Echelon Biosciences Inc.

CDP-MEP Enzymatic Synthesis Kit

K-2000E

Support: echelon@echelon-inc.com

Description: Kit contains compontents necessary for the synthesis of CDP-MEP.

Materials Provided

Catalog #	Description	Amount
I-MO52	CDP-ME (4-diphosphocytidyl-2-C-methyl-D-erythritol)	1 vial
E-2000E	CDP-ME Kinase (IspE)	80 µg
K-2002E	CDP-MEP Synthesis Buffer	25 mL
K-ATP2	10 mM ATP Solution	300 µL
K-LUMa	ATP Detection Reagent	4 mL
K-DTT1	Dried Dithiothreitol (DTT) Powder	50 µmol
---	96-well White Round Bottom Plate	1 plate
---	Microtiter Plate Seal	1 seal

Additional Materials Provided by User:

- Pipettes (capable of delivering between 5 and 1,000 µL)
- Spectrophotometer / Luminometer capable of reading multiwell plates
- Heat block / water bath for boiling samples
- Liquid nitrogen (optional)

Quick Protocol

Prepare Synthesis Buffer and Substrate

Prepare CDP-MEP Synthesis Reaction and controls

Add the CDP-ME substrate to start the reaction

At 1 min, pull 2 µL of Reaction for analysis. Boil to stop the reaction.

At 60 min, pull 2 µL of Reaction for analysis. Boil to stop the reaction.

Analyze the ATP levels of the CDP-MEP synthesis reactions

Storage:
Kit can be stored unopened at -20°C for up to six months. Prepare aliquots of CDP-MEP and freeze. All components and solutions should be protected from light.

Echelon Biosciences Inc.
Ph: 801-588-0455
Fax: 801-588-0497
Echelon-inc.com

Background

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 two unrelated essential pathways exist in nature for their biosynthesis. Until recently it was assumed that all organisms required the mevalonate (MVA) pathway for IPP and DMAPP biosynthesis. Work in the laboratories of Rohmer and Arigoni revealed the existence of an alternate pathway to IPP and DMAPP, the methylerythritol phosphate (MEP) pathway [3,4]. The MEP pathway is utilized by most bacteria, including all Gram-negatives, and plant chloroplasts while the MVA pathway is found in humans, plant cytosol and many Gram-positive bacteria. Due to this natural distribution, the MEP pathway represents a promising target for development of novel antibacterial agents and herbicides [5].

In the fourth step of the MEP pathway, CDP-ME is converted to 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate (CDP-MEP) by CDP-ME kinase (IspE) with the concomitant formation of adenosine diphosphate (ADP). The formation of CDP-MEP occurs at a 1:1 ratio with the consumption of ATP. By measuring the decrease in ATP levels the user can infer the amount of CDP-MEP generated. The user can expect 1,600 µL of > 600 µM CDP-MEP product (> 60% yield).

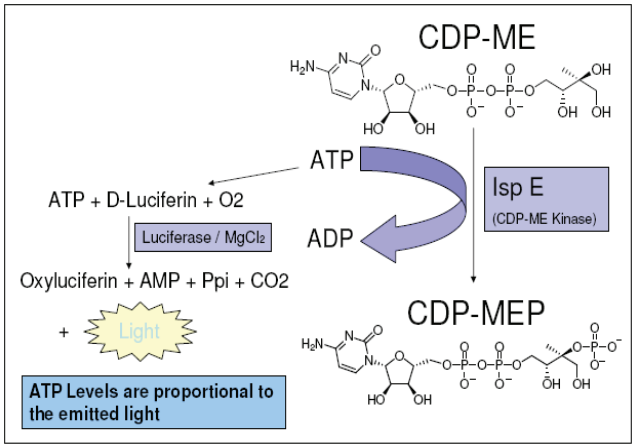

Figure 1

Diagram of CDP-MEP synthesis from CDP-ME using IspE and ATP. CDP-MEP synthesis can be indirectly quantified by monitoring ATP levels.

Reagent Preparations

1. "Complete CDP-MEP Synthesis Buffer" preparation
   a. Prepare 1 M DTT by adding 50 µL ddH2O to the vial of DTT (K-DTT1, 50 µmol). Vortex and proceed to the next step.
   b. Add 25 µL of 1 M DTT (K-DTT1) to the bottle of CDP-MEP Synthesis Buffer (K-2002E). Mix thoroughly.
2. CDP-ME Kinase (IspE) preparation
   a. Prepare 10 µM IspE enzyme for the CDP-MEP synthesis reaction by diluting the provided IspE enzyme (E-2000E) in "Complete CDP-MEP Synthesis Buffer" (Step 1, K-2002E) using the formulas below.

   246 µL - (x) µL = y (µL)

   Refer to label of IspE for vol. 'x'. 'y' is the required volume of "Complete CDP-MEP Synthesis Buffer" needed for 10 µM.
   b. Add the volume of "Complete CDP-MEP Synthesis Buffer" calculated in step 2a to the IspE (E-2000E) vial. Mix gently prior to use.
3. CDP-ME Substrate preparation

   Prepare a 1 mg/mL CDP-ME solution by adding an appropriate amount of "Complete CDP-MEP Synthesis Buffer" (Step 1) to the provided CDP-ME vial (I-M052). Vortex briefly.

CDP-MEP Synthesis Reaction Instructions

4. Pre-incubate "Complete CDP-MEP Synthesis Buffer" (Step 1) at 37 °C.
5. Preheat a heat block or water bath to 90 - 100 °C.
6. "Analysis Tube" Preparation: Label six 1.5 mL microcentrifuge tubes and add 98 µL of the "Complete CDP-MEP Synthesis Buffer" (Step 1) to all six tubes.
   - Analysis Tubes 1, 2 and 3 are for analyzing reactions 1, 2 and 3 at Time = 1 min.
   - Analysis Tubes 4, 5 and 6 are for analyzing reactions 1, 2 and 3 at Time = 60 min.
7. "Reaction Tube" Preparation: Prepare the CDP-MEP synthesis reaction and control reactions according to the table below in three separate 2.0 mL microcentrifuge tubes: *Combine all of the reagents except the substrate (CDP-ME).
8. Start the synthesis reaction by adding the substrate (CDP-ME), prepared in Step 3, to Reaction Tube #3 only, mix all tubes thoroughly and incubate the three reactions at 37 °C. (This is the start of the reaction, T = 0 min.)
9. After 1 minute (T = 1 min.) remove 2 µL from "Reaction Tubes 1, 2, and 3" and add to "Analysis Tubes 1, 2, and 3" prepared in step 3 (containing 98 µL of "Complete CDP-MEP Synthesis Buffer").

Table 1. Reaction Tube Guide

Tube	Synthesis Reactions	ATP (10 mM)	CDP-ME (1.8mM / 1 mg/mL)	IspE (10 µM)	"Complete CDP-MEP Synthesis Buffer" (µL)	Total Vol. (µL)
Reaction Tube #1	ATP Only Control	18.75 µL	-	-	106.25 µL	125 µL
Reaction Tube #2	Buffer Only Control	-	-	-	500 µL	500 µL
Reaction Tube #3	CDP-MEP Synthesis	243 µL	900 µL	162 µL	315 µL	1,620 µL
	Final Concentration	1.5 mM	1.0 mM	1 µM		

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10. Immediately boil "Analysis Tubes 1, 2, and 3" for 2 minutes and store at -80 °C for later analysis. (This step heat inactivates the IspE enzyme; quenching the reaction.)
11. After 1 hour (T=60 min.) remove 2 µL from "Reaction Tubes 1, 2, and 3" and add to"Analysis Tubes 4, 5, and 6" prepared in step 3 (containing 98 µL of "Complete CDP-MEP Synthesis Buffer").
12. Immediately boil "Analysis Tubes 4, 5, and 6" for 2 minutes and store at -80 °C for later analysis. (This step heat inactivates the IspE enzyme; quenching the reaction.)
13. Also after 1 hour (T = 60 min.) aliquot the remaining 1,616 µL from Reaction Tube #3, the "CDP-MEP Synthesis" reaction, into 100 µL aliquots for future experiments. Flash freeze each aliquot in liquid nitrogen and store at -80 °C.
    - DO NOT boil / heat the "CDP-MEP Synthesis" reaction
    - CDP-MEP is unstable and should be stored at -80 °C.
    - Preparing aliquots will help to avoid unnecessary freeze / thaw cycles for future experiments.
    - The kit does not produce pure CDP-MEP. In addition to CDP-MEP, the synthesis reaction contains residual ATP, CDP-ME, and ADP.

CDP-MEP Synthesis Analysis :

Preparation of ATP Standard Curve

Prepare a 100 µM ATP standard solution by diluting the 10 mM ATP solution (K-ATP2) provided in this kit. (ex. Add 5 µL K-ATP2 solution to 495 µL "Complete CDP-MEP Synthesis Buffer".) Prepare the ATP standards as described below. Diluted standards should range between 0 and 100 µM per well in a volume of 200 µL. It is advisable to make the ATP standards in solutions consistent with those to be used in the enzymatic synthesis reaction. See Table 2.

Synthesis Reactions Analysis

1. Thaw Analysis Tubes 1-6 at room temperature. (Stored from steps 9 through 12 in the CDP-MEP synthesis section at -80 °C.)
2. Add 25 µL of the ATP standards prepared above, in triplicate, to the appropriate wells. Refer to Table 3.
3. Add 25 µL from Analysis Tubes 1-6, in triplicate, to the appropriate wells.
4. Add 25 µL of the supplied ATP detection reagent (K-LUMa) to the all of the wells. (Protect the plate from light!)
5. Gently mix the plate for 10 minutes and incubate at room temperature to stabilize the luminescence signal.
6. Record luminescence.
7. Using graphing software:
   a. Plot an ATP standard curve (see Figure 2) and calculate the ATP levels of Analysis Tubes 1-6 by interpolating the values from the ATP standard curve.*Remember that Analysis Tubes 1-6 are a 1:50 dilution so the results must be multiplied by 50 to determine the actual amount of ATP consumed.
   b. Graph and contrast the ATP consumption of the CDP-MEP synthesis reaction and the controls to infer the amount of CDP-MEP formed in the reaction. (See Supporting Figure 1)

Figure 2

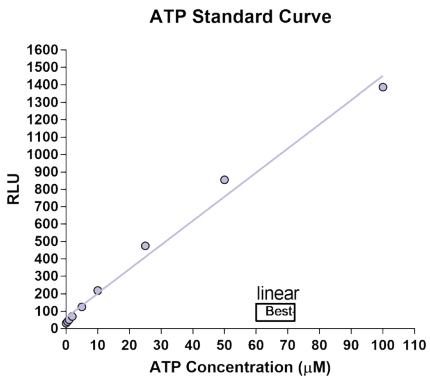
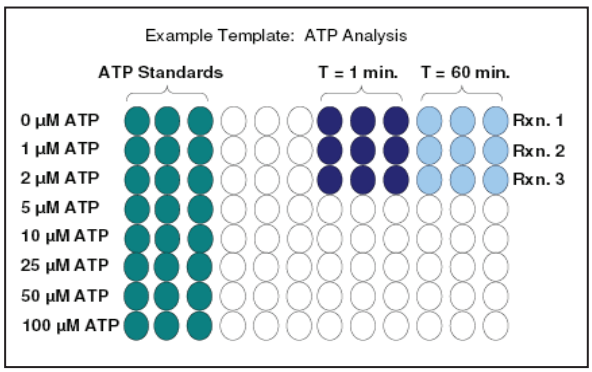


Table 2. ATP Standards

ATP concentration (µM)	Volume of 100 µM ATP solution (µL)	"Complete CDP-MEP Synthesis Buffer" (µL)
0	0	200
1	2	198
2	4	196
5	10	190
10	20	180
25	50	150
50	100	100
100	200	0

Table 3. Suggested Analysis Plate Layout



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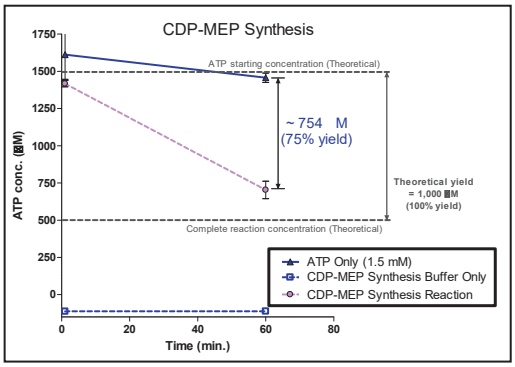
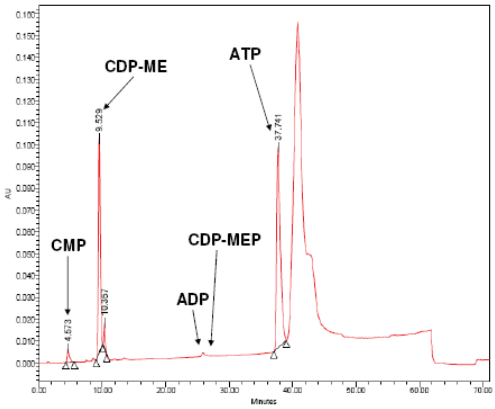
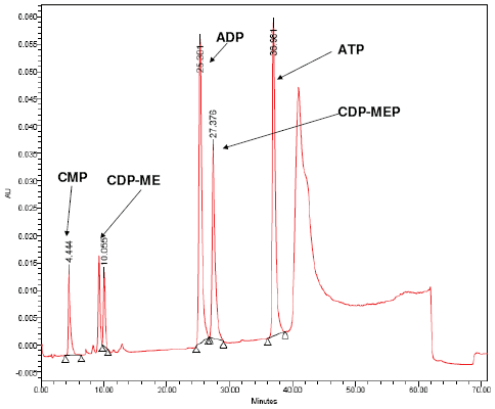


Supporting Figure 1:
Demonstrates the CDP-MEP synthesis reaction utilizing an ATP detection reagent to monitor ATP levels. The formation of CDP-MEP occurs at a 1:1 ratio with the consumption of ATP. By measuring the decrease in ATP levels the user can infer the amount of CDP-MEP generated.



Supporting Figure 2:
HPLC methods demonstrate the presence of the pre-reaction components, CDP-ME and ATP.



Supporting Figure 3:
HPLC methods confirm the formation of CDP-MEP, depletion of ATP and the resultant ADP.

Note: The kit does not produce pure CDP-MEP. In addition to CDP-MEP, the synthesis reaction contains residual ATP, CDP-ME, and ADP.

References (Background):

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5. Brown, M.J. (2003) Current Pharmceutical Biotechnology, 4, 248-259.
6. Bernal, C.; Palacin, C.; Boronat, A. and Imperial, S.(2005) Analytical Biochemistry 337, 55-61.

Related Products

Products	Catalog Number
Assays and Reagents	
MEP Synthase (DXR) Enzyme Inhibitor Screen	K-2000C
CDP-ME Synthase (IspD) Enzymatic Assay	K-2000D
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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