Glycerol-3-phosphate dehydrogenase (GlpD) reporter enzyme assay for *Escherichia coli*

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Method Article

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Abstract

Glycerol-3-phosphate dehydrogenase (GlpD) is a recently introduced reporter enzyme for *E. coli* [1]. GlpD catalyzes the oxidation of Glycerin-3-phosphate (G3P) to dihydroxyacetone-phosphate (DHAP). The oxidation is coupled to the reduction of the artificial yellow substrate tetrazol-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) to a blue-violet formazan, which is mediated by the electron carrier phenazin-methanosulfate (PMS). This leads to an increase in absorption at 570 nm, which is measured to quantify the GlpD activity. To avoid background, it is best to use an *E. coli* strain with a deletion of the chromosomal *glpD* gene, which is available e.g. at the Keio collection [2]. The *glpD* gene can be used for transcriptional fusions, e.g. to characterize promoters, and also for translational fusions, e.g. to study translational regulation. The assay was adapted to the microtiter plate format to enable the parallel handling of a large number of samples. The “procedure” (see below) describes an application with the *glpD* gene in a translational fusion with the gene of interest cloned under the control of the inducible arabinose promoter P_{BAD}.

Introduction

Reporter genes are valuable tools for the analysis of differential gene expression [1, 2]. The encoded reporter enzymes are typically small monomeric enzymes that fold with high efficiency in the cytoplasm of various species and that can be quantified with enzymatic assays that are robust, sensitive, and cheap. Transcriptional fusions are used to study promoter activities or/and transcript stabilities. Translational fusions further report on (differential) translational efficiencies and on protein stabilities. Widely used reporter genes include the *lacZ* gene encoding beta-galactosidase gene, the *gusA* gene encoding beta-glucuronidase, and the CAT gene encoding chloramphenicol acetyltransferase. The *gfp* gene encoding the Green Fluorescent Protein (and many derivatives thereof) is often used not as a reporter for the quantity of a protein of interest, but of its localization within cells or tissues or of its *in vivo* interaction with other proteins. Another, recently introduced reporter gene is *glpD*, which encodes glycerol-3-phosphate dehydrogenase [3]. Here, we describe the application of the *glpD* reporter gene for quantifying translational efficiencies in *E. coli*.

Reagents

- 20% (w/v) arabinose
- 1M Tris-HCl pH 7.4
- GlpD lysis buffer: 50mM Na₂HPO₄, 500mM NaCl, 0.4% (v/v) Triton X 100, pH 8.0

- MTT stock solution: 11.25mM MTT in H₂O bidest. stored at 4°C, protected from light
- PMS stock solution: 56.3mM PMS in H₂O bidest. stored at 4°C, protected from light
- sn-G3P stock solution: 78mg/mL sn-G3P in 0.1M Tris-HCl pH 7.4, prepared freshly
- Triton X 100
- glpD reaction buffer, generated from the stock solutions described above: 1.125mM MTT, 5.63mM PMS, 84mM Tris-HCl pH 7.4, 0.4% (v/v) Triton X 100, 7,8mg/mL sn-G3P

Equipment

- Microtiter plate (MTP) photometer
- 96 well microtiter plates
- Pierce™ BCA Assay Kit (Thermo Scientific)

Procedure

Cell culture and harvesting

1. Inoculate 50 mL medium in 100 ml Erlenmeyer flasks with plasmid-containing overnight cultures to an initial OD₆₀₀ of 0.05. At least three biological replicates of each sample should be used and a negative control (culture with an empty vector) should be added. When the cultures reach an OD₆₀₀ of about 0.6, induce the p_{BAD} promoter with a final concentration of 0.2% (w/v) arabinose.

2. Incubate the cultures for at least 30 minutes to allow expression of the *glpD* fusion gene. Harvest 20mL for the reporter enzyme assay by centrifugation at 4000rpm for 10 minutes at 4°C.

If a transcript analysis is also desired, further 8mL culture can be harvested in parallel (4000rpm, 10 minutes, 4°C). The pellets can be stored at -80°C for later RNA Isolation (compare the Method “Northern blot analysis”).

3. Resuspend the pellets for the reporter enzyme assay in 1mL glpD lysis buffer. Lyse the cells vial shaking with glass beads (500 mg/reaction tube) in a FastPrep™ (MP Biomedicals; 3x 45 seconds at 6.5 intensity with 20 seconds pauses on ice in between)

4. Sediment glass beads and cell debris by centrifugation (13000 rpm, 10 minutes, 4°C). Transfer the cleared lysate to a new reaction tube.

Determination of the specific GlpD reporter enzyme activity

5. Start the MTP photometer and equilibrate it at 37°C for at least 30 minutes.
6. For every reaction pipette 65µL of suitable dilutions of the lysate into a well of a microtiter plate. We recommend 1:10; 1:100 and 1:200 dilutions and technical duplicates for every sample.
7. Add 160µL of the glpD reaction buffer (including the sn-G3P), immediately transfer the MTP to the photometer and start the measurement.
8. The change of absorption is measured at 570 nm for 30 minutes at 20 second intervals with shaking of the plate before each measurement.
9. For the analysis of the volume activity only dilutions can be used that exhibit a linear increase of absorption for at least 3 minutes. The photometer software is used to calculate the change in extinction per minute ($\Delta E/\text{min}$) for all samples.
10. The extinction change in the negative control is subtracted from the extinction changes of all samples to yield the GlpD-specific extinction change.
11. Calculate the GlpD volume activity using the following formula with the $\Delta E/\text{min}$:

$$\text{Volume activity [U/mL]} = \Delta E \cdot \text{min}^{-1} \cdot \epsilon^{-1} \cdot d^{-1} \cdot D$$

U: Enzyme activity ($\mu\text{mol} \cdot \text{min}^{-1}$) 1 U = 16.67 nkat

$\Delta E/\text{min}$: Change in extinction at 570 nm (min^{-1})

ϵ : Extinction coefficient of the formazan product ($17 \text{ mM}^{-1} \cdot \text{cm}^{-1}$)

d: thickness of the cuvette [cm] (300 µl in a 96 well MTP: 0,95 cm)

D: Dilution of the sample

12. To quantify the protein concentration, the PIERCE™ BCA (Bicinchoninic acid) protein assay kit is used with suitable dilutions of the lysates according to the instructions of the manufacturer. The assay has been adapted to 96 well MTPs to enable the parallel processing of many samples. Bovine serum albumin (BSA) is used to generate a standard curve, using concentrations from 0 mg/ml to 2 mg/ml. The colorimetric biuret-reaction results in the formation of a blue color, which can be quantified photometrically at 562 nm. The BSA standard curve is used to calculate the protein concentrations of all samples (mg/ml).

13. The specific GlpD activity [U/mg] is calculated by dividing the volume activity [U/ml] with the protein concentration [mg/ml].

Troubleshooting

Time Taken

Anticipated Results

References

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