Metabolites Measurements with the plate reader

adapted from Paris-Sud: Guillaume Queval, Graham Noctor 2007

This protocol allows you to measure 8 samples together. If Ascorbate is not measured, you can do 16 samples in < 4 hours

Equipment and Reagents

Machine/Product	Reference
Crushed Ice	
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
Plastic UV -96-well plates	Greiner Bio One Cat. No.655 801
Liquid Nitrogen (+ thermos jar)	
2-VP 2-Vinylpyridine 97%	 Aldrich 132292
AO (Ascorbate oxidase from Curcubita) (1000U)	 Sigma A0157-1KU
DMSO Dimethylsulfoxide	 sigma D5879
DTNB 5.5-dithiobis(2-nitro-benzoic acid) ≥ 98%; 396.35 $g.mol^{-1}$	 Sigma D8130
DTT (1,4-Dithiothreitol) ≥ 99 %; 154.24 $g.mol^{-1}$	 Sigma 43815
G6P D-glucose 6-phosphate sodium salt	 Sigma D7879
GR Glutathione reductase from Baker's Yeast	 Sigma G3664
GSH (L-gluthathione, reduced) >98%; 307.3 $g.mol^{-1}$	 Sigma G4251
GSSG (L-glutathione, oxidized) ~98 %; 612.63 $g.mol^{-1}$	 Sigma G4376
HEPES HEPES free acid; 238.31 $g.mol^{-1}$	 Sigma H3375
Hydrochloric Acid concentrated HCl 37 %; 1,19 $g.ml^{-1}$; 36.46 $g.mol^{-1}$ = 12.08 $mol.l^{-1}$	 VWR 20252.290
Na_2-EDTA (disodium salt dehydrate) 99 %; 372.2 $g.mol^{-1}$	 Sigma ED2SS
NADPH >98% (reduced form) tetrasodium salt	 Apollo Scientific BIB3014
Sodium Hydroxide NaOH pellets	 Vel 1735

Sodium Phosphate, monobasic NaH_2PO_4 137,99 $g.mol^{-1}$	 VWR 567545

Prepare Working Reagent Solutions

2-VP: 2-vinyl-pyridine

-  always work in the flow hood and with gloves
- Store at -20°C in 1 ml aliquots

AO: 40 U/ml Ascorbate oxidase

- Prepare a 10X stock solution, use complete bottle (400 U/ml 200 mM NaH_2PO_4 (pH5.6))
- Divide in 280 µl aliquots
- Dilute 10x in 2520 µl 200mM NaH_2PO_4 (pH 5.6)
- Divide in 250µl aliquots (40U/ml 200mM NaH_2PO_4 (pH 5.6))
- Store at -20°C in aliquots: don't forget to order new enzymes when almost finished

GR: 20 U/ml Glutathione reductase

- Centrifugate the complete content of the bottle during 5 min 14000 rpm at 4°C
- Remove the supernatant
- Resuspend the pellet in the correct volume of 200 mM NaH_2PO_4-EDTA (pH 7.5) for 20U/ml
- Divide in 820 µl aliquots
- Store at -20°C in aliquots: don't forget to order new enzymes when almost finished

Following products can be stored for about **~2 months**:

Solution	Weight	Product	in	pH	with	Store
5 M HCl	41.46 ml	HCl 37.5%	100 ml dH_2O	-		4°C
1 M HCl	8.29 ml	HCl 37.5%	100 ml dH_2O	-		4°C
200mM HCl	8.29 ml	HCl 37.5%	500 ml dH_2O	-		4°C
5 M NaOH	20 g	NaOH	100 ml dH_2O	-		4°C
1 M NaOH	4 g	NaOH	100 ml dH_2O	-		4°C
200mM NaOH	3.997 g	NaOH	500 ml dH_2O	-		4°C
120mM NaH_2PO_4	8.25 g	$NaH_2PO_4.2H_2O$	500 ml dH_2O	7.5	NaOH	4°C
200mM NaH_2PO_4	13.8 g	$NaH_2PO_4.2H_2O$	500 ml dH_2O	5.6	NaOH	4°C
200mM NaH_2PO_4 + 10mM EDTA (*)	13.8 g 1.86 g	$NaH_2PO_4.2H_2O$ Na_2-EDTA	500 ml dH_2O	7.5	NaOH	4°C

(*)Put this buffer in the fridge, do not put it on ice because it will crystallize, when crystallization occurs, just mix the solution and you can use it again

Following product can be stored **1 month**:

Solution	Weight	Product	in Vol	Store at
12mM DTNB	47.6 mg	DTNB	10 ml DMSO	RT in dark

Note: when it turns yellow, throw it away because it is oxidized

Following products can be stored 1 week:

Solution	Weight	in Vol	Buffer	Store
10 mM GSH	~3.5 mg	(1000/3.073*weight) µl	200mM NaH_2PO_4 (pH 5.6)	4°C
10 mM GSSG	~7.0 mg	(1000/6.126*weight) µl	200mM NaH_2PO_4 (pH 5.6)	4°C
25 mM DTT	~3.5 mg	(1000/3.856*weight) µl	dH_2O	4°C

Following product can be stored 1 week although Aliquots of 1.5 ml in Eppendorf tubes can be stored for longer periods at -80°C

Solution	Product	MW	Weight	in Vol	Buffer	Store
10 mM NADPH	NADPH	833.4	~10 mg	(1000/8.334*weight) µl	dH_2O	-20°C

Prepare the 1.5mL Eppendorf tubes

- You should harvest samples in 1.5 ml tubes
  - Roots: 1 sample ~50 mg
  - Leaves: 1 sample 100 mg
- Cut pH papers 4x in order to have smaller paper strips that don't absorb too much of your sample
- Label 1.5mL eppendorf tubes (this is for 1 x 8 samples)

GSH1 → GSH3 to dilute the GSH stock
GSH0' → GSH3' to prepare the GSH standard
GSSG1 → GSSG4 to dilute the GSSG stock
GSSG0' → GSSG3' to prepare the GSH standard and incubate with 2-VP for GSSG
GSSG0'' → GSSG3'' to collect the supernatant after incubation with 2-VP for GSSG
1 → 8 to collect the supernatant after centrifugation for AsA and GSH
1' → 8 'to collect the supernatant after centrifugation for NAD and NADP
1' → 8 'to collect the supernatant after centrifugation for NADH and NADPH
1A → 8A to incubate the sample with DTT for total AsA
1G → 8G to incubate the sample with 2-VP for GSSG
1G' → 8G' to collect the supernatant after incubation with 2-VP for GSSG

- Put the samples in liquid nitrogen
- Keep the buffers at 4°C
- Defrost all products on ice
- Turn on centrifuge at 4°C
- Turn on the oven at 20°C
- Prepare 8 x 2 tungsten carbide beads

Prepare a broad standard range of 8 concentrations

GSH broad range (10µl/well for leaves and roots)

Dilute 10 mM GSH stock 100X in 2 steps

- (100µM = 100pmol/µl = 1000pmol/10µl = 1000pmol/well)
- Step 1 = dilute 10X = 1mM: 450µl 200mM NaH_2PO_4 (pH5.6) + 50µl 10mM stock
- Step 2 = dilute 10X = 100µM: 900µl 200mM NaH_2PO_4 (pH5.6) + 100µl 1mM stock

Prepare standards

[1000 pmol* = **100µl** 100µM GSH stock]
500 pmol = **100µl** 200mM NaH_2PO_4 (pH5.6) + **100µl** 100µM GSH stock

250 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 500pmol GSH
125 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 250pmol GSH
62.5 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 125pmol GSH
31.25 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 62.5pmol GSH
15.625 pmol* =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 31.25pmol GSH
[0 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)]	

* All standards are used the first time, but these two can be left out if everything falls within range

GSSG broad range (20µl/well for leaves and roots)

Dilute 10mM GSSG stock 1000X in 3 steps

- (10µM = 10pmol/µl = 200pmol/20µl = 200pmol/well)
- Step 1 = dilute 10X = 1mM: 450µl 200mM NaH_2PO_4 (pH5.6) + 50µl 10mM stock
- Step 2 = dilute 10X = 100µM: 900µl 200mM NaH_2PO_4 (pH5.6) + 100µl 1mM stock
- Step 3 = dilute 10X = 10µM: 900µl 200mM NaH_2PO_4 (pH5.6) + 100µl 100µM stock

Prepare standards

[200 pmol* =		**100µl** 10µM GSSG stock]
100 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 10µM GSSG stock
50 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 100pmol GSSG
25 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 50pmol GSSG
12.5 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 25pmol GSSG
6.25 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 12.5pmol GSSG
3.125 pmol* =	**100µl** 200mM NaH_2PO_4 (pH5.6)	+ **100µl** 6.25pmol GSSG
[0 pmol =	**100µl** 200mM NaH_2PO_4 (pH5.6)]	

* All standards are used the first time, but these two can be left out if everything falls within range

Acidic extraction protocol

Crushing and dividing the samples

- Put 2 **tungsten carbide** beads in the 1.5 ml Eppendorf tubes and shred them ice cold (in liquid nitrogen) during 3.5 minutes at maximum amplitude. Leave the beads (tungsten carbide is non-magnetic).
  ! At this point you can freeze the samples again or you can continue with the extraction !
- Add 800 µl (for *Arabidopsis th.*) or 400 µl (for *Lemna m.*) of 200 mM HCl to the frozen sample and vortex until it is thawed, keep the sample on ice.
- Centrifuge the samples 15 min maximum speed at 4 °C.

Neutralization

*! Apply the same procedure for each sample, always **keep the samples on ice**.*

- take 300µl aliquots (1 → 8) and add 30 µl 200 mM NaH_2PO_4 (pH 5.6)
- vortex and put on ice
- obtain pH 4-5 with 200 mM NaOH

(when > pH 5 the sample gets oxidized): start with 100 µl 200 mM NaOH and vortex immediately
measure pH by putting the pH strip in the solution

afterwards add 100, 50, 20 or 10µl 200mM NaOH
(this should be ca. 200 µl, **write down the exact volume**)
when you passed the pH you can go back with HCl but don't forget to write it in your notes.

Incubate samples for oxidized glutathione measurements

- put 100 µl extract of the ~ 300 µl aliquot (1 → 8) in 1G → 8G
- add 1.3 µl 2-VP (work in flow, use gloves!) and vortex
- add 1 µl 2-VP to the standard (GSSG0'→GSSG3')
- vortex and incubate 30 min at room temperature
- continue with next measurement during incubation
- centrifuge 15 min at 4 °C to precipitate 2-VP
- continue with measurements during centrifugation
- transfer 80 µl supernatant to clean tubes: 1G' → 8G' and GSSG0'' → GSSG3''
- centrifuge again 15 min at 4 °C to precipitate 2-VP

Incubate samples for total ascorbate measurements

- put 140 µl 120 mM NaH_2PO_4 (pH 7.5) in 1A → 8A
- add 10 µl 25 mM DTT
- add 100 µl extract of the ~300 µl aliquot (1 → 8)
- Vortex and incubate 15 min at 20 °C in the cooled microtube block
- continue with next measurement during incubation
- adjust to pH 5.5 with HCl (optimal pH for AO) and write down the exact volume

Reduced Ascorbate measurement

- Start your measurements with ascorbate because it is less stable
- Turn on the plate reader and prepare the runs to measure
- Take a UV 96-well plate
  We always measure the samples in triplicate,
  for 8 samples you have to prepare 8 x 3 = 24 wells
- Add 155 µl diluted 200 mM NaH_2PO_4 (pH 5.6) (= 100 µl dH_2O +55 µl buffer) in each well using the automatic multichannel
- Vortex the extracts and add 40 µl extract to the wells (use the same tip for each triplicate)
- Put the plate in the reader and start the first measurement: M_ASCORBATE ENDPOINT this will measure the total absorbance at 265 nm after 20sec shaking
- Use the automatic pipet and add 5 µl AO to each well (use the same tip)
- Put the plate back into the reader and start the second measurement: M_ASCORBATE KINE+AO after 20 sec shaking, ascorbate oxidation is measured, after 5-10 min the reaction should be completed. Where do the CIG intervene? When the alarm would be given

Total Ascorbate measurement

The protocol is exactly the same for both measuring only reduced ascorbate and total ascorbate, only for total AsA you have to incubate with DTT first in order to reduce all ascorbate that is present in the sample

Glutathione measurement

The protocol is almost the same for both measuring total GSH and only oxidized GSSG, for only GSSG you incubate with 2-VP in order to block all GSH present in the sample and add 20 µl extract instead of 10 µl

- the samples are measured in triplicate, for 8 samples you have to prepare 8 x 3 = 24 wells
- the standard are measured in duplicates, for 8 standards you have to prepare 8 x 2 = 16 wells
- in total these are 40 wells, prepare a mastermix for 50 wells

	Total glutathione (GSH) Mastermix for 50 wells	Oxidized glutathione (GSSG) Mastermix for 50 wells
200mM NaH_2PO_4 - 10mM EDTA (pH 7.5)	5000 µl	5500 µl
dH_2O	3000 µl	2750 µl
10mM NADPH	500 µl	550 µl
12mM DTNB in DMSO	500 µl	550 µl

- Vortex this mastermix and store on ice
- Pipet samples (3x) and standards (2x) in the wells:
    - 10 µl for total glutathione (not incubated) (GSH standard)
    - 20 µl for oxidized glutathione (incubated) (GSSG standard)
- Add 500 µl GR stock (10 µl per sample) to the mastermix and vortex
- Add the mastermix to each well using the automatic multichannel
- 190 µl for total glutathione (GSH standard)
- 180 µl for oxidized glutathione (GSSG standard)
- Put the plate in the reader and start the measurement: M_GLUTATHIONE KINE
- after 20 sec shaking, DTNB reduction is measured during 5 min at 412 nm

Platereader protocols

M_ASCORBATE ENDPOINT Protocol:
Temperature: Setpoint 25°C | Shake: High for 2x 0:10 | Read: (A) 265 | 1 read point per well

M_ASCORBATE KINE+AO Protocol :
endpoint kinetics (total)
Temperature: Setpoint 25°C | Shake: High for 2x 0:10 | Read: (A) 265 | 1 read point per well

number of flashes per well per cycle: 20
kinetic time interval for 4 samples: 11s
number of cycles for 4 samples: 52 cycles
measuring time: 10m02s
kinetic time interval for 8 samples: 18s
number of cycles for 8 samples: 32 cycles
measuring time: 10m06s

M_GLUTATHIONE KINE Protocol:
kinetics (total)
Temperature: Setpoint 25°C | Shake: High for 3x 0:10 | Read: (A) 412 | 1 read point per well

number of flashes per well per cycle: 20
kinetic time interval for 4 samples: 20s

number of cycles for 4 samples:	14 cycles
measuring time:	5m10s
kinetic time interval for 8 samples:	22s
number of cycles for 8 samples:	13 cycles
measuring time:	5m16s

Data analysis

ASCORBATE:

Use the law of Lambert-Beer: $\Delta A = \varepsilon . C . d$

$\Delta A = A_{265 \text{ before addition of AO}} - A_{265 \text{ after 5min addition of AO}}$

$d = 0{,}5\text{cm}$

$\varepsilon_{265} = 14\,000\ M^{-1}cm^{-1} = 0{,}014\ \mu M^{-1}cm^{-1} = 0{,}014\ \left(\mu\frac{\text{mol}}{\text{l}}\right)^{-1} cm^{-1}$

$C = \frac{\Delta A}{\varepsilon . d} = \Delta A . \frac{1}{0.014} \frac{\mu\text{mol.cm}}{\text{l}} \frac{1}{0.5\text{cm}} = \Delta A . \frac{1}{0.014} . \frac{1}{0.5} \frac{\mu\text{mol}}{\text{l}}$

↓(200µl/well instead of 1 liter = 1/5000 dilution)

$C = \Delta A . \frac{1}{0.014} . \frac{1}{0.5} . \frac{1}{5000} . \frac{\mu\text{mol}}{200\mu\text{l}} = \Delta A . \frac{1}{0.014} . \frac{1}{0.5} . \frac{1}{5} . \frac{\text{nmol}}{200\mu\text{l well}}$

Don't forget to take the dilutions into account:

$C = x \frac{\text{nmol}}{200\mu\text{l well}} = x \frac{\text{nmol}}{40\mu\text{l extract}}$

↓(volume after neutralization to correct pH > volume before neutralisation)

$C = x \frac{\text{nmol}}{40\mu\text{l extract}} . \frac{V_{\text{after pH}}}{V_{\text{before pH}}}$

↓(volume extraction buffer > 40µl volume extract)

$C = x \frac{\text{nmol}}{40\mu\text{l extract}} . \frac{V_{\text{after pH}}}{V_{\text{before pH}}} . V_{\text{extraction buffer}}$

↓(total incubation volume > volume incubated extract and again neutralisation) → only for total AsA

$C = x \frac{\text{nmol}}{40\mu\text{l extract}} . \frac{V_{\text{after pH}}}{V_{\text{before pH}}} . V_{\text{extraction buffer}} . \frac{V_{\text{incubation with DTT}}}{V_{\text{incubated extract}}} . \frac{V_{\text{after 2nd pH}}}{V_{\text{before 2nd pH}}}$

↓(calculate per g fresh weight)

$C = x \frac{\text{nmol}}{40\mu\text{l extract}} . \frac{V_{\text{after pH}}}{V_{\text{before pH}}} . V_{\text{extraction buffer}} . \frac{V_{\text{incubation with DTT}}}{V_{\text{incubated extract}}} . \frac{V_{\text{after 2nd pH}}}{V_{\text{before 2nd pH}}} . \frac{1000}{\text{a mg}}$ → expressed in nmol/mg FW

= µmol/g FW

Example:

$$C = x \frac{\text{nmol}}{40\mu\text{l extract}} . \frac{V_{\text{after pH}}}{300\mu\text{l}} . 500\mu\text{l} . \frac{250\mu\text{l}}{100\mu\text{l}} . \frac{V_{\text{after 2nd pH}}}{250\mu\text{l}} . \frac{1000}{\text{a mg}}$$

References:

	Only reduced AsA	Total AsA	Only oxidized DHA	% reduced AsA
Leaves	1.5 µmol/g FW	2.5 µmol/g FW	1 µmol/g FW	60 – 105 %
Roots	0.15 µmol/g FW	0.5 µmol/g FW	0.35 µmol/g FW	60 – 105 %

GLUTATHIONE

Select a range that contains the linear phase of the highest standard
(saturation can occur because limited products in the reaction)
→ normally you take from 0 to ca. 2,5 min

Calculate the slope in minutes, compare with the standard curve

Don't forget to take the dilutions into account:

$$C = x\ \frac{\text{pmol}}{200\mu\text{l well}} = x\ \frac{\text{pmol}}{10 \text{ or } 20\mu\text{l extract}}$$

↓(volume after neutralization > volume before neutralisation)

$$C = x\ \frac{\text{pmol}}{10 \text{ or } 20\mu\text{l extract}} . \frac{V_{\text{after pH}}}{V_{\text{before pH}}}$$

↓(volume extraction buffer > volume extract)

$$C = x\ \frac{\text{pmol}}{10 \text{ or } 20\mu\text{l extract}} . \frac{V_{\text{after pH}}}{V_{\text{before pH}}} . V_{\text{extraction buffer}}$$

↓(calculate per g fresh weight)

$$C = x\ \frac{\text{pmol}}{10 \text{ or } 20\mu\text{l extract}} . \frac{V_{\text{after pH}}}{V_{\text{before pH}}} . V_{\text{extraction buffer}} . \frac{1000}{\text{a mg}}$$ → expressed in pmol/mg

FW = nmol/g FW

Example:

$$C = x\ \frac{\text{nmol}}{10 \text{ or } 20\mu\text{l extract}} . \frac{V_{\text{after pH}}}{300\mu\text{l}} . 500\mu\text{l} . \frac{1000}{\text{a mg}}$$

References:

	Total GSH	Only oxidized GSSG	Only reduced GSH	% reduced GSH
Leaves	150 nmol/g FW	5 nmol/g FW	140 nmol/g FW	90 – 95 %
Roots	200 nmol/g FW	3 nmol/g FW	220 nmol/g FW	90 – 95 %

Principle: spectrophotometrics

Ascorbate assay: measuring decrease in reduced ascorbate at 265 nm

- Non-treated samples: measure only reduced AsA
  - Add AO (ascorbate oxidase)
  - AsA is specifically oxidized to non-absorbing DHA (dehydroascorbate)
- Treated samples: measure total ascorbate
  - Add DTT and its thiols will fully reduce the ascorbate pool to reduced AsA
  - Add AO
  - AsA is specifically oxidized to non-absorbing DHA

Glutathione assay: measuring increase in reduced DTNB at 412 nm

- Non-treated samples: measure total glutathione
  - Add GR (glutathione reductase)
  - Oxidized GSSG is specifically reduced to GSH
  - GSH reduces DTNB to an absorbing molecule
- Treated samples: measure only oxidised GSSG
  - Add 2-VP in order to complexate the GSH that is present in the sample
  - Centrifugate the sample twice in order to remove the 2-VP
  - Add GR
  - Oxidized GSSG is specifically reduced to GSH
  - Only not-complexated GSH reduces DTNB to an absorbing molecule