**Hydroxyproline Assay**

**Protocol is adapted from:**

Hewitson TD, Smith ER, Samuel CS. Qualitative and quantitative analysis of fibrosis in the kidney. Nephrology (Carlton). 2014 Nov;19(11):721-6. doi: 10.1111/nep.12321. PMID: 25196487.

Parsons SA, Millay DP, Sargent MA, McNally EM, Molkentin JD. Age-dependent effect of myostatin blockade on disease severity in a murine model of limb-girdle muscular dystrophy. Am J Pathol. 2006 Jun;168(6):1975-85. doi: 10.2353/ajpath.2006.051316. PMID: 16723712; PMCID: PMC1606625.

**Protocol adapted to a 96-well plate based:**

<https://pubmed.ncbi.nlm.nih.gov/11196318/>

<https://www.abcam.com/ps/products/222/ab222941/documents/Hydroxyproline-Assay-Kit-protocol-book-v2c-ab222941%20(website).pdf>

<https://www.sigmaaldrich.com/US/en/technical-documents/protocol/clinical-testing-and-diagnostics-manufacturing/cytology-and-microscopy/enzymatic-hydroxyproline-assay-protocol>

**Solutions Required:**

Acetate/Citrate Buffer – stock in 4°C fridge.

Working Oxidation Buffer – make fresh on the final day of experiment; from Oxidation Buffer stock in 4°C fridge.

Ehlrich’s reagent – make fresh on the final day of experiment.

Hydroxyproline standards – stock located in MS531 -20°C freezer; thaw and use to make graded dilutions on the final day of experiment.

**Tubes Needed**

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube Type** | **Amount** | **Label with…** | **Contents** |
| Eppendorf Tube (1.5mL) | 2x the # of Samples | Hashcode (2 tubes/hashcode) | 1 for sample chunk  1 for diluted sample |
| Eppendorf Tube (1.5mL) | 1 | “HYP Stand.” | Diluted HYP standard stock |
| Microcentrifuge Tube (0.6mL) | 8 | 0 – 7 | Graded HYP standard dilutions |

**DAY 1**

**Sample Preparation:**

1. Label Eppendorf tubes with the hashcode or sample ID.
2. Weigh and record the weights of **each** tube. The tubes have slightly different weights (~5mg) but this is relatively large when you are only working with ~30mg of tissue.
3. You can’t run more than 40 samples (in duplicate) or 24 samples (in triplicate) on a 96-well plate so it is suggested that you prep no more than 40 or 24 samples. This will become unmanageable otherwise and you are likely to make a mistake.
4. Collect your box of samples from the -80°C freezer or LN2 tank and place on dry ice and get set up with countertop space in front of a balance
5. Identify the hashcode of the first sample you are going to prepare and find the corresponding Eppendorf tube
6. Place a weigh boat on the balance and tare the weight.
7. Using forceps, remove the tissue chunk from the cryogenic vial and use a razor blade to cut a small chunk of tissue off.
8. Place this tissue on the weigh boat and record the rough weight of the tissue. The weight should be ~30mg (± 5mg). Add or subtract tissue as needed to get within the desired range.
9. Return the main tissue chunk to its cryogenic vial and place tissue from the weigh boat into the corresponding Eppendorf tube. Place the cryogenic tubes back on dry ice and the Eppendorf with tissue on ice.
10. Repeat these steps for each of the tissue samples you are analyzing.
11. Return the main tissue samples in the cryogenic vials to the -80°C or LN2 tanks.
12. Either continue to the next steps or stop here for the day and store the tubes in the -80°C freezer until you have time to move forward.

**DAY 1/2:**

1. Turn on the oven to 80°C. You will want to turn this on now so that it is hot by the time you are ready to use it.
2. Turn on the tube warmer to 110°C. You will want to turn this on now so that it is hot by the time you are ready to use it. Set this up in a fume hood for added safety.
3. Move the tissue in Eppendorf tubes from dry ice to normal ice. The tissue will slowly thaw on ice. This is okay. Collagen is hearty and you will boil these in acid, so thawing is not a problem and needed to properly grind the tissue.
4. Open the caps of the Eppendorf tubes and place tubes in the oven (should be ~70°C because it is ~10°C cooler than the set point, on average). Cook the tubes for 1 hour to obtain dry weight of tissue.
   1. NOTE: If any of the tissue samples are under 20mg, cook for ~40-45 mins instead! Tissues can be overcooked and become extremely difficult to pulverize in the next steps.
5. Turn on the tube warmer to 110°C if you haven’t already. You will want to turn this on now so that it is hot by the time you are ready to use it. Set this up in a fume hood for added safety.
6. Place a white plastic tissue pestle into the electric drill and tighten the drill’s chuck.
7. Insert the drill with pestle into the Eppendorf tube and turn on to grind/purée the tissue. Grind for ~10-15 seconds. You may need to move the drill up and down or side to side in order to blend the tissue effectively. It may not completely purée the tissue, you want to try to grind it as much as possible, but this is primarily needed to increase the surface area of the tissue so that the acid can denature the collagen properly.
8. Once the tissue is adequately puréed, remove the drill. Using forceps or the side of the tube, scrape any large bits of tissue that are on the pestle back into the Eppendorf tube. Clean the forceps and pestle with 100% EtOH and a Kimwipe to prevent cross-contamination.
   1. NOTE: to speed things up, keep two pestles in rotation – one sits in the 100% EtOH while the other is in use, then swap.
9. Cap and weigh the tube with pureed tissue. Record the weight alongside the previously recorded empty tube weight. Calculate the difference between the tubes to find the amount of tissue in the tube. This will be used later to calculate the grams of hydroxyproline/collagen per gram of tissue
10. Return the tube to normal ice and repeat this process with the remaining tissue samples.
11. After all samples are pureed and their weights recorded, move to a fume hood.
12. Carefully add 500μL of 6M HCl to each tube and vortex for a few seconds. Ensure all the tissue in the tube is suspended in the acid. You may need to tap the tube, hit it against the table, or lightly centrifuge the tube to pull down the tissue into solution (do not centrifuge too aggressively, you do not want to form a pellet). You just want to ensure that the tissue you measured in the vial actually gets hydrolyzed.
13. Place the filled tubes into the tube warmer set @ 110°C and leave to cook overnight. You will need to place a metal bar (or any heat-resistant, flat object) across the tops of the caps, then add heavy object(s) on top. The metal bar should be smooth such that it makes good contact with every tube cap. This is needed to ensure that some caps don’t slightly open when heated which will lead to uneven hydrolysis. Boiling acid will cause an increase in pressure so add heavy items to ensure tubes don’t pop open.

**DAY 2/3**

1. Once you enter the lab in the morning turn off the tube warmer BUT DO NOT remove the heavy items or the tubes as pressure will still be high in the tubes and if you remove the heavy items the tubes may pop open.
2. Let these tubes cool at room temperature for at least 3 hours.
   1. NOTE: A faster method is to fill two fiberboard freezer boxes with dry ice and set those on top of the metal bar. Place the heavy objects back on top of these because the tubes are still hot enough to pop. Be sure to monitor the temp as it drops – if the tubes freeze then you will have to wait for them to thaw.
3. At this time, you can remove the heavy items and tubes from the tube warmer. Be careful as the acid may be room temperature, but it is still acid. You don’t need to worry about protein degradation, you boiled the samples overnight in acid, so they can stay out at room temperature.
4. You may see a black precipitate in the tubes after heating. This is okay and the next steps should get most of this out of the way.
5. Add 500μL of 6M NaOH into each tube to neutralize the HCl. Vortex the tubes for a few seconds to ensure adequate mixing.
6. Centrifuge the samples at 20,000g for 10 minutes to generate a pellet of the insoluble debris.
7. While those spin down, label a new Eppendorf tube with the hashcode for each sample.
   1. If you have already labeled the second set of tubes, great! Use this time to make the Hydroxyproline standards instead.
8. Remove the tubes from the centrifuge and transfer 20-100 μL of supernatantfrom the initial tube into the new tube. Not all of the black precipitate will pellet, so be sure to transfer the supernatant **without disturbing the pelleted debris or sucking up any of the floating debris**.
   1. (50 μL works well with human myocardium)
   2. It is okay if some supernatant remains in the initial tube, you are calculating concentration of hydroxyproline so the concentration in the supernatant should remain the same regardless of if you take out all or a little of the supernatant.
   3. Decide on the concentration of sample you want to add to the wellplate beforehand and keep this consistent for all samples in the experiment. You may need to empirically determine how to dilute different samples to ensure that the concentration of your unknown sample falls within your standard curve (muscle or disease types might vary). If it is outside your standard curve then you can’t determine HYP concentration of these samples.
9. Adjust final volume in new tubes to **100μL** with diH2O
10. These new tubes should have a light yellow-ish color and be free of any visible debris. The black debris can interfere with your colorimetric assay. These are now stable at room temperature, and you can either pause here and complete the next steps the following day or push on.

**DAY 3/4 (FINAL DAY)**

1. Allow all reagents and samples to come to room temperature.
2. Prepare the standard solutions by mixing 200μL of the stock hydroxyproline standard (1 mg/mL) and with 800μL of diH2O. This will make a 0.2 mg/mL (0.2 μg/μL) solution

|  |  |  |
| --- | --- | --- |
| Chemical | Volume (μL) | Check |
| Stock Hydroxyproline Standard (1 mg/mL) | 200 |  |
| diH2O | 800 |  |

1. Using this diluted stock solution, get 8 0.6mL microcentrifuge/PCR tubes, label them 0-7, and prepare the following standards to generate a standard curve.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Standard # | 0.2 mg/mL HYP standard (μL) | ddH2O (μL) | Final Conc. (μg/μL) | Check |
| 0 | 0 | 100 | 0 |  |
| 1 | 10 | 90 | 0.02 |  |
| 2 | 20 | 80 | 0.04 |  |
| 3 | 30 | 70 | 0.06 |  |
| 4 | 40 | 60 | 0.08 |  |
| 5 | 50 | 50 | 0.1 |  |
| 6 | 60 | 40 | 0.12 |  |
| 7 | 70 | 30 | 0.14 |  |

1. Add 10μL of each standard to the first 3 columns of the wellplate (1A-1G, 2A-2G, and 3A-3G), starting in the first column and working your way down the column (see plate template). Do this in triplicate.
   1. NOTE: avoiding touching the bottom of the wellpate as this may leave marks or spots that will affect the absorbance reading later.
   2. NOTE: doing this under good lighting and laying the wellplate on a dark surface helps with visualization while pipetting.

|  |  |  |
| --- | --- | --- |
| Standard # | Amount added to well (μL) | Mass of HYP in well |
| 0 | 10 | 0 |
| 1 | 10 | 0.2 |
| 2 | 10 | 0.4 |
| 3 | 10 | 0.6 |
| 4 | 10 | 0.8 |
| 5 | 10 | 1.0 |
| 6 | 10 | 1.2 |
| 7 | 10 | 1.4 |

1. Add 10μL of diluted sample to each of the wells in duplicate/triplicate.
2. Evaporate plates to dryness by heating the plate at 65°C in an oven or in a hot plate. Approximately 25-45 minutes.
   1. NOTE: The oven remains ~10°C cooler than the target setpoint (e.g., setpoint of 75°C results in an actual temp of 65°C inside the oven). Adjust temp to account for oven miscalibration. Place a thermocouple probe in the oven to monitor true temperature.
3. Make **fresh** Working Oxidation Buffer and Elrich’s Reagent while wellplate is evaporating/before continuing onto next step.
4. Add 100μL of the Working Oxidation Buffer (containing Chloramine T) to each well, including wells with the standards.
5. Gently shake/tap the plate to mix the samples and leave at room temperature for 5 minutes.
6. Add 100μL of Ehrlich’s Reagent to each well and mix thoroughly by gently shaking/tapping the wellplate.
7. Cover the plate with an adhesive plate seal and incubate in an oven at 65°C for 45 minutes.
8. While keeping the wellplate upright, gently clean off the bottom of the plate with a kimwipe or a paper towel wet with 70% EtOH to remove any streak marks or dirty spots that may be present. Once dry, place the wellplate into the plate reader.
9. Read absorbance at 560nm. Read the absorbance within 20 minutes of heating, ideally immediately after heating.
10. DONE.

**Plate Template**:A screenshot of a test

Description automatically generated

A screenshot of a computer screen

Description automatically generated