Malate Dehydrogenase Assay

Materials:
- LN₂
- Glass Petri dish bottom
- Scalpel with #11 blade
- Flat ended forceps bent at 45°
- Small aluminum foil pouch labeled
- 50 mM Bicine Buffer pH 8.0 (For MDH Extraction)
- Triton X, Mannitol, PVPP, BSA, DTT, PMSF
- 50 mM Bicine Buffer pH 8.0 (For NADP-MDH Activity Assay)
- NADPH, DTT, BSA, OAA, NADH
- 50 mM Bicine Buffer pH 9.0 (For MDH Activation)

Procedure:
Note: Make sure you use freeze clamp to assure “a fast kill” to prevent a change in malate dehydrogenase activity. The following procedure assumes use of the gas exchange fast kill machine, but a hand held freeze clamp can also be used. Sparge all buffers with N₂ overnight.

1. Get Petri dish and cool down and fill with LN₂, have scalpel, forceps, and foil pouch nearby
2. Freeze clamp plates in LN₂ and quickly place in freeze kill apparatus
3. When you are ready, flip air switch to activate freeze clamp, leave clamp pressurized
4. Loosen the wing nut on the leaf cuvette and move cuvette panels out of the way, and cut away excess leaf material and saran wrap with the scalpel.
5. Cool forceps tips in LN₂, Release air pressure on freeze clamp and quickly move freeze clamp plats out of the way, use the forceps to place leaf disk into the petri dish with LN₂ Cut leaf into 2 pieces if need
6. Cool foil pouch in LN₂ and place leaf disk in pouch to store or to break up leaf material and place in microfuge tube.
7. Make extraction buffer in 15 ml tube according to the following table

<table>
<thead>
<tr>
<th>Stock</th>
<th>Volume to add</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicine Buffer* 50 mM pH 8.0 deli case</td>
<td>10 ml</td>
<td>50 mM*</td>
</tr>
<tr>
<td>Triton X-100 shelf</td>
<td>50 μl</td>
<td>0.5 %</td>
</tr>
<tr>
<td>Mannitol 500 mM freezer</td>
<td>200 μl</td>
<td>10 mM</td>
</tr>
<tr>
<td>PVPP powder shelf</td>
<td>0.05 g</td>
<td>0.5 %</td>
</tr>
<tr>
<td>BSA powder frige</td>
<td>0.1 g</td>
<td>1 %</td>
</tr>
<tr>
<td>DTT 1M freezer</td>
<td>50 μl</td>
<td>5 mM</td>
</tr>
<tr>
<td>PMSF 100 mM freezer</td>
<td>100 μl</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

* Bicine buffer also contains 30 mM NaCl, 5mM MgCl₂, and 2mM EDTA

8. When leaf material is in a microfuge tube grind frozen to power leaf matiral, place tube on ice and add 1 ml of extraction buffer, shake or votex tube to mix contents
9. Centrifuge for 5 minutes, place supernatent into new microfuge tube, store on ice
10. Make NADP-MDH Assay buffer in 15 ml tube according to the following formula
   
   1 ml 50 mM Bicine buffer pH 8.0 * X (number of samples)
   + 10 μl NADPH * X (100 μM)
   + 1 μl DTT * X (1 mM)
   + 0.0001 g BSA * X (10 % w/v)

11. Add 800 μl of the mix to each cuvette
12. Add 50 μl of sample to cuvette
13. Once you have a stable baseline add 5 μl of OAA to start the reaction
14. Now we need to fully activate MDH by reducing with DTT to do this add the following to a 500 μl microfuge tube
   
   + 25 μl 50 mM Bicine buffer pH 9.0
   + 25 μl DTT (166.7 mM)
   + 100 μl Sample

15. Allow the MDH to fully activate by incubating for 1 hour on the bench
16. Assay for MDH activity as before starting with step 11, remember you have diluted your sample by 1.5 X, so multiply activity by 1.5 to compare with the unactivated assay
17. Now we need to test for NAD dependent MDH activity to do this make NAD-MDH Assay buffer in 15 ml tube according to the following formula

   1 ml 50 mM Bicine buffer pH 8.0 * X (number of samples)
   + 10 μl NADH * X (100 μM)

18. Add 800 μl of the mix to each cuvette
19. Add 50 μl of sample to cuvette
20. Once you have a stable baseline add 5 μl of OAA to start the reaction
21. You can subtract this activity from the two NADP dependent MDH activities

Reactions:

OAA + NADPH*absorbs at 340nm (MDH) → Malate + NAD

This assay was adapted from:


Malate Dehydrogenase Activity Assay Reagents

50 mM Bicine Buffer pH 8.0 (For MDH Extraction)
In 250 ml dH₂O
+ 2.04 g Bicine, 50mM (fw 163.2; Sigma B-3876)
+ 0.483 g NaCl, 30mM (fw 58.44)
+ 0.254 g MgCl₂•6H₂O, 5mM (fw 203.3; Sigma M-2670)
+ 0.21 g EDTA, 2mM (fw 416.2; Sigma ED4SS)
pH to 8.0 using KOH

500 mM Mannitol
In 50 ml dH₂O
+ 4.56g Mannitol (fw 182.2)
Store in -20°C

1 M DTT
In 10 ml dH₂O
+ 1.54 g DTT (fw 154.2; Sigma D-5545)
Divide into 1.5 ml microfuge tubes and store in -80°C

100 mM PMSF
In 10 ml dH₂O
+ 0.174 g Phenylmethylsulfonyl fluoride (fw 174.2; Sigma P-7626)
Divide into 1.5 ml microfuge tubes and store in -80°C

50 mM Bicine Buffer pH 8.0 (For NADP-MDH Activity Assay)
In 250 ml dH₂O
+ 2.04 g Bicine, 50mM (fw 163.2; Sigma B-3876)
+ 0.11 g EDTA, 1mM (fw 416.2; Sigma ED4SS)
pH to 8.0 using KOH

10 mM NADPH
In 10 ml 100 mM carbonate buffer pH 10.3
+ 0.0833 g HADPH (fw 833.4; Sigma N-1630)
Divide into 1.5 ml tubes and store in -80°C

100 mM Carbonate Buffer PH 10.6
In 100 ml dH₂O
+ 0.848 g Na₂CO₃ (fw 105.99)
+ 0.168 g NaHCO₃ (fw 84.01)
pH to 10.6 using NaOH or HCl

400 mM OAA
In 10 ml dH₂O
+ 0.528 g Oxalacetic Acid (fw 132.1; Sigma O-4126)
Divide into 1.5 ml tubes and store in -80°C

50 mM Bicine Buffer pH 9.0 (For MDH Activation)
In 250 ml dH₂O
+ 2.04 g Bicine, 50mM (fw 163.2; Sigma B-3876)
pH to 9.0 using KOH

50 mM Bicine Buffer pH 8.0 (For NAD-MDH Activity Assay)
In 250 ml dH₂O
+ 2.04 g Bicine, 50mM (fw 163.2; Sigma B-3876)
+ 0.11 g EDTA, 1mM (fw 416.2; Sigma ED4SS)
+ 0.254 g MgCl₂•6H₂O, 5mM (fw 203.3; Sigma M-2670)
pH to 8.0 using KOH

10 mM NADH
In 10 ml 100 mM carbonate buffer pH 10.3
+ 0.0709 g HADH (fw 833.4; Sigma N-8129)
Divide into 1.5 ml tubes and store in -80°C 10 mM NADH