THE ROYAL VETERINARY AND AGRICULTURAL UNIVERSITY KVL
Department of Agricultural Sciences
Agrohydrology & Bioclimatology
Technical Series 1-2000

Laboratory Manual

on

Determination

of

Abscisic Acid

by indirect

Enzyme Linked Immuno Sorbent Assay (ELISA)

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1 The ELISA principle

Today a wide range of immuno assays is available to test for the presence or to determine the amount of a substance in a sample. For a comprehensive description of the the different types of immuno-assays and the principles involved see Kemeny, a practical guide to ELISA (1991). The following brief introduction concentrates on the competitive Enzyme Linked Immuno Sorbent Assay.
The assay described here, follows the principle shown in Diagram 1.

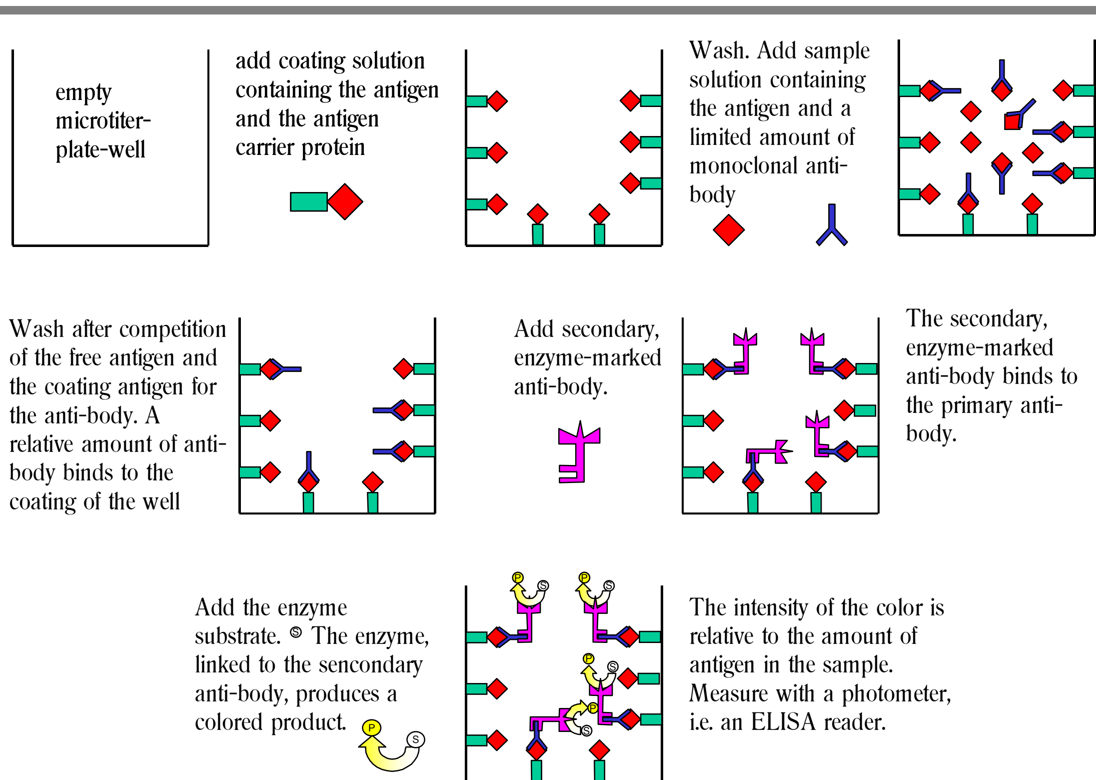


Diagram 1 Schematic presentation of the indirect ELISA principle. Please see explanation in the text.

A new microtiter plate is coated with a solution containing a protein carrier and the antigen. Sample containing the antigen and wall-bound antigen compete for a limited amount of a primary antibody. An amount of antibody relative to the concentration of antigen in the sample binds to the well walls and is marked in the next step with a secondary antibody carrying an enzyme. This enzyme reacts with a substrates and produced a colored product. The intensity of the color reaction is negatively reciprocal to the amount of antigen in the sample. The color intensity is measured with an extinction photometer.

2 Preparation of solutions and chemicals

2.1 Synthesis of ABA-Conjugates

Equipment needed:

centrifuge
freeze drier
glass ware
magnetic stirrer
pH meter
precision balance
TLC – plates and running container
UV lamp
Water bath

2.1.1 Preparation of p- aminohippuracid bound to bovine serum albumine (pAR)

Ingredients used:

p- amminohippuracid	200mg
BSA	200mg
EDC	200mg
H_20	120mL
NaOH	
HCl	
Dialysis tube	

Protocol:
Dissolve BSA and p-amminohippuracid in water. Regulate the solution to pH 8 using 1 molar NaOH. Add 50% of the EDC and regulate the pH to 6.4 using 1 molar HCl. Stir for six hours at room temperature. Add the other 50% EDC and stir again for 14 hours. Discard any precipitate. Dialyze for 24 hours against distilled water, renew the water after 12 hours. Centrifuge the solution and freeze dry the supernatant

2.1.2 Preparation of (+-) Abscisicacid-4'-tyrosolhydrazon (ATH)

Ingredients used:

Abscisic acid	4.5mg
L-Tyrosinehydrazide	15.1mg
Methanol	1.5mL
Acetic acid	0.1mL
Nitrogen gas	

NOTE ! Weigh the round flask before and after to determine the output..

Stir the mixture of ABA, L-tyrosinehydrazide, methanol and acetic acid under nitrogen fumigation at 50°C for 5 days in the dark (Diagram 2). Minimize evaporation and refill solvents when necessary. Refill methanol more often than acetic acid, as it evaporates faster.

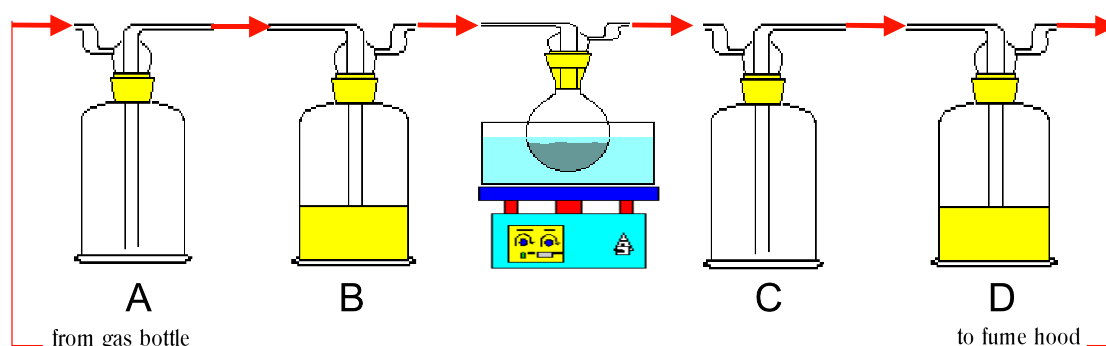


Diagram 2 Experimental set-up for the synthesis of Abscisic acid-4'-tyrosylhydrazon. The reagents are stirred in a 50 ml round flask hanging in a 50° C water bath. nitrogen gas is led through the wash bottles and the round flask to create a oxygen-free reaction environment. Wash bottles B and D are filled with a 95:5 mixture of methanol and acetic acid to minimize evaporation. Wash bottles A and C are security bottles to avoid spill-overs into the gas bottle or the reaction flask.

NOTE ! Often you find two bands which represent two isomers of the product.

Use thin layer chromatography to distinguish the two products (Methanol : Acetic acid 95:5). Use UV-light to identify ATH. The Rf value of ATH is around 0.9. Remove ATH from the plate and dissolve in Methanol. Centrifuge the solution to precipitate the silica gel. Wash the precipitate with methanol and centrifuge again. Repeat this step several times. Combine the methanol supernatants.

TIP The thin layer chromatography may be omitted, if the product is not too contaminated and isomer separation is not desired.

Evaporate the methanol with a rotary evaporator. ATH precipitates as bright yellow, viscous liquid. Store at –20°C.

2.1.3 Azo coupling of pAR

Ingredients used:

p-Amminohippuracid – BSA	29mg
Sodium nitrite	60mg
Ammoniumamidosulfonate	30mg
H2O	6ml
HCl	

NOTE ! Work in an ice bath. In step 2.1.4 make sure the solution stays alkaline pH 8.5-9. If the buffer does not adjust the pH to 9, use a small volume of 1N NaOH to adjust..

Protocol:
Bring 29mg pAR in solution with 5mL H_2O. Regulate to pH 1.5 with 1 N HCl. Dissolve 60mg Sodium nitrite in 0.5mL H_2O and add the solution under constant stirring. Stir for 5 minutes. Dissolve 30mg ammoniumamidosulfonate in 0.5mL H_2O and add to the solution. Stir slowly for 10 min.

2.1.4 Combining the two products

Dissolve 10mg[1] abscisic acid-4-tyrosylhydrazone in 0.5ml methanol and 10ml 0.1 molar borate buffer (pH 9.0). Add through drip feed the solution prepared under 2.1.3 slowly and under constant stirring. Stir slowly for 30 minutes. Dialyze for four days against distilled water at 4°C in the dark, renew the water every 12 hours. Freeze-dry the purified solution.

2.1.5 Stock-solution of ABA-4'-BSA-conjugate

NOTE! This preparation is good for 112 ELISA-plates.

Dissolve 7mg of freeze dried ABA-4'-BSA-conjugate in 1 ml 0.05 molar Sodium hydrogen carbonate solution (pH 9.6). Store in aliquots of 60µL at -20° C as stock solution. Use one aliquot, dilute it again with 140 ml of 0.05 molar sodium hydrogen carbonate solution (see section 2.2.3 for details). For 1 ELISA-plate 20 ml of this solution is needed. Store what is not needed at -20° C.

TIP Before using the conjugate in ELISA tests, check if the concentration of ABA conjugate in the coating solution is sufficient. For that run an ELISA test with different concentrations of conjugate and several dilutions of ABA standards.

[1] If you recover more in step 2.1.2 than the expected output of 100%, this may be due to acetic acid or other residues. Calculate the expected output from the input and adjust the other substances accordingly.

2.2 Preparation of buffers and diluents

Equipment needed:

glass ware
plastic ware
pH meter
magnetic stirrer

2.2.1 Preparation of diluent for secondary antibody (Buffer 1)

Ingredients used:

Tris[hydroxymethyl]aminomethane (TRIZMA)	6.05g
Magnesium chloride hexahydrate	0.2g
Sodium chloride	8.8g
Dist. Water	1000mL
Hydrochloric acid 37%	

Protocol:

Dissolve TRIZMA, MgCl and NaCl in water under constant stirring. Regulate to pH 7.8 with HCl.

2.2.2 Preparation of wash buffer (Buffer 2)

Ingredients used:

NOTE ! Same as Buffer 1

Tris[hydroxymethyl]aminomethane (TRIZMA)	6.05g
Magnesium chloride hexahydrate	0.2g
Sodium chloride	8.8g
Dist. Water	1000mL
Hydrochloric acid 37%	
Bovine serum albumine (BSA)	1g
Tween 20 (Polyoxyethylen sorbit monolaurate	0.5mL

Protocol:

Dissolve TRIZMA, MgCl and NaCl in water under constant stirring. Regulate to pH 7.8 with HCl. Add 1g BSA and 0.5mL Tween 20. Store in 1L bottles at -20°C, warm to room temperature before use.

2.2.3 Phosphate buffer (PBS)

Ingredients used:

Sodium hydrogen phosphate (NaH_2PO_4) solution	50 m<u>M</u>
Di-sodium hydrogen phosphate (Na_2HPO_4) solution	50 m<u>M</u>
Sodium chloride	
Dist. Water	

Protocol:

Prepare 200 ml 0.05 <u>M</u> $NaH_2PO_4 * 2H_2O$ solution: 1.3799 g NaH_2PO_4 fill up to 200 ml with H_2O.

Prepare 50 ml 0.05 M Na_2HPO_4 * $2H_2O$ solution: 0.4450 g Na_2HPO_4 fill up to 50 ml. Adjust the NaH_2PO_4 solution to pH 6 by adding Na_2HPO_4 solution (app. 27-28 ml). Add NaCl to a concentration of 0.1 M; equivalent to 0.5844 g NaCl/100ml solution. Store in the refrigerator up to six months.

2.2.4 Potassium hydroxide solution (optional)[1]

Ingredients used:

Potassium hydroxide (KOH)	28.1 g
Dist. water	100 ml

Protocol:
Dissolve the KOH in the water to prepare a 5 N potassium hydroxide solution.

2.2.5 Sodium hydrogen carbonate solution (pH 9.6)

Ingredients used:

Sodium hydrogen carbonate ($NaHCO_3$)	4.2 g
Dist. water	1000 ml

Protocol:
Dissolve the $NaHCO_3$ in the water to prepare a 0.05 M solution at pH 9.6. Store in 20 ml aliquots at -20° C. Use one aliquot per ELISA-plate.

2.2.6 P-Nitro-phenyl phosphate

Ingredients used:

p- Nitrophenyl phosphate (in tablet form)	20 mg
Sodium hydrogen carbonate solution (pH 9.6)	20 ml

Protocol:
Dissolve the p-nitrophenyl phosphate tablet (20 mg) in 20 ml 0.05 M sodium hydrogen carbonate solution (one aliquot of the solution prepared under 2.2.5).

[1] 5 N KOH was used to stop the color reaction on the plate. Modern readers read the plate so fast, that this is not needed. If you use an older reader the color reaction should be stopped at an absorption value of approximately 1.0

2.3 Antibodies and standard solutions

2.3.1 Primary anti-body (MAC 252)

2.3.1.1 Anti-body buffer

NOTE ! Wear gloves for you own safety.

Ingredients used:

Bovine serum albumin (BSA)	0.5 g
Polyvinylporrolidon (PVP)	0.4 g
PBS buffer	100 ml

Protocol:
Dissolve the BSA and the PVP in the PBS (see section 2.2.3).

2.3.1.2 Dilution and storage of the anti-body

The anti-body is delivered freeze-dried and has to be re-diluted by adding 95 µl H_2O. Give this solution into 10 ml anti-body buffer (see section 2.3.1.1). Rinse thoroughly. Shake thoroughly and freeze immediately in 1 ml aliquots. This stock solution can be stored at -20° C or below for about one year.
For the ELISA the stock solution is diluted further. Dilute 1 ml of the stock solution in 100 ml anti-body buffer and store immediately at -20° C or below in 1ml aliquots for about half a year.
For one ELISA-plate use 1 ml of the second dilution step and dilute again in 11 ml PBS (see section 2.2.3)

2.3.2 Secondary anti-body

The secondary anti-body was produced in goats against rat-albumin and marked with alkaline phosphatase. For one ELISA-plate 20 µl of “Anti-rat IgG [whole molecule] alkaline phosphate conjugate” are diluted in 20 ml Buffer 1 (see section 2.2.1).

2.3.3 Abscisic acid standards

NOTE ! The Antibody has no cross reactivity with (-) ABA.

Weigh out 4 mg (±) ABA. Dissolve the ABA in 100 ml H_2O. Stir for about 12h at 40° C in the dark in a volumetric flask. Refill evaporation losses and stir for a few more minutes. Transfer 2 ml of this solution into a new volumetric 100 ml flask and fill up to the mark (this is the ABA stock solution of 80000 pg (±) ABA /100µl). Store the rest of the original ABA solution at -20° C or below.
Use 5 ml of the ABA stock solution and fill up to 50 ml in a volumetric flask. This solution now holds 8000 pg (±) ABA / 100µl.
Fill 20 ml of this solution into a scintillation vial. Pipette 10 ml from this vial into a second vial add 10ml H_2O, stir thoroughly. Repeat this procedure to prepare (+) ABA standards of 4000, 2000, 1000, 500, 250 and 125 pg / 100 µl. Keep the standards in a refrigerator for no longer than 4 weeks.

3 Indirect ELISA for ABA determination

3.1 Reagents required for one ELISA-plate

Reagents	Quantity	used in step	preparation
ABA conjugate (ABA-4'-BSA)	20 ml	3.3.2	2.1
Wash buffer (buffer 2)	1000 ml	3.3.4-3.3.6	2.2.2
Aqua dest.	300 µl	3.3.4	n.a.
ABA-standards: 125-4000 pg (+) ABA / 100 µl	300 µl	3.3.4	2.3.3
ABA stock solution	300 µl	3.3.4	2.3.3
MAC 252 anti-body solution	1 ml	3.3.4	2.3.1.2
PBS buffer	11 ml	3.3.4	2.2.3
Secondary anti-body (anti-rat IgG)	20 µl	3.3.5	2.3.2
Buffer 1	20 ml	3.3.5	2.2.1
p-nitro phenyl phosphate	20 mg	3.3.6	2.2.6
0.05 M sodium hydrogen carbonate solution	20 ml	3.3.6	2.2.5

3.2 Protocol Overview

Step	Activity	Remark	Time
1	Coating	Prepare the day before	Over night
2	Washing	3x, keep third time for 20 min at 37°C	30 min
3	Loading	Pipette 100 µl standards and samples followed by 100µl primary antibody	About 45 min
4	Incubation	Incubate plates at 4°C	3 hours
5	Washing	3x	10 min
6	Loading	Pipette 200µl of secondary antibody	10 min
7	Incubation	Incubate at 37°C	1 hour
8	Washing	5x	15 min
9	Loading	Pipette 200µl of substrate	10 min
10	Incubation	Incubate at 37°C until Bmax is at 1.0	15-35 min
11	Measurement	Measure the extinction with the reader at 405 nm	5 min
		Total assay-time about	**7 hours**

3.3 Protocol

3.3.1 Equipment

Micro-titer- plates
Water bath or germination oven at 37° C
Blotting paper
Plastic boxes
Refrigerator at 4°C
Multi channel pipette
Micro pipette 5-100 µl
ELISA reader

3.3.2 Coating of the micro-titer-plate

Give in each well of the micro titer plate 200 µl of the ABA-conjugate (ABA-4'-BSA). If not already stored that way, dilute first 60µl of the ABA-conjugate stock solution with 140 ml 0.05 M sodium hydrogen carbonate solution. This solution is sufficient for seven plates.
Incubate the coated plates over night at 4° C.

3.3.3 Sample distribution sheet

Design a sample distribution sheet, or take a copy of the one prepared in the Excel-ELISA calculation file (Table 1). Mark the position and description of each sample on the plate. Make sure the replicate (three per sample) are placed horizontally next to each other. Start with Bmax (maximum binding of the anti-body to the coating antigen), then Bmin (unspecific minimum color reaction) followed by the standards in increasing order of dilution. Use the remaining wells for the samples. Samples should be replicated 3 times horizontally.

sample distribution												
	1	2	3	4	5	6	7	8	9	10	11	12
A	B+	B+	B+									
B	B-	B-	B-									
C	4000	4000	4000									
D	2000	2000	2000									
E	1000	1000	1000									
F	500	500	500									
G	250	250	250									
H	125	125	125									

Table 1: Example of a sample distribution sheet for the ABA-ELISA. Example taken from the Excel calculation spreadsheet for the ABA-ELISA.

3.3.4 Plate loading and addition of primary anti-body

NOTE !
The Antibody may be diluted differently, depending on the activity of the antibody.

The coated micro-titer plate is washed three times with wash buffer 2 using a plastic spray bottle. Keep the third washing solution for 20 min in the wells at 37°C to saturate non-coated parts of the wells with BSA. Hit the micro-titer plate dry on blotting paper. Following the distribution sheet, pipette 100 µl of H_2O for Bmax and 100µl of the ABA stock solution (80000 pg) for Bmin followed by 100 µl of sample or standard into the wells. Dilute 1 ml MAC 252 solution in 11 ml PBS buffer and pipette 100µl of this anti-body solution into each well of the micro-titer plate using a multi-channel pipette. Shake the plate cautiously for 1 min and incubate for three hours at 4° C.

3.3.5 Addition of secondary anti-body

NOTE !
Make sure the temperature (37°C) is accurate at the plate.

Wash the plate three times with wash buffer 2 and hit the plate dry on blotting paper. Dilute 20 µL stock solution of the secondary anti-body in 20 mL buffer 1 and pipette 200 µL of the diluted solution containing the secondary anti-body into each well of the plate using a multi-channel pipette. Incubate the plate for 1 hour at 37° C in a moist plastic box (use wet blotting paper to keep the box moist) in a water bath or a germination oven.

3.3.6 Addition of substrate and extinction measurements

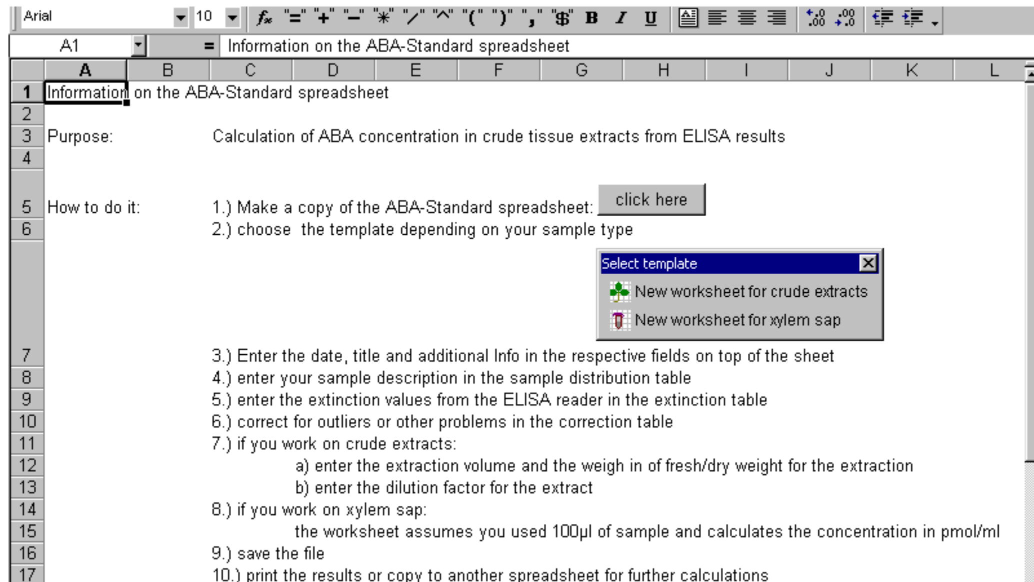
NOTE !
Avoid creating a reaction gradient by too slowly pipetting the substrate.

Wash the plate five times with wash buffer (2) and hit the plate dry on blotting paper. Dilute one tablet (20 mg) of the substrate (p-nitrophenyl phosphate) in 20 ml 0.05 M sodium hydrogen carbonate solution. Pipette 200 µL of this clear solution into each well of the plate. Incubate the plate in a moist plastic box at 37° C in a water bath until the maximal absorption (Bmax) is at 1.0. Measure the entire plate with an ELISA reader at 405 nm.

3.4 Calculation of the results

For the calculation of the results an Excel spreadsheet can be used. The following example explains the calculation based on a spreadsheet developed for this ELISA (see included disk).
Calculations differ slightly between crude extract and xylem sap analyses. Choose the appropriate button.

> ***NOTE!***
> Sometimes, not all the buttons appear. In this case use the Macro command in the Excel menu to create the desired sheet.



A1 = Information on the ABA-Standard spreadsheet

1 Information on the ABA-Standard spreadsheet
3 Purpose: Calculation of ABA concentration in crude tissue extracts from ELISA results
5 How to do it: 1.) Make a copy of the ABA-Standard spreadsheet: click here
6 2.) choose the template depending on your sample type

Select template
New worksheet for crude extracts
New worksheet for xylem sap

7 3.) Enter the date, title and additional Info in the respective fields on top of the sheet
8 4.) enter your sample description in the sample distribution table
9 5.) enter the extinction values from the ELISA reader in the extinction table
10 6.) correct for outliers or other problems in the correction table
11 7.) if you work on crude extracts:
12 a) enter the extraction volume and the weigh in of fresh/dry weight for the extraction
13 b) enter the dilution factor for the extract
14 8.) if you work on xylem sap:
15 the worksheet assumes you used 100µl of sample and calculates the concentration in pmol/ml
16 9.) save the file
17 10.) print the results or copy to another spreadsheet for further calculations

The first table takes the description of the samples assayed and their distribution on the plate. A default standard row is completed automatically, but can be modified. The values entered here will be used later to identify the sample in the spreadsheet calculations.

Calculation sheet for ABA ELISA micro titer plates

Date: | Sample type: ○ Crude extracts ◉ Xylem sap

Title: Enter assay description here

Info: Enter comments, info , remarks here

sample distribution [Extinction entry] [Samples]

	1	2	3	4	5	6	7	8	9	10	11	12
A	B+	B+	B+	S1	S1	S1	S9	S9	S9	S17	S17	S17
B	B-	B-	B-	S2	S2	S2	S10	S10	S10	S18	S18	S18
C	4000	4000	4000	S3	S3	S3	S11	S11	S11	S19	S19	S19
D	2000	2000	2000	S4	S4	S4	S12	S12	S12	S20	S20	S20
E	1000	1000	1000	S5	S5	S5	S13	S13	S13	S21	S21	S21
F	500	500	500	S6	S6	S6	S14	S14	S14	S22	S22	S22
G	250	250	250	S7	S7	S7	S15	S15	S15	S23	S23	S23
H	125	125	125	S8	S8	S8	S16	S16	S16	S24	S24	S24

In the second table the extinction readings from the ELISA reader are entered. This should just be a copy of the results. They should not be altered in any way.

Microsoft Excel - ABA-Standard.xls

File Edit View Insert Format Tools Data Window Help

N23 =

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
13	D	2000	2000	2000	S4	S4	S4	S12	S12	S12	S20	S20	S20	
14	E	1000	1000	1000	S5	S5	S5	S13	S13	S13	S21	S21	S21	
15	F	500	500	500	S6	S6	S6	S14	S14	S14	S22	S22	S22	
16	G	250	250	250	S7	S7	S7	S15	S15	S15	S23	S23	S23	
17	H	125	125	125	S8	S8	S8	S16	S16	S16	S24	S24	S24	
18														
19	Extinction measurements				Corrected readings		Bmax calculation							
20														
21		1	2	3	4	5	6	7	8	9	10	11	12	
22	A	1,241	1,002	1,443	0,384	0,337	0,356	0,304	0,28	0,288	0,304	0,28	0,288	
23	B	0,214	0,179	0,189	0,484	0,48	0,408	0,356	0,334	0,33	0,356	0,334	0,33	
24	C	0,298	0,283	0,285	0,701	0,563	0,523	0,463	0,442	0,459	0,463	0,442	0,459	
25	D	0,34	0,379	0,328	0,653	0,562	0,598	0,56	0,49	0,507	0,56	0,49	0,507	
26	E	0,35	0,378	0,344	0,526	0,567	0,618	0,468	0,498	0,573	0,468	0,498	0,573	
27	F	0,404	0,435	0,422	0,512	0,58	0,529	0,563	0,502	0,517	0,563	0,502	0,517	
28	G	0,552	0,581	0,523	0,38	0,384	0,35	0,346	0,338	0,346	0,346	0,338	0,346	
29	H	0,635	0,644	0,592	0,438	0,415	0,398	0,376	0,393	0,363	0,376	0,393	0,363	
30														
31	corrected readings				Extinction entry		Sample distribution		Bmax calc.					
32														
33		1	2	3	4	5	6	7	8	9	10	11	12	

Info / ABA-zylem (2)

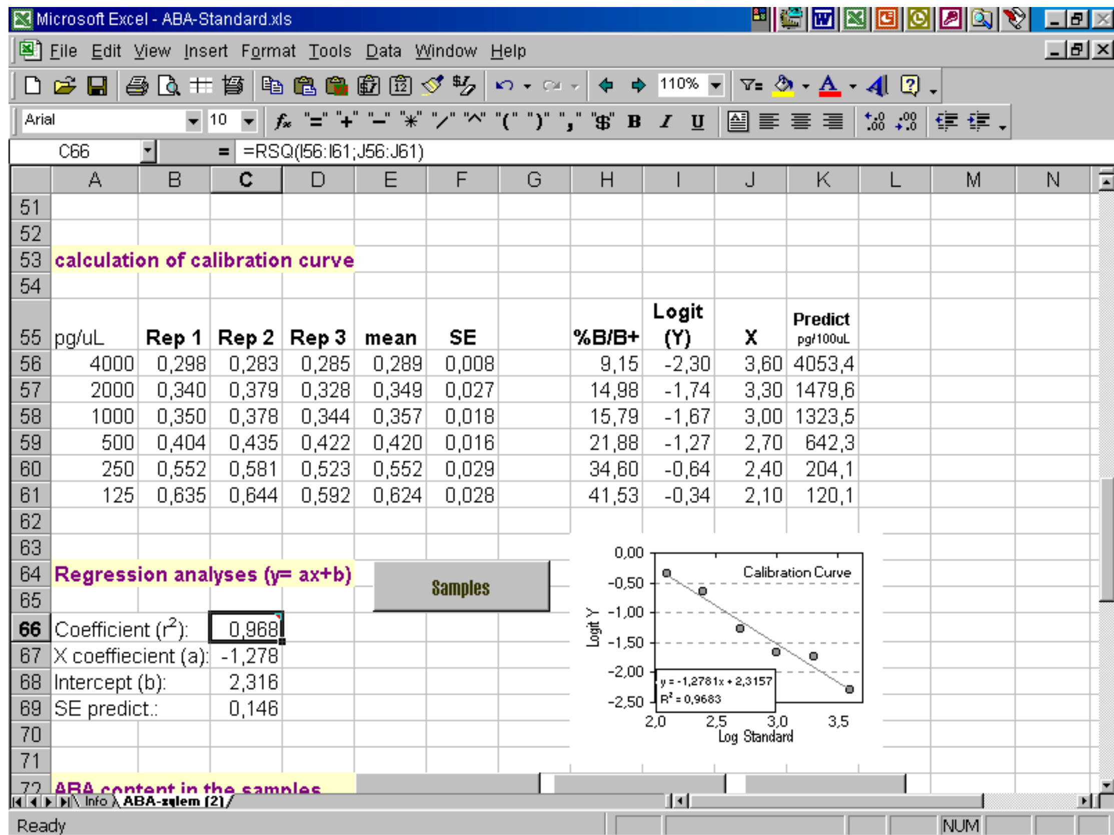
Select destination and press ENTER or choose Paste NUM

The next table allows you to correct for outliers. It is this table that will be used in all further calculations. If the extinction values are outside the range of the standard curve, values will appear on a yellow background. That means they are either to strongly or too weakly concentrated.

	A	B	C	D	E	F	G	H	I	J	K	L	M
30													
31	corrected readings				Extinction entry		Sample distribution		Bmax calc.				
32													
33		1	2	3	4	5	6	7	8	9	10	11	12
34	A	1,241	1,002	1,443	0,384	0,337	0,356	0,304	**0,280**	**0,288**	0,304	**0,280**	**0,288**
35	B	0,214	0,179	0,189	0,484	0,480	0,408	0,356	0,334	0,330	0,356	0,334	0,330
36	C	0,298	0,283	0,285	**0,701**	0,563	0,523	0,463	0,442	0,459	0,463	0,442	0,459
37	D	0,340	0,379	0,328	**0,653**	0,562	0,598	0,560	0,490	0,507	0,560	0,490	0,507
38	E	0,350	0,378	0,344	0,526	0,567	0,618	0,468	0,498	0,573	0,468	0,498	0,573
39	F	0,404	0,435	0,422	0,512	0,580	0,529	0,563	0,502	0,517	0,563	0,502	0,517
40	G	0,552	0,581	0,523	0,380	0,384	0,350	0,346	0,338	0,346	0,346	0,338	0,346
41	H	0,635	0,644	0,592	0,438	0,415	0,398	0,376	0,393	0,363	0,376	0,393	0,363
42													
43													

Based on the corrected readings Bmax (maximum extinction) and Bmin (background noise of the coated plate) are calculated to get Bmax corrected which is Bmax-Bmin. A conversion to percent of Bmax for each value in the standard curve is used to calculated a linear regression line between logit transformed measured values and log transformed assumed concentrations. For details see Quarrie et al. 1988.

Based on the conversions in the last step, the regression coefficient (r^2), the x coefficient (a) and the intercept (b) are calculated. The r^2 should not be as high as possible, preferably not below 0.98.



Microsoft Excel - ABA-Standard.xls

C66 = =RSQ(I56:I61;J56:J61)

calculation of calibration curve

pg/uL	Rep 1	Rep 2	Rep 3	mean	SE	%B/B+	Logit (Y)	X	Predict pg/100uL
4000	0,298	0,283	0,285	0,289	0,008	9,15	-2,30	3,60	4053,4
2000	0,340	0,379	0,328	0,349	0,027	14,98	-1,74	3,30	1479,6
1000	0,350	0,378	0,344	0,357	0,018	15,79	-1,67	3,00	1323,5
500	0,404	0,435	0,422	0,420	0,016	21,88	-1,27	2,70	642,3
250	0,552	0,581	0,523	0,552	0,029	34,60	-0,64	2,40	204,1
125	0,635	0,644	0,592	0,624	0,028	41,53	-0,34	2,10	120,1

Regression analyses (y= ax+b)

Coefficient (r^2):	0,968
X coeffiecient (a):	-1,278
Intercept (b):	2,316
SE predict.:	0,146

The sample mean extinction values are then converted in the same way as the calibration line values and the ABA concentration per sample is calculated using the regression as x=(y-b)/a. After that the concentration is either calculated per g input (either fresh weight or dry weight) in nanogram or pmol per ml xylem sap.

ABA content in the samples — Corrected values — Sample distribution — Regression

Sample	Rep 1	Rep 2	Rep 3	mean	ST Dev	%B/B+	Logit (Y)	log conc.	sample vol	dilution	pmol / ml	SE
S1	0,384	0,337	0,356	0,359	0,024	15,95	-1,662	3,112	100	1	49	10,70
S2	0,484	0,480	0,408	0,457	0,043	25,45	-1,075	2,653	100	1	17	5,71
S3	0,701	0,563	0,523	0,596	0,093	38,82	-0,455	2,168	100	1	6	2,53
S4	0,653	0,562	0,598	0,604	0,046	39,66	-0,420	2,140	100	1	5	1,23
S5	0,526	0,567	0,618	0,570	0,046	36,37	-0,559	2,249	100	1	7	1,68
S6	0,512	0,580	0,529	0,540	0,035	33,47	-0,687	2,349	100	1	8	1,58
S7	0,380	0,384	0,350	0,371	0,019	17,14	-1,576	3,045	100	1	42	7,50
S8	0,438	0,415	0,398	0,417	0,020	21,55	-1,292	2,823	100	1	25	3,67
S9	0,304	0,280	0,288	0,291	0,012	9,34	-2,272	3,590	100	1	147	25,49
S10	0,356	0,334	0,330	0,340	0,014	14,11	-1,806	3,225	100	1	64	8,51
S11	0,463	0,442	0,459	0,455	0,011	25,19	-1,088	2,663	100	1	17	1,32
S12	0,560	0,490	0,507	0,519	0,037	31,41	-0,781	2,423	100	1	10	1,98
S13	0,468	0,498	0,573	0,513	0,054	30,83	-0,808	2,444	100	1	11	3,15
S14	0,563	0,502	0,517	0,527	0,032	32,22	-0,744	2,394	100	1	9	1,60
S15	0,346	0,338	0,346	0,343	0,005	14,43	-1,780	3,204	100	1	61	2,87
S16	0,376	0,393	0,363	0,377	0,015	17,72	-1,535	3,013	100	1	39	4,95

Info | ABA-xylem (2)

3.5 Cross reaction test

To verify the validity of the assay result, in many cases, particularly for crude extracts of flowers, seed, fruits or leaves it is imperative to test if the extract contains compounds which react with the antibody other than the antigen. If this is the case further purification of the extract will become necessary. Any reaction of the antibody other than with the antigen is termed cross reaction and should be tested for as follows:

Conduct a normal ELISA as described in section 3.3. Load the plate with a normal standard row, take 50µl of each standard and add 50µl of dist. H_2O in the plate rows 4-6, and take 50µl of each standard and add 50µl of a sample (preferably with a known ABA concentration) in plate rows 7-9. Follow the remaining protocol.
Calculate the results for both rows as described in section 3.4 and plot the data against the known ABA in-put as shown in Figure 1.

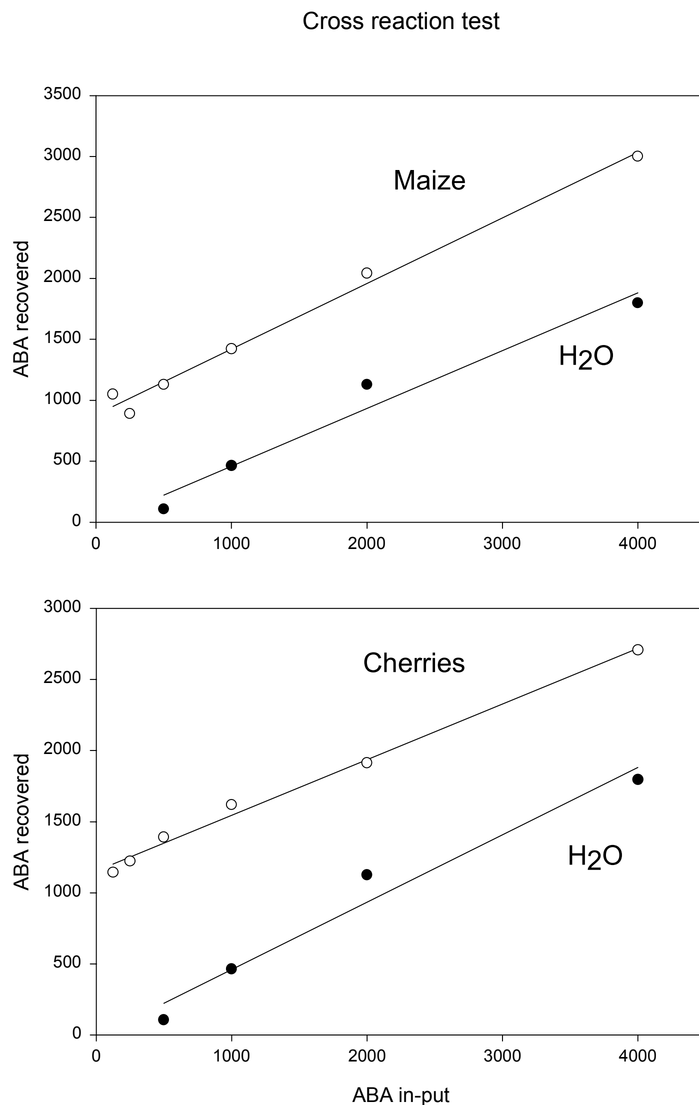


Figure 1 Cross reaction test evaluation on *Zea mays* leaves and cherry fruits. In these examples no cross reaction was detected. ABA values are shown in pg / g dry weight

4 Plant sample extraction methods

4.1 Xylem sap sampling

There are several ways to obtain xylem saps from plants, two of them are described in the following paragraphs.

4.1.1 <u>Xylem sap from field-grown plants</u>

Xylem sap from field grown plants can be obtained by cutting the plant about 10 to 15 cm above the ground (preferably early in the morning, to fully utilize the root pressure). The stump should be rinsed with pure water and dried with plotting paper. A piece of silicon tube is then placed on the stump in a way that the epidermis is not damaged and no leaking occurs (make sure to have several diameters available). Xylem sap collects in the silicon tube through root pressure. If there is risk of too much exposure to light, the tube should be wrapped in aluminum foil. Depending on the plant and the treatment, about 0.5mL should be obtained within 1-2 hours. If the plants have experienced a heavy drought stress, more plants should be cut and the sap combined. The sap is collected from the silicon tube into an Eppendorf-vial, using a pipette, immediately frozen and stored for analysis at -80° C. This method has been used successfully on wheat, oil seed rape, maize and rice.

4.1.2 <u>Xylem sap from potted plants</u>

Xylem sap from potted plants can be collected by the procedure described above under fully irrigated conditions and when no stress is present that significantly reduces the water potential of the plants. Xylem sap of droughted or salt stressed plants can be obtained by pressurizing the potted plant in a Scholander type pressure chamber. The entire pot is sealed into the pressure chamber with branches or tillers protruding the chamber top. Branches or tillers are cut as close to the root system as possible. Pressure is applied until the root water potential is equalized. Use a magnifying glass to observe the xylem vessels and read the pressure gauge when sap droplets form at the cut surface of the xylem vessels. For short time sap sampling (1-4 minutes) increase the pressure to exceed the water potential by about 20% and collect the sap with a micro-syringe into ice-cold Eppendorf-vials. Store the sap immediately at -80 °C for further analysis. For sampling times exceeding 4 minutes the technique can be modified by fixing a silicon tube onto the cut surface which is connected to the capillary top of an Eppendorf glass pipette. A small diameter silicon tube collects the sap through the capillary into an Eppendorf-vial. If this technique is used it should be tested for how long the sap can be sampled without changes in the composition of the xylem sap. The vial should be kept cold and dark.

4.2 Crude extracts

Crude extracts of maize leaves, young maize cobs, and rice leaves have been tested to date with the extraction method describe below.
Weigh out 0.1 g of freeze dried, finely ground material into a centrifuge tube containing 4 ml of H_2O or phosphate buffer (0.02 molar, pH 7.3). Shake the

samples overnight in the cold (4-5 °C) and dark. Spin down the solids and use the supernatant directly or diluted with buffer or H_2O in the ELISA. For materials other than the ones mentioned above, the validity of this extraction method should be tested by both, cross-reaction test and confirming measurements with a HPLC - GC set-up.

5 Acknowledgements and Literature

5.1 Acknowledgements

I would like to thank Lene Korsholm for sharing her experience with me and helping me to establish the ELISA here in Taastrup. Without the patient explanations of Holger Krannich, we would still spin the conjugate down and throw it away. Helga Dörffling and Prof Dr. Karl Dörffling helped understanding the protocol, cross-checking the conjugate and provided more help than anybody could possibly ask for. Finally, I would like to thank Dr. Hauke Hansen for coming up with the ELISA idea in the first place.

5.2 Literature

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6 Annex

6.1 Alphabetical list of chemicals for the ABA-ELISA

Ingredient	Purity	Supplier	Catalogue No	Unit volume
(+-) Abscisic Acid	p.p.p.a.	Fluka	00015	0,100 g
Acetic acid	99-100%	J. T. Baker, Holland	6052	1000 mL
Albumin, Bovine	98%	Sigma	A-3803	50 g
Ammoniumamidosulfonat	p.a.	Merck / Kebo	1.01220.0100 / 15505-100	100 g
ANTI-RAT IgG (whole molecule)		Sigma	A-9654	1 ml
Borate buffer pH 9.00		Merck / Kebo	1.09461.1000 / 18479-1	1000 mL
di-Sodium hydrogen phosphate	p.a.	Merck / Kebo	1.06586.0500 / 1.4561-500	500 g
di-Sodium hydrogen phosphate di-hydrate	LiChropur	Merck / Kebo	1.19753.0250 / 77490-250	250 g
Hydrochloric acid	37%	J. T. Baker, Holland	6081	1000 mL
L-Tyrozine hydrazide	purum	Fluka Chemie AG	93915-5G	5 g
MAC 252		Quarrie		0.100 ml
Magnesium chloride hexahydrate	p.a.	Merck / Kebo	1.05833.0250 / 1.5833-250	250 g
Methanol	p.a.	Merck / Kebo	1.06009.2500 / 52542-25	2500 mL
N-Ethyl-N'(3-dimethyl amino propyl)-carbodiimid hydrochloride		Sigma-Aldrich	E-6383	5 g
Nitrogen		Hede Nielsen		
p-Aminohippur acid	99%	Merck / Kebo	1.00084.0025 / 71292-25	25 g
p-Nitrophenyl Phosphate Disodium (pNPP)		Sigma	N-2765	100 tabs
Polyvinyl pyrrolidon (PVP-40)		Sigma Chemicals		100 g
Potassium chloride	p.a.	Merck / Kebo	1.04933.0500 / 1.4933-500	500 g
Potassium dihydrogene phosphate	p.a.	Merck / Kebo	1.04873.0250 / 1.4873-250	250 g
Potassium hydroxide	p.a.	Merck / Kebo	1.05033.1000 / 1.2402-1	1000 g
Sodium chloride	p.p.a.	Fluka	71381	1000 g
Sodium dihydrogen phosphate monohydrate	p.a.	Merck / Kebo	1.06346.1000 / 1.7157-1	1000 g
Sodium hydrogen carbonate	p.a.	Merck / Kebo	1.06329.1000 / 1.6329-1	1000 g
Sodium nitrit	p.a.	Merck / Kebo	1.06549.0500 /52379-500	500 g
Tris[hydroxymethyl]aminomethane	p.a.	Sigma-Aldrich,	T-1503	500 g
Tween 20 (Polyoxyethylen sorbit monolaurat)	pure	Merck / Kebo	8.22184.1000 / 1.5797-1	1000 mL

6.2 List of equipment

6.2.1 ABA-conjugates

Centrifuge
Freeze drier
Fume hood
Glass ware
Magnetic stirrer
pH meter
precision balance
Refrigerator
Rotation evaporator
TLC-plates and running container
UV-lamp
Water bath

6.2.2 ABA-ELISA

Blotting paper
Computer
ELISA reader (Anthos 2000)
Eppendorf vials
Freezer (-20°C)
Micro-pipette (5-100µL)
Micro-pipette tips
Micro-titer plates (Nunc Immuno plate, MaxiSorp F96)
Multi channel pipette (8)
Plastic boxes
Refrigerator
Scintillation vials
Wash bottles
Water bath