Cobalamin-Dependent Methionine Synthase Assay


The enzyme catalyzes the following reaction:

\[
5\text{-CH}_3\text{-THF} + \text{L-homocysteine} \rightarrow \text{THF} + \text{methionine}
\]

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

\[
\text{THF} + \text{formate} \rightarrow \text{CH}^+\text{-THF} + 2 \text{H}_2\text{O}
\]

At acidic pH, CH\(^{+}\)-THF has an extinction coefficient of 26,500 M\(^{-1}\)cm\(^{-1}\) at 350 nm

1. Add the following to 12x75mm glass tubes:

<table>
<thead>
<tr>
<th>494 µL</th>
<th>H(_2)O</th>
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<tbody>
<tr>
<td>80 µL</td>
<td>1.0 M KPO(_4) (pH 7.2)</td>
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<tr>
<td>40 µL</td>
<td>500 mM DTT</td>
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<tr>
<td>4 µL</td>
<td>3.8 mM AdoMet</td>
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<tr>
<td>4 µL</td>
<td>100 mM L-homocysteine</td>
</tr>
<tr>
<td>50 µL</td>
<td>enzyme sample</td>
</tr>
<tr>
<td>80 µL</td>
<td>500 μM hydroxocobalamin</td>
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</table>

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

2. Initiate reactions with 48 µL 4.2 mM CH\(_3\)THF

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$_2$O 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 X 10$^{-6}$ nM$^{-1}$, the volume of the reaction, and the time:

$$\text{Corrected } A_{350} \times \left( \frac{1.0 \text{ ml}}{26.5 \times 10^{-6} \text{ nM}^{-1} \times 10 \text{ min} \times 10^{-3} \text{ ml/L}} \right) = C_{\text{corrected}} A_{350} \times 3.774$$

Reagents
1. L-homocysteine
   Dissolve 50 mg L-homocysteine thiolactone (Sigma) in 1.7 ml H$_2$O. 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$_2$O to yield ~ 100 mM solution. Store in 1 ml aliquots at –80°C.

• 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. (6R,S)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$_2$O. 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.