ALAS Activity Assay

d-AMINOLEVULINIC ACID SYNTHASE (ALAS)

Mouse liver homogenate

1. Weigh out ~100mg liver.
2. Add four volumes (400µL) of ice-cold 50mM potassium phosphate (KPi) pH7.4.
3. Homogenize with ten up-and-down strokes in a 2-mL glass-Teflon (Potter- Elvehjem) tissue homogenizer in an ice bath.
4. Store the resulting 20%w/v homogenate at −80 °C until needed.

Cultured cell homogenate

1. Wash the cells with phosphate buffered saline (PBS) pH 7.4.
2. Resuspend in about three pellet volumes of 50mM potassium phosphate (KPi) pH7.4.
3. Sonicate (homogenize) while in an ice bath at the lowest practicable power setting for 3 cycles x 5 sec at 50% duty (2.5 sec on, 2.5 sec off).

Preparation of succinyl coenzyme A

1. Prepare these three aqueous solutions and keep on ice:
   - 23.85 mg/mL coenzyme A
   - 10.5 mg/mL succinic anhydride, ground to fine powder
   - 25 mg/mL $NaHCO_3$.
2. Mix equal volumes of these solutions and incubate on ice for 30 min.
3. Use in the ALAS assay.
4. The resulting ~10mM succinyl CoA solution could be also be aliquoted, stored for more than three months at −80 °C and thawed once, only when needed.
5. Succinic anhydride must be dissolved in ice-cold water to minimize conversion to succinic acid before reacting with coenzyme A. Check that the final mixture reaction is maintained at pH 7–7.5 by the $NaHCO_3$ solution.

ALAS assay

1. Adjust enough of each sample to 5-10 mg protein per mL with 50mm potassium phosphate (KPi) pH7.4 to make 6 x 25-µL aliquots for three pairs of live and blank

replicates. Blanks are heat inactivated for 10 min in a boiling water bath before the addition of the ALAS assay buffer, or incubated at 0 min at 37˚C after the ALAS assay buffer has been added.
2. For each 25-µL of sample make 25µL of ALAS assay buffer by mixing 15.5µL 50mM KPi pH 7.4, 2.5µL 1M aqueous glycine pH~7, 2.5µL 10mM succinyl CoA, 0.5µL aqueous succinylacetone and 4.0µL 1mM aqueous pyridoxal 5'-phosphate.
3. Add 25 µL ALAS assay buffer to each 25-µL aliquot of the protein adjusted sample.
4. Incubate the resulting mixture at 37 °C for 30min.
5. Add 450 µL of ice-cold water.
6. Keep the diluted ALAS sample on ice or store frozen until the ALA can be derivatized.

Derivatization of d-aminolevulinic acid (ALA)

1. Prepare derivatizing agent (DA) each sample by mixing water, 37% formaldehyde, ethanol and acetylacetone in a ratio of 107:5:15:23 by volume, respectively. Stir/vortex vigorously for 3 min or more until a clear colorless solution is obtained.
2. Carry out all subsequent steps under minimal lighting.
3. Mix 50-µL aliquot of the diluted ALAS assay sample (above) with 150 µL DA.
4. Incubate in a covered heat block at about 100–103 °C for 5.0min.
5. Cool immediately in an ice bath for 1 to 6h.
6. Centrifuged for 10 min at 14,000 rpm in a microfuge at 4 °C.
7. Collect the supernatant.
8. Keep at 4°C until injection into the UPLC.

Ultra performance liquid chromatography (UPLC) quantitation

1. Inject 10µL of supernatant containing the derivatized ALA into a Waters Acquity UPLC system which includes a binary solvent manager, sample manager, fluorescence detector, column heater and an Acquity UPLC BEH C18, 1.7 µM, 2.1 Å~ 100mm column.
2. Set the fluorescence detector at 370nm excitation and 460nm emission.
3. Keep the sample chamber dark and at 5 °C. Solvent A is 0.2% aqueous formic acid while Solvent B is 100% methanol.
4. Set the flow rate at a constant 0.3 mL/min and the column at 50°C for the total run time of 12min.
5. Use the following gradient schedule with the percent Solvent A at each step as follows:
   - 0 min, 80%
   - 6 min, 60%
   - 7 min, 1%
   - 9 min, 1%
   - 9.5 min, 80%
6. Set the gradient for solvent composition from 0 to 6 min as Waters Gradient 5 (convex with a higher slope at 0 min compared to that at 6 min), and that from 6 to 7 min at Waters Gradient 7, concave.
7. Keep all other gradients in the method linear.

Standard curves

1. Prepare pairs of sample aliquots at the same protein concentration as the unknowns to contain authentic ALA at seven different concentrations in a range similar to those as the sample unknowns, including 0.0µM.
2. Inactivate one of each pair.
3. Put these spiked samples through the ALAS activity assay protocol.
4. Subtract the background UPLC peak readings at 0.0 µM was from those of the spiked samples and use these results to construct a standard curve that to determine the [ALA] in the 25-µL sample aliquots.

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