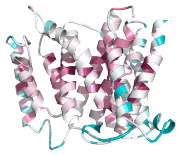
Immunoflourescence - Immuno-Stain Protocol



PEPCK (Wallace et.al)

Assembling Cocktail: for 20 assays

	final conc
8.0 ml – 250 mM Hepes	[100 mM]
1.6 ml – 625 mM $NaHCO_3$ (3.78 g/100 ml)	[50 mM]
0.8 ml – 50 mM $MgCl_2$ + 2.5 mM $MnCl_2$	[2mM, 0.1 mM]
14.4 mg PEP	[2 mM]

0.8 ml – DTT 25 mM DTT (38 -40 mg/10 ml, prepared fresh)
2.5 – 5 mg NADH
25 ul of MDH (use a minimum of 6U/assay)
Dilute to nearly 16 ml and readjust the pH to 7.4 then bring to a final volume of 16 ml.

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.

800 ul cocktail
Incubate and initiate with:
100 ul sample – see the above note for rates
100 ul nucleotide
-10 mM dGDP (21 mg/5ml) – when there is pyruvate kinase contamination
or
-10 mM IDP – when there is no PK
-mM GDP used when the desire is to be as physiological as possible

final volume = 1.0 ml
AU/min conversion to U/ml = 1.61

Trios-Phosphate Isomerase TPI (From Bergmeyer, Meth in Enz, 1974 pp. 515)

Cocktail

10 ml 0.3 M TEA-Cl ph 7.6
200 ul NADH 10 mg/ml
40 ul α Glycerophosphate dehydrogenase (GDH) at least 2 U/assay

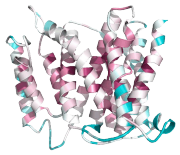
295 ul/assay
2 ul Glyceraldehyde 3-Phosphate (GAP) frozen in 50 mg/ml – 50 ul aliquots
2 ul Sample
Total assay volume = 0.299 ml
AU/minx 24.07 = U/ml

Pyruvate Kinase PK (From Harada et al, BBA, 524 1978 pp. 327-339)

	Stock Solns	Final Conc
880 PK Buffer	50 mM Tric-Cl	50 mM Tris-Cl pH 7.5
	7.45 mg/ ml	100 mM KCl
	1M	5 mM $MgSO_4$
20 ul ADP	48mg/ml PK Buffer	2 mM
20 ul Fru-1,6-P	24 mg/ml PK Buffer	1 mM
20 NADH	7 mg/ml	0.126 mM
20 ul LHD		10U/Assay

Incubate and initiate with:

20 ul Sample		
20 ul PEP	20.6 mg/ml PK Buffer	2mM



ASPARTATE AMINOTRANSFERASE from Enzymes 2.2.1 pp. 160-161

920 ul	Phosphate Buffer	pH 7.4 5 mg/ml Asp
20 ul	a-ketoglutarate	50 mg/ml (1 mg/assay)
20 ul	NADH	8.35 mg/ml (.167 mg/assay)
20 ul	MDH	2.43 U/assay
20 ul	Sample	

Total Assay volume = 1.0 ml
AU/min x 8.05 = U/ml

Nucleoside Diphosophokinase (NDPK)

845 ul	100 mM TEA-Cl	pH 7.6
20 ul	PEP	25.0 mg/ml (0.5 mg/assay) in 0.5 M MgCl/2M KCL
20 ul	NADH	8.35 mg/ml (0.167 mg/assay)
20 ul	ATP	66.5 mg/ml (1.33 mg/assay)
20 ul	LDH	1.0 U/assay
20 ul	PK	1.0 U/assay
20 ul	Sample	
35 ul	dTDP	10 mg/ml (0.33 mg/assay)

Total Assay Volume = 1.0 ml
AU/min x 8.05 = U/ml

Enolase (From Rider et al, BBA 365, 1974 pp. 285 – 300)

860 ul Enolase buffer 50 mM TEA-Cl, 8.75 $MgCSO_4$, 80.4 mM KCl, 3.84 mM EDTApH 7.6

20 ul	NADH	8.33 mg/ml (0.167 mg/assay)
20 ul	ADP	33.32 mg/ml (0.667 mg/assay)
20 ul	LDH	1.0 U/assay
20 ul	PK	1.0 U/assay
20 ul	Sample	
20 ul	G-2-P	16.67 mg/ml (0.333mg/assay)

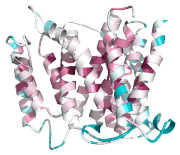
initiate with G-2-P
Total Assay Volume = 1.0 ml
AU/min x 8.05 = U/m

Malate Dehydrogenase

	Stock Solutions
940 ul TEA-Cl	100 mM pH 7.4
20 ul NADH	10 mg/ml in TEA-Cl
20 ul Sample	

Incubate and initiate with:

20 ul OAA	3.2 mg/ml in TEA-Cl



"MICRO ASSAY" FOR P_i FORMATION DURING HEXOSE PHOSPHATE HYDROLYSIS

Burchell' modification of the method of B.N. Ames (1966) (Meth. Enzymol. 8: 115-199).*

STOCK SOLUTIONS:

(A) Acid Molybdate Reagent: 0.42% ammonium molybdate ·4 H_2O in 1N H_2SO_4. Prepare 1N H_2SO_4 by carefully diluting 28.6 ml of concentrated sulfuric acid to 1 liter. (The acid should be added to the water). Dissolve 4.2 g of ammonium molybdate ·4H_2O to 1000 ml with 1N sulfuric acid. The reagent is stable indefinitely at room temperature.

(B) 10% SDS (sodium dodecylsulfate): Dissolve 20 g of SDS in H_2O and adjust volume to 200 ml. Stable at room temperature. (High grade needed) – No phosphate!

(C) 10% Ascorbic Acid: 10 g in 100 ml of water. Keep in refrigerator (4°C). Should be stable for about one month. Be sure to label date of preparation.

PROCECURE:

WORKING SOLUTION:

Combine stock solutions (A), (B), and (C) in the following proportions:

6 volumes (A) (acid molybdate reagent)
2 volumes (B) (SDS solution)
1 volume (C) (ascorbic acid)

The Working Solution should be stable for one day if kept on ice.

Terminate the phosphatase assay by addition of 0.9 ml of Working Solution to 0.1 ml of assay medium.

Develop color by incubating for 20 min at 45 °C or one hour at 37 °C. The blue reduced phosphomolybdate comples should be stable for several hours. Netiher glucose-6-P nor mannose-6-P (or other hexose-6-P) should undergo significant hydrolysis under these conditions. However, 5 percent or more of PP_i will be hydrolyzed.

Read absorbance at 820 nm. 0.01 micromole of P_i should yield an $O.D._{820}$ = 0.26. The method should permit assay of between 1 and about 50 nmoles of P_i.

PHOSPHATASE ASSAY MEDIA:

50mM Tris/Cacodylate or suitable alternate buffer
1 to 30 mM hexose-6-phosphate
Other additions (e.g. inhibitors)
Final volume = 80 microliters

Initiate phosphatase assays by addition of 20 microliters of appropriately diluted enzyme preparation. Run reagent blanks (i.e. "zero-time" controls).

* Use of SDS to clarify (solubilize) microsomal protein.