

Spectrophotometric Determination of Glutamine Synthetase Activity in Cultured Cells

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[Abstract] Glutamine synthetase (GS), which catalyzes the conversion of glutamate and ammonia to glutamine, is widely distributed in animal tissues and cell culture lines. The importance of this enzyme is suggested by the fact that glutamine, the product of GS-catalyzed de novo synthesis reaction, is the most abundant free amino acid in blood (Smith and Wilmore, 1990). Glutamine is involved in many biological processes including serving as the nitrogen donor for biosynthesis, as an exchanger for the import of essential amino acids, as a means to detoxifying intracellular ammonia and glutamate, and as a bioenergetics nutrient to fuel the tricarboxylic acid (TCA) cycle (Bott *et al.*, 2015). The method for the assay of GS enzymatic activity relies on its γ -glutamyl transferase reaction by measuring γ -glutamylhydroxamate synthesized from glutamine and hydroxylamine, and the chromatographic separation of the reaction product from the reactants (Deuel *et al.*, 1978). An overview of the GS glutamyl transferase reaction can be found in Figure 1. GS activity was measured by a spectrophotometric assay at a specific wavelength of 560 nm using a microplate reader. The method is simple, and has a comparable sensitivity with those methods applying radioactively labelled substrates. This modified procedure has been applied to assay/determine GS activity in cultured cell lines including the human mammary epithelial MCF10A cells and the murine pre-B FL5.12 cells, and could be used to measure GS activity in other cell lines.

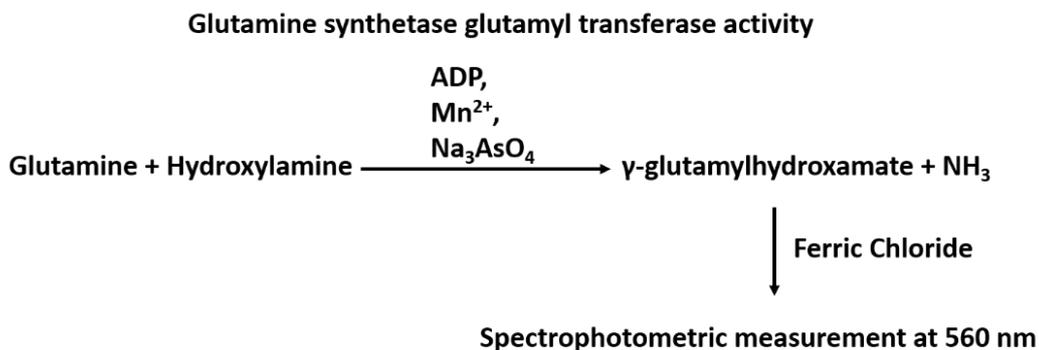


Figure 1. An overview of the GS glutamyl transferase reaction

Materials and Reagents

1. 0.05% trypsin-EDTA (1x) (Thermo Fisher Scientific, Gibco™, catalog number: 25300-054)
2. PBS (1x) (Corning, catalog number: 21-031-CV)
3. Imidazole (Sigma-Aldrich, catalog number: I0250)
4. L-glutamine (Sigma-Aldrich, catalog number: G3126)
5. Hydroxylamine hydrochloride (Sigma-Aldrich, catalog number: 55459)
6. Sodium arsenate dibasic heptahydrate (Sigma-Aldrich, catalog number: S9663)
7. Manganese(II) chloride tetrahydrate (MnCl₂·4H₂O) (Sigma-Aldrich, catalog number: 221279)
8. Adenosine 5'-diphosphate sodium salt (ADP) (Sigma-Aldrich, catalog number: A2754)
9. Iron(III) chloride hexahydrate (FeCl₃) (Sigma-Aldrich, catalog number: 31232)
10. Trichloroacetic acid (Sigma-Aldrich, catalog number: 522082)
Note: This product has been discontinued.
11. Hydrochloric acid (HCl) (Sigma-Aldrich, catalog number: 258148)
12. L-glutamic acid γ-monohydroxamate (γ-glutamylhydroxamate) (Sigma-Aldrich, catalog number: G2253)
13. Lysis buffer (see Recipes)
14. 1x assay buffer (see Recipes)
15. 1x stop buffer (see Recipes)
16. γ-glutamylhydroxamate standard stock (see Recipes)

Equipment

1. Accumet™ AB15 Basic and BioBasic™ pH/mV/°C Meters (Thermo Fisher Scientific, Fisher Scientific, catalog number: 13-636-AB15B)
2. Sonicator (model: CL-18/120) (Thermo Fisher Scientific, Fisher Scientific™, catalog number: FB120110)
3. -80 °C freezer
4. 37 °C incubator
5. Microcentrifuge (Eppendorf, model: 5418)
6. Microplate (96-well plate clear bottom) reader (Molecular Devices, model: SpectraMax M5)

Software

1. SoftMaxPro software

Procedure

1. Collect ~8 x 10⁶ cells in a 10 cm dish cells by using 1x trypsin.
2. Rinse the cells with cold 1x PBS and then remove the PBS twice.
3. Add certain amount (*i.e.*, 300-500 µl) of lysis buffer (50 mM imidazole-HCl, pH 6.8) and lyse the cells with sonicator at level 4 output control, 50% duty cycle for 30 sec and pause for 30 sec for 5 cycles.
4. Incubate the cells at -80 °C for at least 4 h for the freeze/thaw cell lysis procedure.
5. Thaw the cells and centrifuge the cells at 16,000 x g at 4 °C for 15 min.
6. Collect the supernatant and measure the protein concentration by BCA protein assay (He, 2011).
7. Add 20-40 µg of protein, then add lysis buffer to raise volume to 50 µl.
8. Add equal volume (50 µl) of 1x assay buffer and then incubate at 37 °C for 2-6 h (Please see the notes).
9. Add equal volume (100 µl) of 1x stop buffer to observe brown color products and centrifuge at 16,000 x g at 4 °C for 10 min.
10. The absorbance of γ-glutamylhydroxamate in the supernatant of samples was measured at 560 nm. The blank was 50 µl lysis buffer + 50 µl 1x assay buffer + 100 µl 1x stop buffer.
11. A serial dilution of 25, 12.5, 6.25, 3.125, 1.5623, 0.781, and 0.391 mM γ-glutamylhydroxamate (100 µl) were added with 100 µl of 1x stop buffer and centrifuge at 16,000 x g at 4 °C for 10 min. The absorbance of γ-glutamylhydroxamate in the supernatant was measured at 560 nm at room temperature as a standard curve (Figure 2).

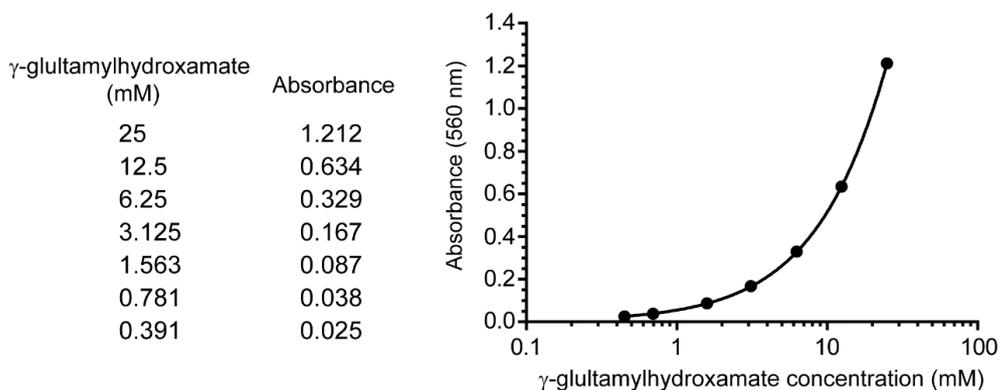


Figure 2. A representative standard curve. The standard curve is generated by performing a serial dilution of γ-glutamylhydroxamate. The upper boundary of the standard starts at a known concentration of 25 mM, and this is diluted by half to reach the lower boundary at 0.39 mM. A blank with no standard is included. These concentrations are then input into SoftMaxPro software and the absorbance is read at 560 nm. The readings from known concentrations are determined and a 4 parameter fit is applied. In this way the standard curve is generated within a large working range, unknown samples can be tested, and the absorbance of unknowns can be plotted to determine concentration.

Representative data

The GS activity was reported as $\text{nmol min}^{-1} \text{g protein}^{-1}$. The GS activity was expressed by the formula: γ -glutamylhydroxamate concentration (mM) \times 100 μl /incubation time (min)/protein amount (g). For example, the concentration of γ -glutamylhydroxamate calculated from MCF10A vector cells was 2.378 mM as compared with standard curve. The added protein amount was 10.36 μg , and the incubation time at 37 °C was 120 min. The GS activity would be $2.378 \times 100/120/10.36 \times 10^{-6} = 1.91 \times 10^5 \text{ nmol min}^{-1} \text{g protein}^{-1}$ as indicated (Figure 3).

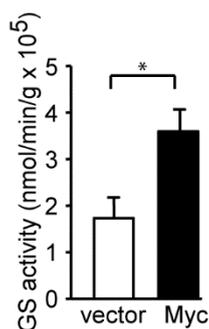


Figure 3. Myc induces GS activity. c-Myc was stably expressed in MCF10A cells. GS activity was determined and is shown as the mean plus SEM of at least five independent experiments. * $P < 0.05$. Student's *t*-test was used to determine the *P* value (Bott *et al.*, 2015).

Notes

The various incubation time (2-6 h) at 37 °C was determined by the amount of active GS expressed in different cell lines. Higher active GS expressed leads to shorter incubation time to reach the optimal amount of γ -glutamylhydroxamate. The optimal incubation time used in the assay may be adjusted for different cell lines.

Recipes

1. Lysis buffer (50 mM imidazole-HCl, pH 6.8)
Add 170.2 mg imidazole in 50 ml ddH₂O and adjust pH to 6.8 with HCl.
2. 1x assay buffer
 - a. 5x stock 250 mM imidazole-HCl, pH 6.8
Add 851 mg imidazole in 50 ml ddH₂O and adjust pH to 6.8 with HCl.
 - b. 5x stock 250 mM L-glutamine, 250 mM imidazole-HCl
Add 1.8275 g L-glutamine in 50 ml 5x stock 250 mM imidazole-HCl.
 - c. 5x stock 125 mM hydroxylamine
Add 434.3 mg hydroxylamine in 50 ml ddH₂O.
 - d. 5x stock 125 mM sodium arsenate

Add 1.95 g sodium arsenate in 50 ml ddH₂O.

- e. 5x stock 10 mM MnCl₂

Add 99.2 mg MnCl₂ in 50 ml ddH₂O.

- f. 5x stock 0.8 mM ADP

Add 17.09 mg ADP in 50 ml ddH₂O.

Then mix equal amount of (b)-(f) to get 1x assay buffer (final concentration: 50 mM imidazole-HCl, 50 mM L-glutamine, 25 mM hydroxylamine, 25 mM sodium arsenate, 2 mM MnCl₂ and 0.16 mM ADP).

3. 1x stop buffer

3x stop buffer (270 mM FeCl₃, 5.4 N HCl, 4.35% trichloroacetic acid)

Use 7.3 g FeCl₃, 45 ml 12 N HCl as well as 4.35 ml 100% trichloroacetic acid and add ddH₂O to 100 ml to get 3x stop buffer.

Dilute 3x stop buffer with ddH₂O to get 1x stop buffer (final concentration: 90 mM FeCl₃, 1.8 N HCl and 1.45% trichloroacetic acid) when doing the assay.

4. 100 mM γ -glutamylhydroxamate standard stock

Add 16.214 mg γ -glutamylhydroxamate in 1 ml ddH₂O.

Acknowledgments

This protocol was adapted and modified from a previous study (Deuel *et al.*, 1978). It was described in (Bott *et al.*, 2015). This work was supported by grants from NIH (R01CA129536 and R01GM97355 to WXZ).

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