

Precipitation, digestion, and quantitation of glycogen from cultures of primary hepatocytes

Reagents

0.75% SDS

100% TCA

0.2M NaAc (pH 4.4-4.6@RT--equal volumes NaAc and AcAc)

1N NaOH

70-75% and 100% EtOH (undenatured)

Glucoamylase (Fluka); 100x = 30mg/mL in 0.2M NaAc (stable at 4C for ~2 months)

Materials

96-well flat-bottom (clear) assay plate

1.7mL microfuge tubes

Microcentrifuge @ 4°C

Plate reader @405nm

Note: Instructions assume 12-well plates

A. Precipitation

1. Dump media and lyse cells with 200uL 0.75% SDS. You may wash cells with cold PBS once or twice before lysing so dead cells do not interfere with protein measurements. Residual glucose is not an issue in this assay due to precipitation and washing.

Note: Make sure cells are well-lysed; tap plate and place on shaker for 10-15 minutes if necessary.

Note: Due to collagen coating, it may appear that some of the lysate is still on the bottom of the well, even after shaking and standing plate on-edge to allow lysate to flow to bottom of well. Ignore.

Note: 0.75% SDS is optimal for cell lysis without interfering with protein precipitation; higher SDS percentages (>1.0%) tend to reduce the efficiency of TCA protein precipitation, while lower SDS percentages (<0.50%) are not sufficient to lyse cells completely.

2. Transfer lysate to 1.7mL centrifuge tubes. Scraping is not necessary- simply pipette the lysate out.

Note: If measuring protein, remove a 5uL aliquot and add to pre-labeled tubes containing 25uL H₂O. Take care when aliquoting for protein, due to stickiness of lysate. Try to take lysate from edge of tube to avoid DNA mass. You may quickly sonicate the lysate to aid pipetting (1 second at low power) – sonication has no adverse effects on glycogen recovery.

3. Add 25uL of 100% TCA to precipitate protein (5-10% final TCA content). Vortex and allow to precipitate at 4°C for 20-60 minutes. Occasionally check on tubes and gently agitate/flick to speed up process.

Note: We have found that lower temperatures actually result in less efficient precipitation. Tubes should remain at 4°C until a fluffy white precipitate can be seen; the remaining supernatant should look clear.

This step is CRITICAL for full recovery. Inefficient precipitation will inhibit digestion.

4. Centrifuge at top speed (13000 RPM) at 4°C for 10 minutes.

Note: You should have a tightly-packed pellet with a clear supernatant

5. Transfer all supernatant to clean 1.7mL centrifuge tubes. Total volume should be ~210-230uL.

6. Add 750uL (2.5-3.0 volumes) of 100% EtOH (RT or cold) to supernatant. The final concentration of EtOH should be between 65-80%.

7. Vortex and place tubes at -80°C for \geq 2 hrs to precipitate glycogen.

Note: For this step, you may leave samples for >1 week at -80°C without affecting precipitation efficiency.

B. Wash

8. Take tubes from -80C and centrifuge at max speed (13000 RPM) at 4°C for 15-20 minutes to pellet glycogen.

9. Remove (simply invert tubes or carefully use a bench vacuum) supernatant and place tubes cap-down (and open) on clean paper towel to allow excess ethanol-TCA to drain. You may or may not see a small glycogen pellet (clear) at the bottom of the tube. We have found that with cells, the pellet is very tightly packed at the bottom, and you do not need to worry about it coming loose.

10. Wash with 1mL of 70-75% EtOH. Add ethanol, invert tubes, and re-centrifuge at max speed at 4°C for 10 minutes.

Note: This second centrifugation is probably not necessary, but should be performed as a precaution.

11. Remove supernatant and allow to blot on paper towel for ~5 minutes.

12. Place tubes into speedvac and allow to dry for ~1-3 minutes at low or medium heat. Allow all liquid to evaporate; you do not need to be concerned about over-drying the pellet.

13. Remove tubes from speedvac and add 350uL 0.2M NaAc. You should make your blanks and standards at this time, in the same volume of 0.2M NaAc. A (high) glycogen standard is recommended, to control for digestion efficiency. You may wish to run this standard +/- glucoamylase.

14. Sonicate samples at low power for 5 seconds.

Note: Residual protein will be broken up during this step, which aids in digestion. Depending on how efficiently you got rid of protein, sonication can significantly tighten your data, as excess protein appears to interfere with the ability of glucoamylase to access/digest glycogen. If you precipitated the protein well, sonication is optional- testing is required to determine this. Most often, we sonicate as a precaution, unless we are working with radiolabeled samples.

15. After sonication, there should be no visible residue on the bottom or side of the tube. Place tubes in centrifuge (4°C or RT) and briefly spin down to bring liquid to bottom of the tube (5 seconds at 5000 RPM works fine).

C. Digestion

16. Add 1x glucoamylase to all samples and blanks/standards. [Do NOT add any glucoamylase to your (-) glucoamylase glycogen standard, if you have one]. Vortex briefly at low speed, to mix.

17. Allow to digest at 37-45°C (45°C is preferred) in the dark for 1.5hrs, with shaking.

18. When finished digesting, spin at top speed at 4°C for 10 minutes. Allow samples to come to room temperature before pipetting.

19. Pipette 200uL aliquots of supernatant in a 96-well plate.

Note: Generally, 200uL/350uL will give ODs that are within range of the machine. If glycogen content is especially high, you may need to reduce the volume assayed (we have had to use as little as 15-25 uL). The limit of most spectrophotometers is OD = 4.0, which corresponds to approximately 8ug of glucose (depending on how long you allow the assay to react).

20. Neutralize the NaAc with NaOH. Add 10uL of 1N NaOH per 200uL 0.2M NaAc. For more or less NaAc, scale NaOH concentration and/or volume accordingly.

D. Quantitation

22. Add GOD-PERID rxn mix, at 100uL/well. Allow to react in dark for 5-10 minutes.

Note: The final assay pH should be ~7.0-7.5.

24. Read at 405nm.

Reagent Sources and additional information

Sigma	A1888	ABTS; [2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)- diammonium salt]
Sigma	10115	Amyloglucosidase from <i>Aspergillus niger</i> ; ~70 units/mg, lyophilized powder; pH optimum = 4.6-4.8, 50-55°C (also known as glucoamylase)
Sigma	P6782	Type VI-A, essentially salt-free, lyophilized powder, ~1000 units/mg solid (using ABTS), 250-330 units/mg solid (using pyrogallol) ; pH optimum for ABTS = 5.0, 25°C
Sigma	G7141	Glucose Oxidase from <i>Aspergillus niger</i> . Type X-S, lyophilized powder, 100,000-250,000 units/g solid; pH optimum = 5.5, 35°C

Stock solutions

1. ABTS = 50 mg/mL in H₂O. (4°C, >2 months)
2. HRP (PERID) 10x = 5.0 mg/mL in 50/50 0.2M Na-phosphate/glycerol (-20°C, >1yr)
3. Glucose Oxidase (GOD) 10x = 20mg/mL in 50/50 0.2M Na-phosphate/glycerol (-20°C, >1yr)
4. 1M Na-phosphate, pH 6.3-6.5@RT = 50/50 1M Na-phosphate Monobasic/1M Na-phosphate Dibasic
5. Glucoamylase, 100x = 30mg/mL in 0.2M NaAc (stable at 4C for ~2 months)
6. 2M NaAc @pH 4.6@RT = 50/50 2M NaAc/2M AcAc (dilute 10x to 0.2M for assay)
7. 1N NaOH
8. Glucose standards in H₂O, top standard @20ug/uL --> 0.3125ug/uL
9. Glycogen standard in H₂O @20ug/uL

Note: Make 1x stocks of HRP and GOD in 50/50 0.2M Na-phosphate/glycerol.

Master mixes

GOD-PERID rxn mix, per sample:	H ₂ O:	73uL
	1M Na-phosphate, pH 6.3-6.5@RT:	20uL
	Add 93uL of GOD-PERID buffer +	3uL of ABTS, 50mg/mL
		2uL of 1x HRP
		2uL of 1x GOD

100uL final volume = sufficient for 1 sample (enough substrate and enzyme to max out absorbance (>10ug glucose oxidizing capacity))