# δ-Aminolevulinic Acid Synthase

April 6, 2000


Summary

δ-Aminolevulinic acid synthase catalyzes the pyridoxal phosphate-dependent condensation of succinyl coenzyme A and glycine to δ-Aminolevulinic acid. This protocol describes a coupled enzyme assay for determining δ-aminolevulinic acid synthase activity.

The method relies on a chemical condensation of δ-aminolevulinic acid (ALA) with acetylacetone at pH 4.7 to yield 2-methyl-3-acetyl-5-propionic acid pyrrole (ALA-pyrrole). In the presence of modified Ehrlich's reagent, a colored complex of ALA-pyrrole is formed which is quantitated at 556nm in the spectrophotometer.


Solutions required

1. 1.0 M sodium acetate buffer pH = 4.7
   adjust with 20% HCl to a pH of 4.7

2. 1.0 M tris-HCl buffer pH = 7.5
   adjust with 20% NaOH to a pH of 7.5

3. 50 mM potassium phosphate buffer pH = 7.0
   prepared by mixing 8 mL of 50 mM $KH_2PO_4$ and 20 mL of 50 mM $K_2HPO_4$

4. 1.0 M glycine
   prepare 10 mL

5. 1.0 M succinate
   adjust with 20% NaOH to a pH of 7.0
   prepare 20 mL

6. 0.1 M $MgCl_2$
   prepare 20 mL

7. 0.2 M ATP
   adjust with 20% NaOH to a pH of 7.0
   prepare 10 mL

8. 0.01 M pyridoxal phosphate
   prepare 6.75 mL

9. 0.01 M coenzyme A
   prepare 8.75 mL

10. Modified Ehrlich's reagent
    Prepared by adding 1.0 g of *para*-dimethyl-aminobenzaldehyde to 30 ml of glacial acetic

acid, adding 8 ml of 60 % perchloric acid and diluting to 50 ml with glacial acetic acid. Must be prepared fresh at the time of an assay.

11. ALA standard solutions
Make standard solutions containing 2.5 µM, 10 µM and 40 µM ALA.

12. 10% trichloroacetic acid

Preparation of Solutions

1a. Prepare *enzyme substrate cocktail* by combining:

10 mL 1.0 M glycine
10 mL 1.0 M succinate
10 mL 0.1 M $MgCl_2$
5 mL 1.0 M tris-HCl buffer

This solution may be stored in the refrigerator at 4°C. The solution should be prepared fresh once a month.

b. Prepare *enzyme control cocktail* by combining:

10 mL DI water
10 mL 1.0 M succinate
10 mL 0.1 M $MgCl_2$
5 mL 1.0 M tris-HCl buffer

This solution may be stored in the refrigerator at 4°C. The solution should be prepared fresh once a month.

2. Prepare *stock cofactor cocktail* by combining:

10.0 mL 0.2 M ATP
8.75 mL 0.01 M CoA
6.75 mL 0.01 M pyridoxal phosphate
10.0 mL DI water

This solution should be thoroughly mixed and then 2-3 mL distributed into each of numerous Eppendorf tubes. These solutions must be stored at –20°C, and the amount removed for an assay when needed.

Preparation of Cell Extract

Follow general protocol **Preparation of Cell Extract.**

1. Centrifuge cultures (100 mL) at 10,000 × g for 10 min.

2. After first pelletization of cells, resuspend the cells at 4°C with 10 mL of 50 mM potassium phosphate buffer (pH 7.0).
3. After second pelletization of cells, resuspend the cells at 4°C with 2 mL of 50 mM potassium phosphate buffer (pH 7.0), and break with French Press.

Procedure (Enzyme Reaction)

1. For each assay, prepare the enzyme reaction mixtures shown in the following table into separate 1.5 mL Eppendorf tubes, and then place them in a water bath at 37°C for 20 min. One "control" is required (using solution 1b above). Prepare one "Experimental" for each sample solution to be examined (using solution 1a above).

| Solution | Volume (µL) added to: | |
|---|---|---|
| | Control | Experimental |
| DI $H_2O$ | 480 | 480 |
| Control/Substrate cocktail | 350 | 350 |
| cofactor cocktail | 150 | 150 |

4. Initiate the enzyme reaction by adding 20 µL of cell extract to each tube†, and then promptly mixing the contents of the Eppendorf tube (1000 µL total volume).
5. After 20 minutes of reaction, withdraw a 300 µL sample and immediately transfer to a 1.5 mL Eppendorf tube containing 150 µL of 10% trichloroacetic acid.

† If necessary, the cell extract may be diluted with tris-HCl buffer, and a final volume of 20 µL still used for the reaction mixture. For example, mixing 10 µL of the cell extract diluted with 1000 µL of buffer would result in a dilution of 101×, and the final activity calculated for the reaction mixture should be multiplied by this dilution to obtain the activity of the cell extract.

Procedure (Colourimetric Quantification of ALA)

1. Centrifuge each 450 µL sample at 13,000 rpm for 5 min.
2. Transfer 300 µL of each supernatant to a glass tube containing 400 µL of 1.0 M sodium acetate (pH 4.7).
3. To prepare standard solutions, in a glass tube mix 300 µL of each ALA standard (i.e., 2.5 µM, 10 µM and 40 µM) with 400 µL of 1.0 M sodium acetate (pH 4.7). Also mix 300 µL of DI water with 400 µL of 1.0 M sodium acetate as a "blank" for the spectrophotometer.
4. Add 35 µL acetylacetone to each 700 µL sample and standard.
5. Heat each tube in a water bath at 80°C for 15 min.
6. Turn on the visible bulb on the spectrophotometer (Beckman DU50). Select the fixed-wavelength window on the instrument. Load the method “A:/ALAS”. This method has a temperature of 30 $^{o}$C, a wavelength of 556 nm.
7. Cool tubes to room temperature, add 700 µL of modified Ehrlich’s reagent to tubes and

immediately vortex.

8. After (exactly) 10 minutes, measure the absorbance for each of the reaction mixtures.

Calculation of Activity

One unit (U) of δ-aminolevulinic acid synthase activity is defined as the amount of enzyme required to produce 1.0 μmole of aminolevullinic acid in one minute.

1. Record the absorbance of the three ALA standards (see following table).

| C | A |
|---|---|
| 2.5 | |
| 10 | |
| 40 | |

where C = concentrations of ALA standards (μmol/L)
A = observed absorbance at 556 nm

2. Prepare a calibration curve using the results from the standards. Plot C versus A and obtain the best-fitting line (determine two parameters, $\alpha$ and $\beta$):

$$C = \alpha A + \beta$$

3. Calculate the corrected absorbance for each sample by subtracting the absorbance for the control at the same reaction time from the absorbance from each experimental at the same reaction time. Use the calibration curve to determine the concentration of ALA in each sample. If the calculated ALA concentration in a sample is greater than 40 μmol/L, then the enzyme assay will need to be repeated by diluting the cell extract. If the calculated ALA concentration in a sample is less than 2.5 μmol/L, then the enzyme assay will need to be repeated by concentrating the cell extract.

4. Calculate activity using the following formula

$$\text{Activity} = \frac{TV \times C \times D_1}{V \times T \times CF}$$

Activity: Volumetric Activity (U/L)
TV: Total volume in enzyme reaction mixture (1000 μL)
V: Volume of cell extract used in enzyme reaction mixture (20 μL)
T: Time of enzyme reaction (10 or 20 minutes)

$D_1$: Dilution of sample with trichloroacetic acid (i.e., 450 µL/300 µL = 1.5)

CF: Dilution of cell extract (For example, if a 100 mL sample is concentrated to a 2 mL volume for the French Press, then CF=50)

5. Specific Activity = $\frac{Activity}{Protein\ Concentration}$

Activity: Volumetric Activity, as calculated in #2 above (U/L)

Protein Concentration: Protein concentration, as calculated in protocol **Total Protein Concentration** (mg/L)

Specific Activity: (U/mg protein)


Reference

B. F. Burnham (1970) D-aminolevulinic acid sythase. Methods in Enzymology, 17A, 195-200.

M. J. Van der Werf, J. G. Zeikus (1996) 5-aminolevulinate production by *Escherichia coli* containing the *Rhodobacter sphaeroides hemA* gene. Appl. Env. Microbiol. 62(10), 3560-3566.