample for 3 times, including more than 2 approximate values.
	Cross-contamination during sample addition	Try to avoid cross-contamination when adding samples
The colour development on the plate is irregular	Cross-contamination from manual plate washing	When washing the plates by hand, the first 3 injections of the lotion should be discarded immediately, use timer to keep washing times
	Cross-contamination when drying the plate	Use a suitable and clean absorbent paper towel when drying the plate and do not dry it on the same spot
	If using automatic washing device, there can be a blockage or other mechanical error contributing to varied volume release into the wells	Check that automatic ELISA plate washer is functioning properly
	Incomplete centrifugation of the sample, resulting in coagulation in the reaction well or interference of sediment or residual cellular components	Always use specified centrifugation conditions for the samples.
	The sample is stored for too long time, resulting in contamination.	Samples should be processed fresh or stored as recommended prevent contamination
	Incorrect preparation of Washing Solution or direct misuse of concentrated Washing Solution	Redo according to the manual