Enzymatic Assay of OROTATE REDUCTASE
(EC 1.3.1.14)

PRINCIPLE:
Orotate + β-NADH > Dihydroorotate + β-NAD

Abbreviations used:
β-NADH = β-Nicotinamide Adenine Dinucleotide, Reduced Form
β-NAD = β-Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS:  T = 25°C, pH = 6.5, A\textsubscript{340nm}, Light Path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 400 mM Sodium Phosphate Buffer, pH 6.5 at 25°C
   (Prepare 200 ml in deionized water using Sodium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. S-0751. Adjust to pH 6.5 at 25°C with 1 M NaOH.)

B. 400 mM Cysteine Solution (Cys)
   (Prepare 10 ml in deionized water using L-Cysteine Hydrochloride, Monohydrate, Sigma Prod. No. C-7880. Adjust to pH 6.5 at 25°C with 1 M NaOH.)

C. 10 mM Orotic Acid Solution (Orotic)
   (Prepare 10 ml in 10 mM NaOH using Orotic Acid, Monohydrate, Sigma Prod. No. O-2625. Adjust to pH 6.5 with 10 mM HCl.)

D. 1.6 mM β-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (β-NADH)
   (Prepare 2 ml in Reagent A using β-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Prod. No. N-8129, or dissolve the contents of one 5 mg vial of β-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-105, in the appropriate volume of Reagent A. PREPARE FRESH.)
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REAGENTS:  (continued)

E. 3 mM Flavin Adenine Dinucleotide Solution (FAD)  
(Prepare 1 ml in deionized water using Flavin Adenine  
Dinucleotide, Disodium Salt, Sigma Prod. No. F-6625.  
PREPARE FRESH.  PROTECT FROM LIGHT.)

F. Orotate Reductase Enzyme Solution  
(Immediately before use, prepare a solution containing  
1 - 2 units/ml of Orotate Reductase in cold Reagent  
A.)

PROCEDURE:

Prepare a reaction cocktail by pipetting (in milliliters)  
the following reagents into a suitable container:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>6.40</td>
<td></td>
</tr>
<tr>
<td>Reagent A (Buffer)</td>
<td>10.00</td>
<td></td>
</tr>
<tr>
<td>Reagent B (Cys)</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>Reagent C (Orotic)</td>
<td>6.00</td>
<td></td>
</tr>
<tr>
<td>Reagent E (FAD)</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

Mix by stirring and adjust to pH 6.5 at 25°C with either  
1 M HCl or 1 M NaOH.

Pipette (in milliliters) the following reagents into  
suitable cuvette:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Cocktail</td>
<td>2.75</td>
<td>2.75</td>
</tr>
<tr>
<td>Reagent F (Enzyme Solution)</td>
<td>0.05</td>
<td>------</td>
</tr>
<tr>
<td>Reagent A (Buffer)</td>
<td>------</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Mix by inversion and incubate for 5 minutes at 25°C to  
activate the enzyme. Monitor the $A_{340nm}$ until constant,  
using a suitably thermostatted spectrophotometer. Then add:

Reagent D ($\beta$-NADH)    | 0.20  | 0.20  |

Immediately mix by inversion and record the decrease in  
$A_{340nm}$ for approximately 5 minutes. Obtain the $r$ $A_{340nm}$/minute  
using the maximum linear rate for both the Test and Blank.
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CALCULATIONS:

\[
\text{Units/ml enzyme} = \frac{(r_{A_{340nm}}/\text{min Test} - r_{A_{340nm}}/\text{min Blank}) \times (3) \times (\text{df})}{(6.22)(0.05)}
\]

3 = Total volume (in milliliters) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of β-NADH at 340 nm
0.05 = Volume (in milliliter) of enzyme used in the assay

\[
\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}
\]

\[
\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}
\]

UNIT DEFINITION:

One unit will convert 1.0 µmole of orotic acid to dihydroorotate per minute at pH 6.5 at 25°C.

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 167 mM sodium phosphate, 67 mM cysteine hydrochloride, 2.0 mM orotic acid, 0.01 mM FAD, 0.11 mM β-NADH and 0.05 - 0.1 unit orotate reductase.

REFERENCE:


NOTES:

1. This assay is a modification of the enzyme assay described in the cited reference.

2. All product and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.

This procedure is for informational purposes. For a current copy of Sigma’s quality control procedure contact our Technical Service Department.