

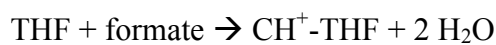
Cobalamin-Dependent Methionine Synthase Assay

This protocol is based on the method developed by the Matthews lab [Drummond, J. T., Jarrett, J., Gonzalez, J. C., Huang, S., and Matthews, R. G. (1995) *Anal. Biochem.* **228**, 323-329; Jarrett, J. T., Goulding, C. W., Fluhr, K., Huang, S., and Matthews, R. G. (1997) *Methods Enzymol* **281**, 196-213]

The enzyme catalyzes the following reaction:



The product, THF, is detected spectrophotometrically following its conversion to 5,10-methenyl-THF by heating with formate in acid:



At acidic pH, CH⁺-THF has an extinction coefficient of 26,500 M⁻¹cm⁻¹ at 350 nm

1. Add the following to 12x75mm glass tubes:

| | |
|--------|---------------------------------|
| 494 μL | H ₂ O |
| 80 μL | 1.0 M KPO ₄ (pH 7.2) |
| 40 μL | 500 mM DTT |
| 4 μL | 3.8 mM AdoMet |
| 4 μL | 100 mM L-homocysteine |
| 50 μL | enzyme sample |
| 80 μL | 500 μM hydroxocobalamin |

mix well, then preincubate at 37°C for 5 minutes

2. Initiate reactions with 48 μL 4.2 mM CH₃THF
mix well, incubate at 37°C for 10 minutes.
3. Stop reactions with 200 μL 5N HCl/60% formic acid
mix well, then incubate at 80° C for 10 minutes in a heat block. Cool to room temperature.
4. Transfer each 1 ml reaction to 1.5 mL microcentrifuge tube and centrifuge at room temperature at top speed for 5 minutes to pellet precipitated protein, then measure absorbance at 350 nm (zero the instrument against water). This centrifugation step is not necessary for samples with low protein concentration.

Blanks -- two kinds of blanks can be used:

- (i) The simplest is a “no enzyme” blank. Use 50 μl of whatever buffer your sample was in. This is fine for monitoring column fractions or when all the samples have the same protein content/concentration (e.g. kinetics). This single blank can be subtracted from all of the

samples in the assay. The no enzyme blank is typically 0.280 – 0.300 A_{350} in our hands.

(ii) “minus homocysteine” blank. For every enzyme sample, a second set of tubes contains the same amount of enzyme, but minus homocysteine. Use 4 μl of H_2O instead. This blank is necessary when measuring activity in crude extracts.

All samples and blanks should be performed in duplicate or triplicate. A standard curve for THF can be prepared under a given set of assay conditions to correct for variations in yield and linear range. For following activity in column fractions, etc., a standard curve is not necessary.

Calculations

Activity in nmol/min (milliunits, mU) is calculated from the extinction coefficient of CH^+ -THF in acid = $26.5 \times 10^{-6} \text{ nM}^{-1}$, the volume of the reaction, and the time:

$$\text{Corrected } A_{350} \times \left(\frac{1.0 \text{ ml}}{26.5 \times 10^{-6} \text{ nM}^{-1} \times 10 \text{ min} \times 10^3 \text{ ml/L}} \right) =$$

$$\text{Corrected } A_{350} \times 3.774$$

Reagents

1. L-homocysteine
Dissolve 50 mg L-homocysteine thiolactone (Sigma) in 1.7 ml H_2O . Add 0.83 ml 0.8 M NaOH, bubble with argon for 5 min and incubate at 45°C , 6 min. Acidify with 5.0 M acetic acid to pH 5 (check with pH paper); dilute to 3.3 ml with argon-bubbled H_2O to yield ~ 100 mM solution. Store in 1 ml aliquots at -80°C .

Check actual concentration by titration with DTNB (see pp. 155 and 220 of Chemical Modification of Proteins, by Means and Feeny, Holden-Day, Inc., 1971):

- prepare 10 mM DTNB stock in 50 mM K•Phos (7.0) (39.6 mg/10 ml)
 - dilute 100 mM hcy stock 1:100 and add 50 μl to 900 μl 0.1 M Na•Phos (8.0)
 - add 50 μl DTNB reagent. Stand 2-3 min, read at 412 nm.
 - subtract buffer blank, divide corrected A_{412} by the extinction coefficient for the liberated thionitrobenzoate anion (13.6 mM^{-1}) to calculate actual L-homocysteine concentration in stock
2. (6*R*,5)-methyl-THF (monoglutamate form; calcium salt, Schircks Laboratories, Jona, Switzerland): prepare 4.2 mM stock in 8 mM Na•Ascorbate. Store in dark under argon at -20°C
 3. hydroxocobalamin (Sigma H-8017 or ICN 157408): dissolve to 500 μM in H_2O . Store in dark at 4°C .
 4. S-adenosyl-L-methionine (chloride salt, Sigma or *p*-toluensulfonate salt, BASF): dissolve to 3.8 mM in 1 mM HCl. Store at -70°C .
 5. formate/HCl: slowly add 41.6 ml concentrated HCl (12 N) to 58.4 ml of 88% formic acid.