PEPCase Assay without Radioactivity (Cytosol Marker)

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5:20 PM

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Materials:
100 mM bicine buffer pH 7.8
Malate Dehydrogenase (Sigma M-2634)
1 M $NaHCO_3$
50 mM PEP
10 mM NADH
Clear 96-well plate

Procedure:
1. Turn on plate reader. Set incubator for 37 ºC
2. Select "Protocols" and choose GAPDH-PEPCase Enzyme Assay
    This will allow for easy data analysis
3. Refer to plate set-up on next page to determine how to set up your wells. Following this set up will allow you to cut and paste your data into an excel template
4. Prepare Master Mix of:
+187 µl NAF Hepes Buffer
+ 10 µl NADH
+ 1 µl 1 M $NaHCO_3$
+ 2 µl malate deydrogenase
    Per sample
5. Add 200 µl of buffer to each well
6. Add 10-100 µl extract to each well
7. Start kinetic assay, verifying that you have a stable baseline
8. Start the reaction by adding 10 µl PEP using hedgehog tool
9. Immediately begin assay
    Kinetic absorbance assay
    Wavelength= 340 nm
    Run time= 20 min
    Choose minimum interval between readings
        To do this you must:
        Select settings and choose kinetic assay
            Adjust the minimum time interval
            Exit settings
            Delete existing assay data
            Select settings again and re-adjust minimum time interval

*****These amounts assume 0.25 mg plant material in 500 µl buffer

Data Analysis
1. Look at the graph of the data and determine between which time points the slope of the data is linear
2. Click on reduction and change the lag time and the end time so that only the linear portion is included in the calculations
3. Copy the "Slope" column from the data table from SoftMax Pro and paste the data in the excel template

Plate Set-up

	1	2	3	4	5	6	7	8	9	10	11	12

A	Gradient 1 Part 1 Replicate 1	Gradient 1 Part 2 Replicate 1	Gradient 1 Part 3 Replicate 1	Gradient 1 Part 4 Replicate 1	Gradient 1 Part 5 Replicate 1	Gradient 1 Part 6 Replicate 1	Gradient 2 Part 1 Replicate 1	Gradient 2 Part 2 Replicate 1	Gradient 2 Part 3 Replicate 1	Gradient 2 Part 4 Replicate 1	Gradient 2 Part 5 Replicate 1	Gradient 2 Part 6 Replicate 1
B	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2
C	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3
D												
E												
F												
G												
H												

By using this plate set-up you can cut and paste your data into an excel template

PEPCase Assay Reagents

100 mM Bicine pH 7.8 (same as GAPDH assay)
In 250 ml dH_2O
+ 4.08 g bicine (fw 163.2; Sigma B-3876)
+ 1.02 g $MgCl_2 \bullet 6H_2O$, 20mM (fw 203.3; Sigma M-2670)
+ 0.37 g KCl 20mM (fw 74.55; Sigma P-4504)
+ 0.21 g EDTA 2mM (fw 416.2; Sigma ED4SS)
pH to 7.8 using KOH

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

1 M $NaHCO_3$:
In 100 ml dH_2O
+ 8.4 g $NaHCO_3$ (fw 840.01)

50 mM PEP:
In 10 ml dH_2O
+ 0.117 g PEP (fw 234.0; Sigma P-7002)
Divide into 1.5 ml microfuge tubes and store in -80°C

2 M HCl:
In 418 ml of dH_2O
+ 82 ml HCl 37.5%, 12.2 M
Dilute in fume hood, ****Remember Acid into water never the reverse*******

Malate Dehydrogenase:
1. Pipette 1.2 ml of enzyme into 2 ml tube
2. Centrifuge at max speed for 10 min
3. Remove supernatant and resuspend in 1.6 ml 30% glycerol bicine buffer
   (This makes 5 U/µl malate dehydrogenase)