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EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: Evaluation of kinase type enzymes via ADP-dependent ATP depletion assay via NADH-dependent enzyme cascade

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Instruction

Evaluation of kinase type enzymes via ADP-dependent ATP depletion assay via NADH-dependent enzyme cascade

1 Introduction / Purpose

Since the two kinase steps in the “kinase/kinase/synthase” cascade for isobutene production involve ATP consumption, we established an enzymatic coupled assay for kinase activity in order to study the ATP dependent phosphorylation of the substrates involved in the cascade. Here, an ADP-dependent ATP depletion assay via NADH-dependent enzyme cascade is used for the detection and quantification of ATP consumption in order to follow indirectly the phosphorylation of substrates.

2 Equipment and chemicals

2.1 Equipment

Branson SONIFIER 250
Polarstar Omega plate reader

2.2 Chemicals

Isobutanol (2-methyl-1-propanol) (Fluka Chemika 99.5 % GC) Cat. No. 58450
Piruvate kinase(PK) (Roche, Cat. No. 10128155001)
L-Lactate Dehydrogenase (L-LDH) (Roche, Cat. No. 10127230001)
Micro BCA protein assay KIT (Thermo Scientific Cat. No. 23235)
Triethanolamine HCl (Sigma Cat. No. T-1502)
Phosphoenol pyruvate (Sigma Cat. No. P7252)
Magnesium sulfate (Sigma Cat. No. M-1880)
Potasium Chloride (Sigma Cat. No. P-4504)
B-Nicotinamide Adenine Dinucleotide (Reduced form) (β-NADH) (Roth >98% Cat. No. AE 12.2)
ATP (Sigma >99% Cat. No. A2383)
Imidazol (Sigma Cat. No. I5513)

2.3 Bacterial strains

E. coli BL21(DE3)

3 Media and buffers

3.1 LB agar

10 g L^{-1} tryptone

5 g L^{-1} yeast extract

5 g L^{-1} sodium chloride

15 g L^{-1} *agar agar*

3.2 LB *medium*

10 g L^{-1} tryptone

5 g L^{-1} yeast extract

5 g L^{-1} sodium chloride

3.3 TB medium

12 g L^{-1} tryptone

24 g L^{-1} yeast extract

5 g L^{-1} glycerol

10 % (v/v) KPi buffer (170 mM KH_2PO_4, 720 mM K_2HPO_4)

3.4 Reaction master mix (final assay concentrations)

Triethanolamine HCl 100 mM, pH 7.6
Phosphoenol pyruvate 2 mM
Magnesium sulfate 10 mM
Potasium Chloride solution 10 mM
B-Nicotinamide Adenine Dinucleotide 0.2 mM (Reduced form) (β-NADH)
Pyruvate kinase 40 U
Lactic dehydrogenase 100 U

3.5 Substrates (final assay concentrations)

Isobutanol 10 mM (in reaction buffer)
ATP 10 mM DTT solution

3.6 Additional chemicals

Micro BCA protein assay KIT
Imidazol: 20-500 mM

4 Procedures

This protocol is suitable to evaluate kinase/phosphotransferase enzymatic activity using protein kinase lysates or with purified kinase proteins as well.

4.1 Expression

Precultures: from single colony, *E. coli* BL21(DE3) harboring any of the kinase plasmids or the empty vector were grown o/n at 37°C and 180 rpm in 4 mL LB media.

Cultures: cultures were inoculated with 1% (v/v) preculture in 4 mL TB media for protein lysates or in 50 mL for His-Tag protein purification.
Induction: IPTG (0.1 mM) was added after 4 h of incubation a 37 °C and 180 rpm.

Expression: After induction, cultures were incubated o/n at 25°C and 180 rpm.
Crude protein extracts: Cells were harvested from 4 mL, resuspended in 1 mL reaction buffer, and cell solutions were sonicated (output control, 1.5; duty cycle 35).

Protein purification: Cells were harvested from 50 mL cultures, resuspended in 10 mL binding buffer, and sonicated (output control, 2; duty cycle 40). Lysates were used for further purification by IMAC using His GraviTrap™ TALON columns and imidazole gradient (20-500 mM). Purification process was assessed by SDS-PAGE.
Protein quantification: Protein quantification was performed according to Micro BCA protein assay KIT instructions.

4.2 ADP-dependent ATP depletion assay via NADH-dependent enzyme cascade

The ADP-dependent ATP depletion assay via NADH-dependent enzyme cascade involves three stages. The first one is the enzymatic reaction using isobutanol (10 mM) as substrate, kinase (cell lysate 100 µg or purified enzyme10 µg) as phosphotransferase and ATP (10 mM) as phosphate donor. The second reaction consist in coupling the ADP generated during the first reaction, with a PK phosphotransferase and with phosphoenolpyruvate to regenerate ATP and to produce pyruvate. Finally, during the third reaction the pyruvate generated during the second reaction is coupled with a LDH oxidoreductase to catalyze the conversion of pyruvate into lactate using NADH as cofactor. In this way, the NADH consumption is equimolar to the ADP generated during our target reaction, reporting that phosphorylation of isobutanol occurred. The assay is performed by triplicated in 96 well plates adding 270 µL of master mix, 10 µL of pure protein or lysate (10 µg or 100 µg), 10 µL of isobutanol (final concentration 10 mM), and 10 µL of ATP (final concentration 10 mM). Total volume reaction per well is 300 µL.

4.3 Analysis

The 96 well plates were measured directly in a plate reader at 340 nm, using end-point fashion to monitor NADH consumption. We determined that 20 min reaction time is enough to reach saturation for end point assays.

5 remarks/troubleshooting

It is highly recommended using phosphate free buffers to avoid high background levels when this protocol is used to determine ATP consumption. Since isobutanol is very volatile, it is recommended to use always a freshly prepared substrate stock.

6 Biosafety

No biosafety issues were associated with this protocol. The protocol was developed and performed at an S1 laboratory. The pipetting of isobutanol was performed in the fume hood due to the harmfulness of isobutanol vapors (H226-315-318-335-336). For protection against isobutanol, nitrile gloves are to be worn at all time when handling the samples.

7 Acknowledgements

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