PEPCK Enzyme Assay Protocol


Measuring reverse reaction using MDH coupled assay
OAA + NADH -> Malate + NAD^+

Use 1.0 ml polystyrene cuvettes. Measure absorbance at 340 nm (NADH absorbance) for 5 min.
Rate MUST be linear for 2-3 min. and NOT change without dGDP

Prepare all reagents in 100 mM HEPES pH 7.0 (make a 100 ml stock to use, store at RT)

Stock Reagent Preparation:
- 450 mM NaHCO\textsubscript{3} Prepare 10 ml, Store at room temp in capped vial/tube
- 200 mM PEP Prepare 1 ml, Store in 100 µl aliquots at -20oC
- 300 mM MgSO\textsubscript{4} Prepare 50 ml, Store at room temp in capped vial/tube
- 300 mM MnCl\textsubscript{2} Prepare 50 ml, Store at room temp in capped vial/tube
- 1200 mM NaF Prepare 50 ml, Store at room temp in capped vial/tube
- 1 M DTT Use frozen aliquots from Lysate Preparation
- 100 mM dGDP Prepare 1 ml, Freeze in 100 µl aliquots
- 150 mM NADH Prepare 1 ml, Freeze in 100 µl aliquots

MDH is sold as concentrate; calculate how many units to add per 1.0 ml assay for 6 total units

<table>
<thead>
<tr>
<th>Stock Reagent</th>
<th>µl per stock per assay</th>
<th>Final Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>450 mM NaHCO\textsubscript{3}</td>
<td>100 µl</td>
<td>45 mM</td>
</tr>
<tr>
<td>200 mM PEP</td>
<td>10 µl</td>
<td>2 mM</td>
</tr>
<tr>
<td>300 mM MgSO\textsubscript{4}</td>
<td>10 µl</td>
<td>3 mM</td>
</tr>
<tr>
<td>300 mM MnCl\textsubscript{2}</td>
<td>10 µl</td>
<td>3 mM</td>
</tr>
<tr>
<td>1200 mM NaF (fluoride)</td>
<td>10 µl</td>
<td>12 mM</td>
</tr>
<tr>
<td>1 M DTT</td>
<td>1 µl</td>
<td>1 mM</td>
</tr>
<tr>
<td>150 mM NADH (abs at final should be ~1.2-2)</td>
<td>10 µl</td>
<td>0.15 mM</td>
</tr>
<tr>
<td>6 Units MDH</td>
<td></td>
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</tbody>
</table>

QS with HEPES buffer to 800 µl ~ 650 µl

Assemble Cocktail for 5-10 assays Multiply using numbers above
- Add 100 µl sample and incubate at 30oC for 2 min
- Read total absorbance.
Initiate assay with 100 µl 100 mM dGDP - follow absorbance at 340 nM

Conducting the assay:
During the early stages of the purification it is important to use dGDP and to obtain a rate in the absence of dGDP and then the rate with dGDP.

AU/min conversion to U/ml = 1.61
**Homogenization Base Buffer:**
*Prepare and aliquot in 10 ml fractions at -20°C – check to see for any growth or precipitate before using.*

- 300 mM Sucrose
- 10 mM Tris-Cl pH 8.0
- 1 mM EDTA
- 0.5% Fatty acid free BSA

**1 M DTT** 0.155 g DTT powder in 1.0 ml miliQ water. Freeze (-20°C) in 0.020 ml aliquot

**100 mM PMSF** 17.4 mg/ml dissolved in ethanol. Prepare 100 ml, store in tight fitting bottle at -20°C (add fresh just prior to use)

**20% Sodium Deoxycholate** 2 g in 10 ml water. This will take some time to prepare, use a glass bottle, apply gentle heat while using stir bead. Deoxycholate is an ionic detergent to reduce protein interactions and solubilize mitochondria membrane – release CS from mito into homogenate

**Tissue Homogenization**
- Prepare Homogenization Buffer: KEEP ICE COLD. Assemble buffer just before use.
  - 10 ml base buffer
  - 10 µl 1M DTT final conc [1.0 mM]
  - 100 µl 100 mM PMSF – add right before use. Slowly pipet with tip immersed in solution. Mix while adding. Half-life is about 30 min.
- Excise 0.5 -1.0 g of tissue. Record the exact mass of tissue
- Mince and homogenize in in 2ml of homogenization buffer per gram of tissue.
- Using a Potter-Elvhjem glass homogenizer pass 5 times (one pass = up and down through sample) Keep ice cold.
- Divide the solution in half. Tube 1: cytosolic fraction; Tube 2: total lysate
  - Cytosolic Fraction half will be left as is and centrifuged for PEPCK activity
  - Total lysate fraction: add deoxycholate and sonicate to second half to solubilize mitochondria for CS
- Total Lysate Fraction (tube 2)
  - Add 20 µl of 20% deoxycholate per 1.0 ml homogenate, rotate to mix.
  - Sonnicate (3-5 x 5-10 second bursts) on ice.
- Leave Cytosolic fraction (tube 1) as is.
- Centrifuge both total lysate fraction and cytosolic fraction at 18,000 x g (max speed on microcentrifuge) for 30 min. at 4oC. Carefully balance each tube with a blank water tube
- Pipet / collect each supernatant and transfer to a new tube.
  - Cytosolic – Divide into 3 similar volumes, label and freeze.
  - Total lysate – Divide into 3 similar volumes, label and freeze.