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Glucosyl extraction from glycogen, by acid hydrolysis (v1)

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Protocol status: In development
We are still developing and optimizing this protocol

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Disclaimer

Abstract

This protocol is designed to extract glycogen from samples and hydrolyze glycogen to glucose monomers that can be detected using an enzymatic system or using liquid chromatography-linked mass spectrometry. It has not been directly compared to other systems for measuring glycogen

Guidelines

1. Advice corresponding to individual steps is placed in the step's "notes" section
2. There are alternatives to some of the materials used herein. Glycogenolysis can also be performed enzymatically using amyloglucosidase or amylase.

Materials

Reagents

Hydrochloric acid
Ammonium bicarbonate
Methanol
Ethanol
Dry ice & bucket

Equipment

Centrifuge (capable of 17,000 x g at 4°C)
Heat block or water bath capable of maintaining temperature at 80°C
Fume hood
Ultrasonic homogenizer
Speedvac / Lyophilizer / system to dry samples

Consumables

1.5 mL Eppendorf tubes

Troubleshooting

Safety warnings

Hydrochloric acid is a strong acid. Be careful when handling and use in a fume hood.

Methanol is volatile and flammable. Do not use near an open flame.

Make buffers and prepare heating element

1 The extraction buffer is 80% methanol, 20% ethanol.

The hydrolysis buffer is 2N HCl (hydrochloric acid) in H_2O.

The neutralization buffer is 2N NH4OCOOH (ammonium bicarbonate) in H_2O.

Notes:
- Plan to make at least 1.5 mL of extraction buffer per sample.
- If the extracted tissue will be used for mass spectrometry, this buffer should be supplemented with an internal standard.
- If the extracted tissue will be used for mass spectrometry, be sure to us HPLC-grade buffer components.

Homogenize tissue

2 We normally begin with tissue that was flash-frozen in a plastic 1.5 mL Eppendorf tube and stored at -80°C. The tissue is moved from the -80°C freezer to dry ice and 500 µL of extraction buffer is added per tube.

Homogenize the tissue using a technique optimized for the tissue of interest. Specific "notes" regarding homogenization of samples from mouse retina and RPE-choroid-sclera.

Notes:
- I am not providing more specific detail because there are a large variety of tissue disruption techniques and each is can be optimal for different applications types.
- To disrupt retina tissue we use an ultrasonic homogenizer at 10% of it's maximal power output. 5 pulses (each pulse is a duty cycle) per tube of retina tissue is usually sufficient to fully disperse the tissue, and to ensure complete homogenization we use 10 pulses. I acknowledge that there will be lab-to-lab differences in how the power output affects the actual sonication, and a lab should determine their own optimal settings for tissue disruption.
- RPE-choroid-sclera is more durable than retina tissue, and the extracellular matrix components of the tissue are not homogenized. We use the same settings to homogenize RPE cells

Extract glucose and other metabolites

3 Incubate tissue extract on dry ice for >45 minutes.

Notes:
- Proteins and glycogen should be poorly soluble in methanol/ethanol and should precipitate

4 Centrifuge sample at maximum speed to pellet protein and glycogen. For us, maximum centrifuge speed is 17,000 x g, and we pellet samples at this speed over 15 minutes at 4°C.

Transfer supernatant containing unpolymerized glucose and other metabolites to a new 1.5 mL Eppendorf tube.

Notes:
- Centrifuge sample at maximum speed to pellet protein and glycogen. For us, maximum centrifuge speed is 17,000 x g, and we pellet samples at this speed over 15 minutes at 4°C.

5 Resuspend the tissue pellet (containing glycogen) in 500 µL of fresh extraction buffer then repeat step 4. Repeat this once more.

Notes:
- I will normally combine the supernatant(s) in step 5 with the supernatant in step 4. I dry the combined sample and later resuspend it as needed for further analysis
- At this point the protein/glycogen pellet should have been washed in 80% methanol, 20% ethanol three times.

Hydrolyze glycogen

6 In a fume hood, resuspend protein/glycogen pellet in 50-100 µL of HCl 'hydrolysis buffer'. Incubate sample at 80°C for 1 hour on a heat block or in a water bath.

Notes:
- Ensure that lids are kept securely in place, some varieties of tubes may otherwise 'pop'.
- Following this hydrolysis step, what was previously a molecule of glycogen should now exist as glucose monomers.

7 Remove tubes from heat and add (dropwise) an equal volume of neutralization buffer as you had hydrolysis buffer. Vortex samples and centrifuge at 17,000 x g to pellet large debris.

Notes:

- The solution will bubble up quite a bit as the acid-base reaction occurs. The more base is added at once, the more bubbles will appear (and potentially overflow from the tube). So smaller drops are recommended.

8 Transfer the supernatant to a new tube. This supernatant contains the glucosyl residues formerly on glycogen. It can be assayed enzymatically to determine glycogen content, or using mass spectrometry.

Note:

- If directly supplied to a liquid chromatography-mass spectrometry HPLC column, samples could add too much salt to the system and ruin the column. A sample clean-up step is recommended.
- For sample clean-up, we suggest drying samples, adding 90% methanol, vortex the sample, pellet samples at 17,000 x g for 30 minutes and 4°C, and transfer supernatants to new tubes. Salts are not highly soluble in methanol and should be retained in a pellet following the spin step. Glucose is also poorly soluble in methanol, so depending on glycogen/glucose content the clean-up step may not retain 100% of the glucose. We have not tested glucose yield using this method.

Protocol references

Glycogen isolation is sensitive to acid concentration and time:
https://www.sciencedirect.com/science/article/pii/S0960852412008401?via%3Dihub#b0075

There are more protocols worth citing, and I will add them later:
https://www.sciencedirect.com/science/article/pii/S000326970800208X?via%3Dihub

used multiple methods:
https://www.sciencedirect.com/science/article/pii/S1550413113001484?via%3Dihub#sec4