Methenyl-THF Cyclohydrolase Assay

Assay monitors the hydrolysis of 5,10-methenyl-THF to 10-formyl-THF by following the decrease in absorbance at 355 nm. The substrate, 5,10-methenyl-THF, has a high non-enzymatic buffer-catalyzed hydrolysis rate, which must be subtracted from the experimentally-measured rate. This non-enzymatic hydrolysis can be minimized by using potassium maleate as the buffer, which has a 40-fold lower rate than phosphate buffer (Rabinowitz, J. C. (1960) in *The Enzymes* (Boyer, P. D., ed) Vol. 2, 2nd Ed., pp. 185-252, Academic Press, New York).

Procedure:

Prepare fresh cocktail:

5 ml 1 M K•maleate (pH 7.4) (use KOH to adjust pH of maleic acid)
34 µl 2-mercaptoethanol (14.6 M stock)
14.2 ml H_2O
19.375 ml = ~50 assays at 375 µl/tube

Leaves room for 100 µl sample/buffer + 25 µl substrate = 0.5 ml total reaction volume. Samples should be diluted in the buffer cocktail to minimize non-enzymatic hydrolysis.

Substrate: stock solution is ~2 mM. Dilute 10 µl into 1 ml 0.36 N HCl and measure A_{350} to calculate actual concentration:

$$A_{350}/24.9\ mM^{-1} \times 100 = mM$$

Final assay concentrations are:

0.2 M K•maleate (pH 7.4)
20 mM 2-mercaptoethanol
~100 µM (R,S)5,10-methenyl-THF

Blank the spec at 355 nm with 20 µl substrate in 0.5 ml 20 mM 2-mercaptoethanol/0.1 N HCl (13.5 µl BME into 10 ml 0.1 N HCl)

Assay:

- add 375 µl cocktail to cuvette
- add 100 µl sample/buffer
- initiate rxn with 25 µl substrate stock; cap cuvette with patch of parafilm and invert 3X to mix.
- place in spec and start recording

Data reduction: calculate ΔA_{355} over 15-60 sec (may need to ignore first 10-15 sec to get stable slope), then convert to ΔA_{355}/min by multiplication.

Need to do a no-enzyme blank to determine the non-enzymatic rate of substrate hydrolysis – this will be substracted from each enzyme sample. Typical rate for no-enzyme blank is 0.03-0.06 ΔA_{355}/min

For the SpectraMax, find the Cyclo file in the Protocol Folder of the SoftMax folder. Choose CuvetteSet; Kinetics; Vmax. To blank the instrument, read the cuvette with substrate + BME/HCl using the REF button. Samples can then be read using the READ button. They can each be named using the Template function, and a table can be printed out with all the data. The software reports the the V_{max} as change in milli A_{355}/min [i.e. $\Delta(A_{355} \times 10^{-3})$ per min].

$$\frac{1uM}{.0249A355} \times \Delta A355/\min = \frac{1umol}{l} = \frac{nmol}{ml} \times 0.5ml = nmol product/\min$$

ΔA_{355}/min X 20.1 = nmol/min = mU