Carbo Assay Plate Reader

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Sean Weise

Materials:
G6P, Glucose, Sucrose, F6P, Fructose Assay:
150 mM Hepes Buffer pH 7.2
50 mM NADP
50 mM ATP
1 u / 4 µl Glucose-6-phosphate dehydrogenase, G6PDH (Sigma G-8529)
1 u / 4 µl Hexokinase, HEX (Sigma H-4502)
1 u / 1 µl Phosphogucose isomerase, PGI (Sigma P-9544)
5 u / 1 µl Invertase, INV (Sigma I-4504)
MDS M2 Plate Reader with path check

Procedure:
G6P & Glucose Assay

1. To help insure consistency and eliminate some pipetting errors pre-mix the Hepes buffer, NADP, ATP, and in cases of assays needing only glucose measurements (starch, sucrose, glucose) add G6PDH as well in a falcon tube according to the following formulas
   + 180 µl Hepes buffer * # of samples (110 mM Hepes in well)
   + 10 µl NADP * # of samples (500 nmol in well)
   + 10 µl ATP * # of samples (500 nmol in well)
   + 2 µl G6PDH * # of samples (0.4 unit in well)
2. Add 5 – 50 ul of sample (40 µl works well more than that and it gets noisy) to each well (use repeating pipette) do in triplicate if possible
3. Without touching sample add 200 µl of the buffer mix to each well (use multi channel pipette)
4. Place plate in plate reader, First do a kinetic assay at λ = 340 nm, use mixing option to thoroughly mix before assaying, don't worry about a second wavelength or using path check we just want a quick read to ensure there is a stable baseline
5. One you have a stable baseline do an endpoint assay, at 340. If trying to determine metabolites such as G6P or maltose that are in low concentration do not use path check but instead record the OD's and determine the pathlength at the end. For metabolites such as glucose, sucrose and starch turn path check on so that the OD will be normalized to a 1 cm path length, check the box marked water constant since we are using a aqueous system. It is not necessary to specify a background OD
6. While you are obtaining a baseline reading add 5 µl of HXK (1 units in well) to each prong of the hedge hog tool (use repeating pipette)
7. After obtaining the basline OD record data in an open Excel spreadsheet and set up plate reader for a kinetic assay at 340 nm only as we did in step 4
8. Eject plate and add HXK using the hedge hog tool, quickly start the kinetic assay
9. Allow reaction to proceed and when you have a stable plateau stop kinetic assay and do another end point assay as we did in step 5.
10. To assay fructose and F6P add 2 µl of PGI using the hedge hog tool and allow reaction to go to completion.

NOTE: If you with to separate the Fructose and F6P, you will need to perform a second assay adding G6PDH first to get rid of any G6P, then add PGI this will tell you how much F6P you can

then subtract this from you combined fructose F6P measurement. If your extra paranoid you can then add hexokinase and this will tell you how much Glucose and Fructose combined you have.

11. To assay sucrose add 10 µl of Invertase using the hedge hog tool and allow the reaction to go to completion.

NOTE: The invertase has a pH optimum ≈ 5 and a temperature optimum of 55°C however the enzyme will work at pH 7 and room temp, just slower so it will work to add it directly to the plate, we just have to add a little more enzyme i.e. 50 units instead of 1 unit. The reaction can take as long as 1.5 hr.

NOTE: Since sucrose breaks down to glucose and fructose if you have previously added PGI you will be measuring both the glucose and the fructose from the sucrose, so make sure to divide your calculated sucrose concentration by two.

NOTE: Total well volume of most 96 well plates is 370 µl, recommended working volume is between 200 – 250 µl

Reactions:

Sucrose (*INV*) → **Glucose** + Fructose

Glucose + (*HXK* + *ATP*) → **G6P** (*G6PDH* + *NADP*) → **6-Phosphogluconate** + NADPH (absorbs at 340nm)

Fructose (*HXK* + *ATP*) → **F6P** (*PGI*) → **G6P**

Math:
ΔOD / 6220 (extinction coefficient at 340 nm) * 240e-6 (volume of well) / 40e-6 (amount of sample added to the well) = Molarity of sample

These assays were adapted from:

Lowry, Oliver H., and Passonneau, Janet V., (1972) A flexible system of enzymatic analysis. Academic Press Inc.

CARBOHYDRATE ASSAY REAGENTS

Hepes buffer pH 7.2:
In 500 ml of distilled H_2O
+ 17.9 G Hepes, 150 mM (fw 238.3)
+ 1.5 g $MgCl_2$, 15 mM (fw 203.3)
+ 0.6 g EDTA, 3 mM (fw 416.2)
pH to 7.2 using KOH or HCl

50 mM ATP:
In 10 ml of distilled H_2O
+ 0.276 g ATP (fw 551.1; Sigma A-2383)
pH to 7.0 using KOH

50 mM NADP:
In 10 ml distilled H_2O

+ 0.383 g NADP (fw 765.4; Sigma N-0505)

30% Glycerol solution for enzymes:
In 7 ml 150 mM Hepes buffer, use citrate buffer when making up Invertase
+ 3 ml Glycerol
usually X units of enzyme per Y μl of 30% glycerol solution

20 mM Glucose Standard
In 10 ml dH_2O
+ 0.036 g Glucose (fw 180.16)
Aliquot and store in 2 ml tube

50 mM Citrate buffer pH 4.5:
In 250 ml dH_2O
+ 2.63 g citric acid (fw 210.15)
pH to 4.5 using KOH pellets