**Hydroxyproline Assay**

(Assay Kit purchased from Chondrex #6017)

*Time: ~ 28 hours completion*

This protocol is for one (1) sample. Before starting you will need:

* Razorblade – 11C
* Weighboat – 11B
* Forceps – 11C
* Safety glasses – 1K
* Gloves – lab bench
* Lab coat – MN508 behind door
* Bullet Blender – MN508 lab bench
* DI H2O – MN508 lab bench, if needed MN507
* 2 mL longneck pre-scored ampoule – 8D
* Bunsen burner – 8C
* Two sets of pliers
* 96 well plate – 7E

I. Sample Preparation & Homogenization

1. Obtain tissue sample from vapor phase nitrogen storage.
2. Cut and weigh sample to ~100 mg
3. Using forceps place sample in dounce homogenizer. Dilute sample:
   1. Add 50 uL DI H2O to the dounce homogenizer for every 1 mg tissue i.e. 500 uL – 10 mg, 5000 uL – 100 mg etc.
4. Homogenize until all visible tissue disappears. Time will vary.
5. Transfer 400 uL of tissue homogenate into 2 mL brown pre-scored longneck ampoules
6. Transfer leftover homogenate into Eppendorf tube. Place into a labeled sample box and store in 4 degree Celsius refrigerator.
7. Apply appropriate PPE and add 400 uL of 12M HCl to each ampoule using a fume hood. *\*Note: 12M HCl is very corrosive. Handle with care. View MSDS for more details.*
8. Using appropriate PPE setup the Bunsen burner by connecting the supply hose to a gas valve. Avoid leaving excess supply tubing around the base of the Bunsen burner.
9. Using 2 sets of pliers tilt the ampoule to ~45 degree angle and place the neck of the ampoule between the sets of pliers directly in the light blue portion of the flame. Wait ~15-30 seconds for the glass to become malleable and pull the pliers apart to create a sealed ampoule. *\*Note: melting the newly formed tip of the ampoule will help strengthen the tip to avoid a break in the seal. The neck of the ampoule will be very hot to the touch as will the pliers. Handle with care and avoid placing these objects on the benchpaper.*

II. Hydrolysis

1. Place the sealed ampoule inside the oven and bake at 120° C for 24 hours
2. Sketch 96-well plate diagram in lab notebook adding wells for 7 standards and sample spikes used in the assay.
3. Take 100 uL of Hydroxyproline (HP) Standard and add to 900 uL of distilled water to make 400 g/mL of the diluted HP standard; then serially dilute it with distilled water. For example, mix 500 uL of the standard (400 ug/ml) with an equal volume of distilled water to make a 200 ug/ml solution, and then repeat it five more times to make 100, 50, 25, 12.5, and 6.3 ug/ml standards.

III. Post-Hydrolysis

1. Open oven door and allow sample to cool for 15-30 minutes. Remove the ampules and break at the score using multiple kim wipes to create a barrier between the glass ampule and the user’s hand.
2. Pipette ampule’s entire contents to a clear 2 ml Eppendorf tube and spin at 10,000 rpm for 3 minutes. You should see a black pellet at the bottom of the tube. *\*Note: Ensure the centrifuge is balanced*
3. Pipette 10 uL of supernatant from each Eppendorf tube to a separate well for each sample in a 96 well plate
4. Store the remaining supernatant in a 2 mL Eppendorf tube and place in 4 degree Celsius refrigerator for use again.

**Assay**

1. Immediately prepare 100 uL of the chloramine T/Solutuion A for each reaction well in the 96-well plate by adding 6 uL of chloramine T to 94 uL of solution A. Pipette 100 uL of the solution to each reaction well and lavage 10 times to mix thoroughly. Each mixture is stable 2-3 hours after preparation.
2. Incubate at room temperature for 20 minutes.
3. Prepare 100 uL of Diluted DMAB reagent to each well by adding 50 uL of DMAB concentrate to 50 uL of Solution B. Add 100 uL of this solution to each reaction well and lavage 10 times to mix thoroughly.
4. Incubate at 60° C for 30 minutes.
5. Read the optical density values at 560 nm using the spectrometer located on the 6th floor.

III. Colorimetric Analysis

1. Using the spectrometer located on the 6th floor in the Esser lab record the absorbance at 560 nm.
2. Set the protocol by going to: protocol > protein quant > Default protocol
3. Perform calculations using the optical density values presented by the SoftMax Pro program