PROVOST & WALLERT RESEARCH

PEPCK Enzyme Assay

Protocol



Investigating the Biochemistry & Cellular Physiology of NHE1 EST. 1998

PEPCK ASSAY (*Wiese, Lambeth, Ray, Comp Biochem Physiol Vol 100B No 2 pp 297-302 1991*)

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 NaHCO₃ 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 MgSO₄ Prepare 50 ml, Store at room temp in capped vial/tube 300 mM MnCl₂ 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

Stock Reagent	µl per stock per assay	Final Conc
450 mM NaHCO ₃	100 µl	45 mM
200 mM PEP	10 µl	2 mM
300 mM MgSO₄	10 µl	3 mM
300 mM MnCl2	10 µl	3 mM
1200 mM NaF (fluoride)	10 µl	12 mM
1 M DTT	1 µl	1 mM
150 mM NADH (abs at final should be ~	-1.2-2) 10 µl	0.15 mM
6 Units MDH	<i>,</i> .	

QS with HEPES buffer to 800 μ l ~ 650 μ l

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 -20oC – 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 (-20oC) in 0.020 ml aliquot
- **100 mM PMSF** 17.4 mg/ml dissolved in ethanol. Prepare 100 ml, store in tight fitting bottle at -20oC (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.
 - o 10 ml base buffer
 - ο 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
 - o 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.