Echelon Biosciences Inc.

MEP Synthase (DXR) Enzyme Inhibitor Screen

K-2000C

Support: echelon@echelon-inc.com

Description: 96-well medium throughput assay for identifying potential antimicrobials and specific inhibitors of the MEP Synthase (DXR) enzyme

Materials Provided

Catalog #	Description	Amount
E-2000C	MEP Synthase (DXR) Enzyme (50 μg)	1 vial
K-2001C	1-deoxy-D-xylulose-5-phosphate (DXP) Substrate	1 bottle
K-2002C	DXR Reaction Buffer	1 bottle
K-2003C	β-NADPH	1 bottle
K-2004C	Fosmidomycin Inhibitor	1 vial
---	Reaction Incubation Plate (UV transparent flat bottom)	1 plate
---	Microtiter Plate Seal	1 seal

Additional Materials Provided by User:

- Microtiter plate reader capable of reading absorbance, specifically at 340 nm.
- Pipettes (capable of delivering between 5 and 1,000 uL)
- Microcentrifuge tubes

Storage:

Kit can be stored unopened at -20°C for up to six months. All components and solutions should be protected from light.

Quick Protocol

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Background:

The methylerythritol phosphate (MEP) pathway is used by most bacteria, including all Gram-negative bacteria, for isoprenoid biosynthesis. Isoprenoids comprise one of the most diverse classes of compounds found in nature. With over 50,000 different isoprenoids identified to date, they exhibit a broad range of structural complexity and are involved in a variety of biological functions. [1] Electron transport (quinones), stabilization of cell membranes (hopanoids and sterols), cell wall biosynthesis (dolichols), signal transduction (prenylated proteins), photosynthesis (chlorophylls) and modification of tRNAs are among the processes that involve isoprenoids. [2] Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the precursors for all isoprenoid compounds and the MEP pathway is one of two unrelated essential pathways existing in nature for their biosynthesis. These two precursors are produced by either the mevalonate (MVA) or MEP pathway. The MVA pathway is found primarily in eukaryotes, including humans, plant cytosol, Archaea, and some Gram-positive bacteria, while the MEP pathway is utilized by most bacteria and plant chloroplasts. Due to this natural distribution, the MEP pathway represents a promising target for development of novel antibacterial agents and herbicides. [3]

In the first pathway-specific reaction of the MEP pathway for isoprenoid biosynthesis, MEP Synthase (DXR) catalyzes the rearrangement of 1-deoxy-D-xylulose-5-phosphate (DXP) to generate 2-C-methyl-D-erythritol-4-phosphate (MEP) in the presence of β-nicotinamide adenine dinucleotide phosphate (NADPH) and a divalent cation. [4, Figure 1] Fosmidomycin is a natural product inhibitor of MEP synthase and has validated the MEP pathway as an antibiotic target.

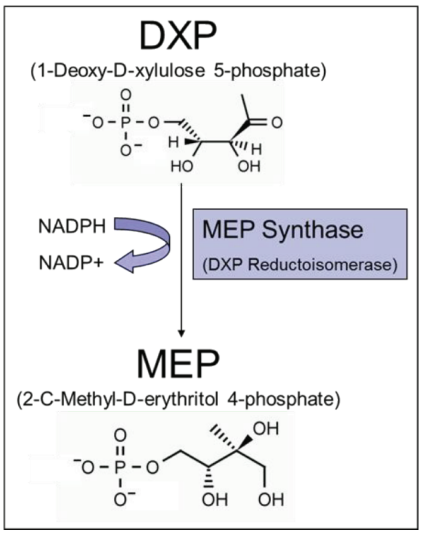

Figure 1:
Diagram of MEP synthesis from DXP using MEP synthase, a.k.a. DXP.

Assay Design:

Echelon's MEP Synthase (DXR) assay, provided in a medium throughput screening format, will evaluate compounds for inhibition of DXR activity. The assay monitors the depletion of β-NADPH which is detected spectrophotometrically. By measuring this depletion of β-NADPH one can infer the conversion of the DXP substrate to the MEP product. Fosmidomycin is supplied in this assay as a control inhibitor.

Assay Kit Notes:

1. The concentration of MEP Synthase utilized in the enzymatic reaction may be adjusted in order to further optimize and monitor enzyme activity.
2. DMSO concentrations of up to 5% can be utilized for compound preparation without any effect on assay performance.
3. If the user's spectrophotometer is unable to read the plate in a continuous kinetic mode you may read the plate at a single time point, suggested time is 10 minutes, to calculate percent inhibition. If this method is utilized, we suggest running additional controls for each compound to account for potential inhibitor related interference of absorbance at 340 nm, which may result in false positives and/or negatives. This should be done by running the test compound reactions with and without the DXR enzyme, allowing the user to subtract any potential background signal that may be related to individual inhibitors.
4. The MEP Synthase Enzyme Inhibitor Screen is designed to be a one-time use assay. If the inhibitor screen is to be run on separate occasions, the remaining kit reagents can be stored at -20 °C; however some degradation of the β-NADPH reagent has been observed with multiple freeze-thaw cycles.

Assay Procedure:

Please read this entire section and the assay notes section before beginning the assay.

1. Bring reagents to room temperature before use. Place the MEP Synthase enzyme (E-2000C) on ice.

2. Prepare 1.2 mM DXP Substrate by adding 2,079 µL DXR Reaction Buffer (K-2002C) to DXP Substrate bottle (K-2001C). Vortex to mix. Keep at room temperature.

3. Prepare 15 mM β-NADPH by adding 240 µL DXR Reaction Buffer (K-2002C) to β-NADPH vial (K-2003C). Vortex to mix. Keep at room temperature.

4. Preparation of the β-NADPH Standard Curve (see Table 1):
   a. Using the chart above, prepare the β-NADPH Standards in microcentrifuge tubes.
   b. Add 200 µL of each β-NADPH standard to the appropriate wells (Rows A-D, Columns 1&2).

5. Preparation of DXR Reaction Controls:
   a. Add 90 µL of DXR Reaction Buffer (K-2002C) to the "No Enzyme Control", "No Substrate Control", and "Full Rxn (-Inhibitor)" wells (Rows E-G, Columns 1&2).
   b. Prepare the Fosmidomycin Inhibitor control by adding 20 µL of 1 mM Fosmidomycin Inhibitor (K-2004C), 10X the final desired concentration, followed by 70 µL DXR Reaction Buffer (K-2002C) to each of the "Full Rxn (+ Inhibitor)" wells (Row H, Columns 1&2).

6. Preparation of test compounds (Rows A-H, Columns 3-12):
   a. Prepare the compounds to be tested at 10X their final desired concentration.
   b. Add 20 µL per well, of the above 10X preparation, followed by 70 µL of DXR Reaction Buffer (K-2002C) per well.

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7. Prepare 2.5 µM MEP Synthase by adding 444.6 µL of DXR Reaction Buffer (K-2002C) minus the volume stated on the E-2000C vial to the 50 µg size of MEP Synthase (DXR) Enzyme (E-2000C). Mix thoroughly by pipetting up and down 5-10 times. Place on ice until needed.

8. No Enzyme Control Solution: Prepare 250 µL of "No Enzyme Control Solution" by combining the following. Mix thoroughly.
   a. 246 µL DXR Reaction Buffer (K-2002C).
   b. 5.6 µL of 15 mM β-NADPH solution (Prepared in step 3).

9. DXR Reaction Solution: Prepare 8 mL of "DXR Reaction Solution" by combining the following. Mix thoroughly.
   a. 7,466 µL DXR Reaction Buffer (K-2002C).
   b. 178 µL of 15 mM β-NADPH solution (Prepared in step 3).
   c. 356 µL of 2.5 µM MEP Synthase (Prepared in step 7).

10. Add 90 µL of "No Enzyme Control Solution" to the "No Enzyme Control" wells (Row E, Columns 1&2).

11. Add 90 µL of "DXR Reaction Solution" to the "No Substrate Control", "Full Rxn (- Inhibitor)", "Full Rxn (+ Inhibitor)", and all of the "Test Compounds" wells (Rows F, G, and H, Columns 1&2; Rows A-H, Columns 3–12).

12. Cover the plate with a plate seal and incubate the controls and test compounds for 10 minutes at room temperature with shaking.

Initiation of the DXR Inhibitor Reactions

13. Prepare the plate reader and then add 20 µL "DXR Reaction Buffer" (K-2002C) to the "No Substrate Control" wells (Row F, Columns 1&2).

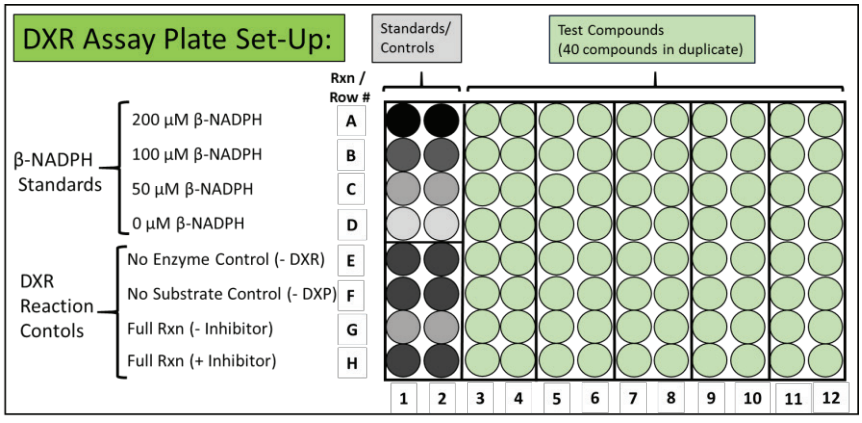
14. Add 20 µL 1.2 mM DXP Substrate (Prepared in step 2.) to the "No Enzyme Control", "Full Rxn (- Inhibitor)", "Full Rxn (+ Inhibitor)", and all of the "Test Compounds" wells to start the reactions (Rows E, G, and H, Columns 1&2; Rows A-H, Columns 3–12).

15. Read the absorbance at 340 nm in kinetic mode, reading every 30 seconds for ten minutes

Table 1, β-NADPH Standards

β-NADPH Standard (µM)	Volume of β-NADPH solution (µL)	Volume of DXR Reaction Buffer (µL)
200	12 (15 mM solution)	888
100	450 (200 µM above)	450
50	450 (100 µM above)	450
0	0	900

Table 2. Suggested DXR Plate Layout



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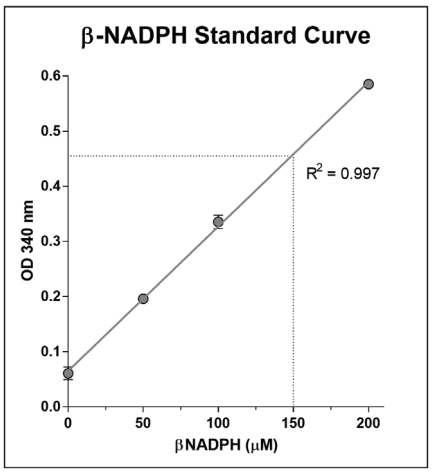
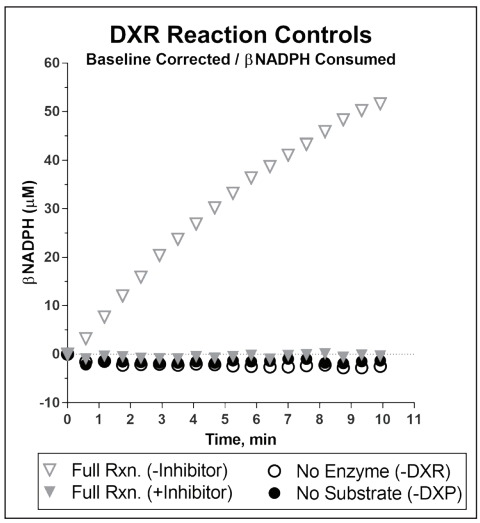
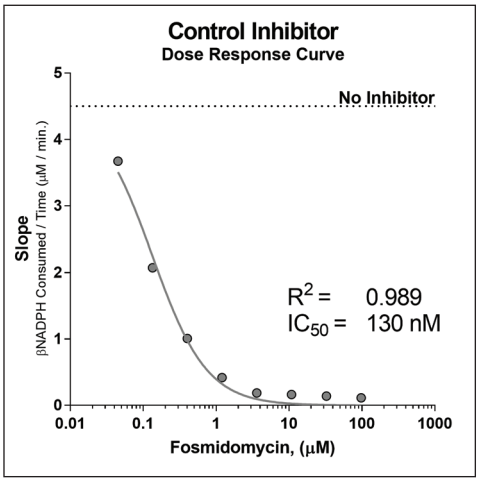
Supporting Figures:



Supporting Figure 1:
β-NADPH standard curve measuring β-NADPH in solution. Use computer software to generate a linear regression curve. Using this curve one can interpolate the amount of β-NADPH consumed in the enzyme reactions.



Supporting Figure 2:
DXR Reaction Controls. A concentration of 100 μM was utilized for the control inhibitor.



Supporting Figure 3:
Control Inhibitor's IC50 Curve. Fosmidomycin demonstrates an IC50 value of 130 nM utilizing the K-2000C assay conditions. The reactions were run for 10 minutes and corrected for baseline during analysis. The IC50 is analyzed using the log (inhibitor) vs. response – variable slope (four parameter) curve fit using GraphPad Prism Software.

References (Background):

1. Bochar, D.A.; Freisen, J.A.; Stauffacher, C.V. and Rodwell, V.W.(1999) in Comprehensive Natural Products Chemistry, (Cane, D. Ed.) Pergamon Press, Oxford, pp. 15-44.
2. Sacchettini, J.C. and Poulter, C.C. (1997) Science, 277(5333), 1788-9.
3. Testa, C.A.; Brown, M.J. (2003) Current Pharmceutical Biotechnology, 4, 248-259.
4. Koppisch, A.T.; Fox, D.T.; Blagg, B.; Poulter, C.D. (2002) , Jan 8;41(1), 236-43.

Related Products

Products	Catalog Number
Assays and Reagents	
IspD Assay	K-2000D
CDP-MEP Synthesis Kit	K-2000E
Isoprenoid Diphosphate Reagents	I-0050 I-0051
Malachite Green Assay	K-1500
MEP Pathway Intermediates	
1-Deoxy-D-xylulose 5-phosphate (DXP) 1-Deoxy-D-xylulose (DX) 2-C-Methyl-D-erythritol 4-phosphate (MEP) 2-C-Methyl-erythritol (ME) 4-Diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME)	I-M050 I-M050A I-M051 I-M051A I-M052
MEP Pathway Inhibitors	
FR900098 5-Ketoclomazone IspF Inhibitor 1	B-4202 B-4101 B-4102

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