

## **Leaf Amino Acid Assay Protocol**

Development of his method is described in the manuscript Gu, L., A.D. Jones and **R.L. Last**. 2007. Rapid LC-MS/MS assay for protein amino acids and metabolically related compounds. Anal. Chem. <http://pubs.acs.org/cgi-bin/abstract.cgi/ancham/asap/abs/ac070938b.html>. Please cite this paper if you publish data derived from all or part of this protocol.

### **Reagents:**

1 mM Phenylalanine-d8  
9  $\mu$ M Valine-d8 (Cambridge Isotope Laboratories, DLM-488-0.5 and DLM-372-1)  
10 mM DTT

### **Harvesting tissue:**

1. Flats containing the plants to be harvested are kept under 80-100 uE light during the harvest.
2. A fully-expanded leaf (approximately leaf #7 counting from the smallest visible rosette leaf), without the petiole, is harvested, weighed, cut into two pieces, and placed into a 2 ml microfuge tube containing one 3 mm stainless steel ball (CCR Products, 302SS).
3. Tubes containing harvested tissue are put directly into a pre-frozen rack nestled deeply in dry ice. Using this system, harvested leaf tissue freezes completely within seconds.
4. Store leaf tissue at  $-80^{\circ}\text{C}$ .

### **Processing AA samples:**

#### **Equipment:**

Refrigerated microfuge,  $-9^{\circ}\text{C}$   
Eppendorf 5810R tabletop centrifuge,  $4^{\circ}\text{C}$   
Water bath,  $90^{\circ}\text{C}$   
Pre-frozen ( $-80^{\circ}\text{C}$ ) 96-tube rack  
Pre-chilled ( $4^{\circ}\text{C}$ ) 96-tube racks (2)  
Hero 2200 Dual Head paint shaker (Hero Products Group)  
MicroRacks (Dot Scientific Inc, RN0946-08R)  
Millipore filter plate (300  $\mu$ l, Millipore, MSRL N0450)  
Millipore collection plate (300  $\mu$ l, Millipore, MSCP NPP00)  
Aluminum sealing film (Dot Scientific Inc, AF-100)  
AXYMAT silicone sealing mat (VWR, 10011-130; AXYGEN, AM-96-PCR-RD)

#### **Tissue grinding:**

Harvested tissue is kept frozen on dry ice or in refrigerated centrifuge during all procedures until extraction reagent is added.

1. Shake frozen tissue, in secured rack, 2 min. with paint shaker.
2. Centrifuge tubes 10 seconds, 13,000 rpm, refrigerated microfuge ( $-9^{\circ}\text{C}$ ).

**Extraction reagent:**

45 ml distilled deionized dH<sub>2</sub>O

45 µl of 1 mM Phe-d8

45 µl of 10 mM DTT

Keep chilled on ice.

**Extraction:**

1. Add 400 µl of extraction reagent to each sample tube; mix by hand. Keep sample tubes on ice.
2. Centrifuge 10 seconds, 13,000 rpm, refrigerated microfuge (-9°C).
3. Incubate at 90°C for 5 min.
4. Place the racks of tubes on ice; centrifuge 10 seconds, 13,000 rpm, refrigerated microfuge (-9°C). Keep on ice.

**Transfer and filter:**

1. Transfer all of the supernatant to MicroCaps 8-tube strips (Dot Scientific Inc, RN0946-08C) in a rack.
2. Centrifuge at 4,000 rpm, Eppendorf 5810R tabletop centrifuge, 4°C, 5 min.
3. Prewet filter plate by adding 100 µl dH<sub>2</sub>O and centrifuging at 2000xg, Eppendorf 5810R tabletop centrifuge, 4°C, 5 min.
4. Place the prewetted filter plate on the collection plate, add 300 µl extraction supernatant. Centrifuge at 2000xg at 4°C for 5 min.
5. 10 µl of 9 µM Val-d8 is added per 90 µl filtered extraction supernatant for mass spec analysis.
6. Store extractions in plates at -80°C, sealed with aluminum sealing film.

**Set up LC-MS standards and samples**

1. Carefully peel off the sealing film from sample plate and replace with a clean or new silicon mat.
2. Take one aliquot of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 µM 21 amino acid standard stock from -80°C (Sigma, LAA-21), thaw in room-temperature water bath, vortex, and keep on ice.
3. Take used LC vials (water, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 µM, water) from -20°C.
4. Add 1 ml of fresh ddH<sub>2</sub>O to vial 1 and 11. Transfer amino acid stocks from 0.5 ml tubes to 100 µl LC vial inserts and place the inserts in the designated vials. Keep on ice.
5. Bring samples and 11 standard vials, on ice, to Mass Spec facility with protocols for LC-MS.

**Reference:**

Jander, G., Norris, S.R., Joshi, V., Fraga, M., Rugg, A., Yu, S.X., Li, L.L., and Last, R.L. 2004. Application of a high-throughput HPLC-MS/MS assay to Arabidopsis mutant

screening; evidence that threonine aldolase plays a role in seed nutritional quality. *Plant J.* 39 (3): 465-475.