**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:**

Chloramine T solution

Acetate/Citrate Buffer

Working Oxidation Buffer

Ehlrich’s reagent

Hydroxyproline standards

**Tubes Needed**

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube Type** | **Number** | **Label** |  |
| Eppendorf Tube | 2 times # Samples | Hashcode |  |
| Eppendorf Tube | 1 | Diluted Standard |  |
| Microcentrifuge Tube | 8 | 0 – 7 |  |

**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 ~20mg of tissue.
3. You can’t run more than 40 samples (in duplicate) on a 96-well plate so it is suggested that you prep no more than 40 samples. This will become unmanageable otherwise and you are likely to make a mistake.
4. Collect your box of samples from the -80C 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 ~ 30 mg. 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 -80C or LN2 tanks.

**DAY 1/2:**

1. 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 is actually needed to properly grind the tissue.
2. Place a white plastic tissue pestle into the electric drill and tighten.
3. Turn on the tube warmer to 110C. 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.
4. Insert the drill with pestle into the Eppendorf tube and turn on to grind/purée the tissue. You may need to move the drill up and down or side to side in order to blind 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.
5. 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
6. 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 per gram of tissue
7. Return the tube to normal ice and repeat this process with the remaining tissue samples.
8. After all samples are pureed and their weights recorded, move to a fume hood.
9. Carefully add 500 uL of 6M HCl to each tube and vortex. After all tubes have been filled with the acid move on to the next step.
10. Place the filled tubes into the tube warmer. You will need to place a metal bar 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 4 hours.
3. At this time you can remove the heavy items and tubes from the tube warmer. Be careful as there acid may be room temperature but it is still acid. You don’t need to worry about protein degradation, you just 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 rid of this
5. Add 500 uL of 6 M NaOH into each tube to neutralize the HCl. Vortex the tubes.
6. Centrifuge the samples at 10,000 g for 5 minutes to generate a pellet of this insoluble debris.
7. Label a new Eppendorf tube with the hashcode and transfer the supernatant from the initial tube into the new tube without disturbing the debris. 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.
8. These new tubes should have a light yellow-ish color and be free of any visible debris. 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**

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

|  |  |  |
| --- | --- | --- |
| Chemical | Volume (uL) | Check |
| Stock Hydroxyproline standard (1 mg/mL) | 200 |  |
| ddH2O | 800 |  |

1. Using this diluted stock solution get 8 eppendorf tubes and label them 0-7 and prepare the following standards to generate a standard curve.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Standard # | 0.2 mg/mL HYP standard (uL) | ddH2O (uL) | Final Conc. (ug/uL) | 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 10 uL of each standard to the wells starting in the first column and working your way down the column (see plate template). Do this in duplicate or triplicate.

|  |  |  |
| --- | --- | --- |
| Standard # | Amount added to well (uL) | 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 2-10 uL of sample to each of the wells in duplicate/triplicate (adjust the final volume to 10 uL per well with ddH2O. You will need to know the amount of H2O added to back calculate the concentration and ultimately ug of HYP per mg of tissue). (We will need to determine what works best for human cardiac tissue in terms of the volume of sample added. We can’t be outside the limit of detection)(**We may also need to dilute down the samples, 1:4 then adding 10 uL would be consistent with Brown et al which dilutes 1:20 then adds 50uL**)
2. Evaporate plates to dryness by heating the plate at 65C in an oven or in a hot plate. (Not sure on the time but we should determine this so that we can say “approximately X minutes”.
3. Add 100 uL of the Oxidizing Solution (containing Chloramine T) to each well (Brown says not to add to the blanks but I think you should definitely add to blanks. They add oxidizing solution without the chloramine T to the blanks)
4. Gently shake the plate to mix the samples and leave at room temperature for 5 minutes (abcam leaves it for 20 minutes which is different from most other protocol which call for 5 mins)
5. Add 100 uL of Ehrlich’s reagent to each well and mix thoroughly.
6. Cover the plate with an adhesive plate seal and incubate in an oven or hotplate at 65C for 45 minutes (Sigma kit says 65C for 60minutes but abcam and Brown both use 65C for 45mins. We might just use the the plate lid or we can try buying these seals which might help to prevent evaporation/contamination)
7. Read absorbance at 560 nm(Brown et al says 570, most others say 560, 558, or 550. We will probably go to with 560) Read the absorbance within 20 minutes of heating, ideally immediately after heating.
8. DONE

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

Description automatically generated