Enzyme Analysis by Spectrophotometry - Plate Reader method

Enzyme Extraction

Equipment and reagents:

Machine/Product	**Reference (Company, Type, …)**
Centrifuge – cooled	Eppendorf 5415R with F45-24-11 rotor
Mixer Mill / Cryo Mill	Retsch MM 400 with 2x PTFE Adapter rack for 10 reaction vials 1.5 and 2.0 ml.
Microtube Vortex	Ika Vortex 1
Plate reader	BioTek PowerWave HT with Gen 5 software
Microcentrifuge tubes	Safe-lock, 1.5 and 2.0 ml
Pipettes (+Multistep / Multichannel)	1-5 ml; 0.1-1 ml; 5-100 µl
8-tube strip PCR tubes	
Crushed Ice	
Liquid Nitrogen (+ thermos jar)	
PVP (Polyvinylpyrrolidone, PVP40)	 Sigma PVP40T
TRIS (Tris(hydroxymetyl)-aminoethane) 99 %, 121.14 g.mol^{-1}	 Sigma T1378
Na_2-EDTA (disodium salt dehydrate) 99 %; 372.2 g.mol^{-1}	 Sigma ED2SS
DTT (1,4-Dithiothreitol) ≥ 99 %; 154.24 g.mol^{-1}	 Sigma 43815
Hydrochloric Acid concentrated HCl 37 %; 1,19 g.ml^{-1}; 36.46 g.mol^{-1} = 12.08 mol.l^{-1}	 VWR 20252.290

- Most buffers can be prepared in advance and kept at 4°C
- Requirement: the samples must be collected in 1.5 or 2.0 ml Eppendorf tubes at harvest.

Prepare reagent working solutions:

- **Extraction buffer** (0.1 M TRIS ; 1 mM Na_2-EDTA; 1 mM DTT, pH 7.8) Dissolve 12.114 g TRIS (121.14 g/mol) + 0.3722 mg EDTA (372 g/mol) + 0.1542 g DTT (154.2 g/mol) in 900 ml distilled water, adjust to pH 7.8 with HCl (use 5M HCl) and dilute to 1 liter. Keep the extraction buffer on ice.

Extraction protocol

- Transfer the plant samples (±100 mg) from the low temperature freezer (-80 °C) to the liquid nitrogen. Use expanded polystyrene floats to keep them organized.
- Pre-cool Eppendorf adaptors of the mixer mill in liquid nitrogen. The adaptors are cool when they don't "bubble" anymore.
- Add two stainless steel beads and a spatula tip of polyvinylpyrrolidone (PVP) to each sample tube.
- Place in Mixer mill adapters, and shred for 3,5 minutes at 30Herz. You can shred 10 samples at once (5 per block, see figure). If more tubes are placed, the risk exists that the caps of the outer tubes will snap.

- Tap down the sample powder and add 1.5 ml extraction buffer; vortex immediately.
- Remove the steel beads from the test tubes with a magnet (slide it up on the outside); vortex again.
- Centrifuge at 13000 rpm for 10 minutes at 4 °C
- Carefully transfer 0.5 ml of the supernatant extract solution to a pre-cooled 1.5 ml sample tube with 0.5 ml extraction buffer, vortex, and keep on ice for the duration of the measurements.
- Note: Transfer ~150 µl extract in a 8x tube PCR-strip for easy dispensing with the automatic pipette.
- Note: Make a technical replicate for each measurement.

CAT (Catalase; EC 1.11.1.6)

Equipment and reagents:

Machine/Product	**Reference (Company, Type, …)**
Plastic UV -96-well plates	Greiner Bio One Cat. No.655 801
Crushed Ice	
Potasium Phosphate, monobasic KH_2PO_4 136.09 g.mol^{-1}	 Fluka 60221
Potasium Hydroxide KOH p.a	 Baker 0222
Hydrogen Peroxide H_2O_2 30 % 1110 g.l^{-1}, 34.01 g.mol^{-1}	 Sigma H1009

Prepare reagent working solutions:

- **Phosphate Buffer:** Dissolve dilute 3.403 g KH_2PO_4 in water; adjust to pH 7.0 with KOH; bring to 250 ml Keep on ice. (This is the same buffer used for GPOD)
- **CAT H2O2 Buffer (prepare freshly)**: dilute 25 µl 35% H_2O_2 in 5 ml phosphate buffer.

Measurement protocol

In a 96-well **UV-plate** Wavelength: λ = 240 nm

CAT H2O2 Solution:	190 µl
Sample Extract:	10 µl

Shake for ~15 seconds

Calculation:

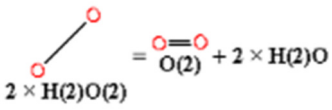
$$Enzyme\ \ capacity\ \left(U/g\right) = \frac{\Delta A}{\Delta t}\ \frac{V_c V_b}{\varepsilon\ d\ V_e\ m}$$

*ε = millimolar extinction coefficient (l*mmol^{-1}*cm^{-1}). For the catalase measurement the specific extinction coefficient is ε= 40 mM-1*cm-1*

d = light path (cm)= 0,56

V_b *= extraction buffer volume (ml)*= 3 V_c *= reaction volume (ml) in well*= 0,2

V_e *= extract volume (ml) in well*= 0,010 *m = fresh weight (g) of extracted sample*



Reaction of catalase:

GPOD (Guaiacol peroxidase; EC 1.11.1.7)

Equipment and reagents:

Machine/Product	**Reference (Company, Type, …)**
Plastic 96-well plates	Greiner Bio One Cat. No.655 101
Crushed Ice	
Potasium Phosphate, monobasic KH_2PO_4 136.09 $g.mol^{-1}$	 Fluka 60221
Potasium Hydroxide KOH p.a	 Baker 0222
Hydrogen Peroxide H_2O_2 30 % 1110 $g.l^{-1}$, 34.01 $g.mol^{-1}$	 Sigma H1009
Guaiacol 124.14 $g.mol^{-1}$	 Sigma G5502

Prepare reagent working solutions:

- **Phosphate Buffer 0.1M:** Dissolve dilute 3.403 g KH_2PO_4 in water; adjust to pH 7.0 with KOH; bring to 250 ml. (This is the same buffer used for CAT)
- **H2O2 (prepare freshly) 8mM**: dilute 50 µl 30% H_2O_2 in 3 ml H_2O.
- **Guajacol 90 mM**: 50µl stock solution in 5 ml H_2O. Freeze in 5 ml aliquots.
- **Prepare the Guajacol-H_2O_2 Master Mix:** Mix on a 1:1 ratio. Keep on ice.

Measurement protocol

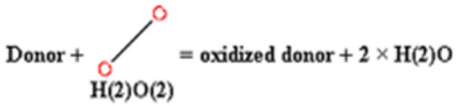
In a 96-well plate Wavelength: λ = 436 nm

Phosphate Buffer	150 µl
Sample Extract:	10 µl
Guajacol MasterMix:	40 µl

Calculation:

$$Enzyme\ \ capacity\ \left(U/g\right) = \frac{\Delta A}{\Delta t}\ \frac{V_c V_b}{\varepsilon\ d\ V_e\ m}$$

*ε = millimolar extinction coefficient (l*mmol^{-1}*cm^{-1}). For tetra-guaiacol at 436 nm, the specific extinction coefficient is ε= 25.5 mM-1*cm-1*
d = light path (cm)= 0,56
V_b = extraction buffer volume (ml)= 3
V_c = reaction volume (ml) in well= 0,2
V_e = extract volume (ml) in well= 0,010
m = fresh weight (g) of extracted sample



General reaction of Peroxidase where in this case the donor consists of 4 Guaiacol and is oxidized to tetraguaiacol

The appearance of tetraguajacol is measured spectrophotometrically at 436 nm.

SPOD (Syringaldazine peroxidase; EC 1.11.1.7)

Equipment and reagents:

Machine/Product	**Reference (Company, Type, …)**
Plastic UV -96-well plates	Greiner Bio One Cat. No.655 801
Crushed Ice	
TRIS (Tris(hydroxymetyl)-aminoethane) 99 %, 121.14 g.mol^{-1}	 Sigma T1378
Hydrochloric Acid concentrated HCl 37 %; 1,19 g.ml^{-1}; 36.46 g.mol^{-1} = 12.08 mol.l^{-1}	 VWR 20252.290
SAZ Syringaldazine 99 %, 360.36 g.mol^{-1}	 Sigma S7896
Methanol 99,8 %; 0,79 g.ml^{-1}; 32.04 g.mol^{-1}	 Merck 1.06009
Dioxane 1,4-dioxaan	 Sigma 443557
Hydrogen Peroxide H_2O_2 30 % 1110 g.l^{-1}, 34.01 g.mol^{-1}	 Sigma H1009

Prepare reagent working solutions:

- **TRIS buffer 0.1M, pH 7.5:** Dissolve 3.0285 g TRIS in water; adjust to pH 7.5 with HCl 5M; bring to 250 ml
- **Syringaldazine (SAZ):** Dissolve 3.6 mg SAZ in 1 ml methanol . Add 2 ml dioxane. Mix well and freeze in small quantities (1000 µl) in Eppendorf tubes. Keep on ice.
- **H_2O_2 10 mM:** Dilute 50 µl mg H_2O_2 in 5 ml H_2O. Prepare freshly. Keep on ice.

Measurement protocol

In a 96-well **UV-plate** Wavelength: λ = 530 nm

TRIS Buffer	155 µl
H2O2 10 mM	20 µl
Plant Extract	20 µl
SAZ	5 µl

Add extract first, then the SAZ substrate to prevent precipitation.

Calculation

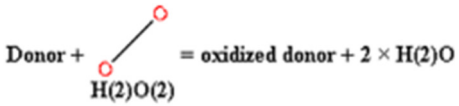
$$Enzyme\ \ capacity\ \left(U/g\right) = \frac{\Delta A}{\Delta t}\ \frac{V_c V_b}{\varepsilon\ d\ V_e\ m}$$

*ε = millimolar extinction coefficient (l*mmol^{-1}*cm^{-1}). For oxidized syringaldazine at 530 nm, the specific extinction coefficient is ε= 11.6 mM-1*cm-1*

d = light path (cm)= 0,56

V_b = extraction buffer volume (ml)= 3 *V_c = reaction volume (ml) in well*= 0,2

V_e = extract volume (ml) in well= 0,020 *m = fresh weight (g) of extracted sample*



General reaction of Peroxidase where in this case the donor consists of Syringaldazine and is oxidized to oxidized syringaldazine

The appearance of oxidized syringaldazine is measured spectrophotometrically at 530 nm.

APOD (Ascorbate peroxidase; EC 1.11.1.11)

Equipment and reagents:

Machine/Product	**Reference (Company, Type, …)**
Plastic UV -96-well plates	*Greiner Bio One Cat. No.655 801*
Crushed Ice	
Hydrogen Peroxide H_2O_2 30 % 1110 g.l-1, 34.01 g.mol-1	 Sigma H1009

Prepare reagent working solutions:

- **HEPES-EDTA buffer at pH 7.0 (HEPES 0.1 M, EDTA 1 mM)** Dissolve 5.958 g HEPES [*Sigma H3375*] and 0.093 g Na2-EDTA [*Sigma ED2SS*] in water; adjust to pH 7.0 with KOH; bring to 250 ml.
- **Na Ascorbate 30 mM** Dissolve 0.297 g Na Ascorbate [*Sigma A7631*] in 50 ml water. Prepare FRESH regularly and KEEP IN A DARK BOTTLE.
- **H_2O_2 20 mM** Dilute 200 µl mg H_2O_2 [*Sigma H1009*] in 10 ml. (~160 measurements) Prepare FRESH daily.
- **Make Ascorbate-H2O2 MasterMix.** Mix 350 µl H2O2 solution with 1 ml Ascorbate solution. Keep in the DARK. Keep on ice.

Measurement protocol

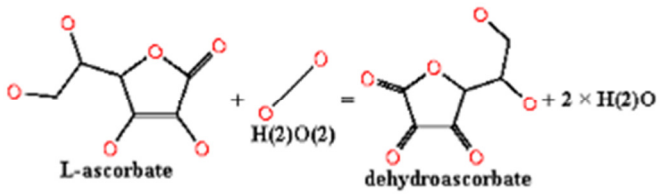
In a 96-well **UV-plate** Wavelength: λ = 298 nm

HEPES-EDTA (pH 7.0)	155 µl
Plant Extract	18 µl
Ascorbate MasterMix	27 µl

Calculation

$$Enzyme\ \ capacity\ \left(U/g\right) = \frac{\Delta A}{\Delta t}\ \frac{V_c V_b}{\varepsilon\ d\ V_e\ m}$$

*ε = millimolar extinction coefficient (l*mmol^{-1}*cm^{-1}). For dehydroascorbate at 298 nm, the specific extinction coefficient is ε= 11.6 mM-1*cm-1*
d = light path (cm)= 0,56
V_b = extraction buffer volume (ml)= 3
V_c = reaction volume (ml) in well= 0,2
V_e = extract volume (ml) in well= 0,010
m = fresh weight (g) of extracted sample



Reaction between L-ascorbate and H_2O_2 to form dehydroascorbate and H_2O

The appearance of dehydroascorbate is measured spectrophotometrically at 298 nm.

SOD (Superoxide Dismutase; EC 1.15.1.1)

Machine/Product	**Reference (Company, Type, …)**
Plastic 96-well plates	Greiner Bio One Cat. No.655 101
Crushed Ice	
Potasium Phosphate, monobasic KH_2PO_4 136.09 $g.mol^{-1}$	 Fluka 60221
Potasium Hydroxide KOH p.a	 Baker 0222
Xanthine 2,6-Dihydroxypurine 99 %, 152.11 $g.mol^{-1}$	 Sigma X7375
XOD Xanthine Oxidase from bovine milk	 Sigma X4500
Na_2-EDTA (disodium salt dehydrate) 99 %; 372.2 $g.mol^{-1}$	 Sigma ED2SS
Cytochrome C Cytochrome C from horse heart ≥ 95 %	 Sigma C2506

Prepare reagent working solutions:

- **KH_2PO_4 buffer (50 mM) at pH 7.8:** Dissolve dilute 3.402 g KH_2PO_4 in water; adjust to pH 7.8 with KOH; bring to 500 ml.
- **Xanthine 0.5 mM in Phosphate buffer (pH 7.8)** Dissolve 1.9 mg Xanthine in 25 ml Phosphate buffer. Boil to dissolve. Adjust the volume and freeze in 5 ml aliquots.
- **XOD in Phosphate buffer (pH 7.8)** Dilute 25 µl mg XOD in 500 µl Phosphate buffer.
- **Na_2-EDTA 1 mM in Phosphate buffer (pH 7.8)** Dissolve 37.6 mg Na2-EDTA in 100 ml Phosphate buffer (pH 7.8)
- **Cytochrome C 0.1mM in Phosphate buffer (pH 7.8)** Dissolve 18.576 mg Cytochrome C in 15 ml ml Phosphate buffer. Freeze in 5 ml aliquots.
- **SOD MasterMix:** Mix equal parts (650+650+650µl) of EDTA, Xanthine and Cytochrome C to make the SOD mastermix

Measurement protocol

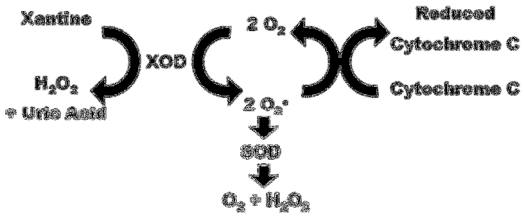
In a 96-well plate Wavelength: λ = 550 nm

SOD measurement relies on an inhibition reaction, blanks must be measured first.
Measure 4 wells with blanks separately first, then measure your 8 samples in the next separate run.

	Blank	Sample
Phosphate buffer (pH 7.8)	135 µl	130µl
SOD MasterMix	60 µl	60 µl
Plant Extract	-	5 µl
XOD	5 µl	5 µl

Calculation

Reaction: 2 superoxide + 2 H(+)➔ $O_2 + H_2O_2$



Superoxide dismutase catalyzes the conversion of radicals ($O_2°$) into di-oxygen and hydrogen peroxide. The superoxide-generating system consists of xanthine oxidase and xanthine. Addition of the plant extract results in a disproportionation of the superoxide radicals and hence an inhibition of the reduction of cytochrome c, which is a measure to define SOD capacity.

In the blank cuvette, cytochrome c will be reduced by the formed superoxide radicals and this reaction is followed at 550 nm

$$\%inhibition = \frac{\left(\frac{\Delta A}{\Delta t}\right)_{REF} - \left(\frac{\Delta A}{\Delta t}\right)_{SAMPLE}}{\left(\frac{\Delta A}{\Delta t}\right)_{REF}} \times 100\ \%$$

This is the equation needed to calculate the relative inhibition (%) of cytochrome C reduction, where ($\Delta A/\Delta t$) = the slope of the enzyme-induced absorption change (min^{-1}) IMPORTANT: the slope is given per minute!!

SOD UNIT DEFINITION:

$$z(U) = \frac{\%\ inhibition}{50\%}$$

One unit will inhibit the rate of reduction of cytochrome C by 50 % in a coupled system, using xanthine and xanthine oxidase at pH 7.8 at 25 °C.

In relation with the extract volume, the extraction buffer volume, and the weight of the extracted sample:

$$SOD\ capacity\left(\frac{U}{g}\right) = z(U) \times \frac{V_b}{V_e\ m}$$

z(U) = is calculated above
V_b *= extraction buffer volume (ml)*= 3
V_e *= extract volume (ml) in well*= 0,005
m = fresh weight (g) of extracted sample

GR (Glutathione Reductase; EC 1.8.1.7)

Machine/Product	**Reference (Company, Type, …)**
Plastic UV -96-well plates	Greiner Bio One Cat. No.655 801
Crushed Ice	
TRIS (Tris(hydroxymetyl)-aminoethane) 99 %, 121.14 $g.mol^{-1}$	 Sigma T1378
Na_2-EDTA (disodium salt dehydrate) 99 %; 372.2 $g.mol^{-1}$	 Sigma ED2SS
Hydrochloric Acid concentrated HCl 37 %; 1,19 $g.ml^{-1}$; 36.46 $g.mol^{-1}$ = 12.08 $mol.l^{-1}$	 VWR 20252.290
GSSG (L-glutathione, oxidized) ~98 %; 612.63 $g.mol^{-1}$	 Sigma G4376
NADPH >98% (reduced form) tetrasodium salt	 Apollo Scientific BIB3014

Prepare reagent working solutions:

- **TRIS-EDTA buffer at pH 8 (TRIS 0.1 M, Na_2-EDTA 1 mM)** Dissolve 3.059 g TRIS and 93 mg Na_2-EDTA in water; adjust to pH 8 with HCl; bring to 250 ml.
- **GSSG 82 mM** Dissolve 25 mg GSSG in 500 µl water. Freeze per 1 ml in Eppendorf tubes. Keep on ice.
- **NADPH 6 mM** Dissolve 2.5 mg NADPH in 500 µl water. Deep-freeze per 1 ml in Eppendorf tubes. Keep on ice.
- **GR Mastermix:** Mix GSSG and NADPH on a 1:1 ratio. Keep on ice.

Measurement protocol

In a 96-well **UV-plate** Adjust wavelength to λ= 340 nm

TRIS Buffer (pH=8)	165 µl
GR MasterMix	7 µl
Plant Extract	28 µl

Calculation

Analysis of GR capacity is based on the reduction of GSSG (glutathione disulfide), using NADPH. In this reaction the decrease of NADPH is followed at 340 nm

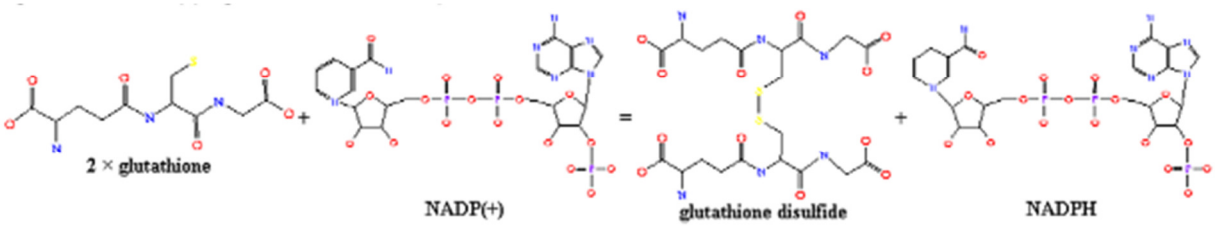
$$Enzyme\ \ capacity\ \left(U/g\right) = \frac{\Delta A}{\Delta t}\ \frac{V_c V_b}{\varepsilon\ d\ V_e\ m}$$

ε = millimolar extinction coefficient ($l*mmol^{-1}*cm^{-1}$). For for ß-NADPH at 340 nm, the specific extinction coefficient is **ε**= *6.22 mM-1*cm-1*

d = light path (cm)= 0,56

V_b = *extraction buffer volume (ml)*= 3 V_c = *reaction volume (ml) in well*= 0,2

V_e = *extract volume (ml) in well*= 0,028 *m = fresh weight (g) of extracted sample*



Reaction between glutathione disulfide and NADPH to form 2 glutathione and NADP(+)