O-GlcNAcase Activity Assay

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Based on: Macauley MS et al., 2005. O-GlcNAcase uses substrate-assisted catalysis: kinetic analysis and development of highly selective mechanism-inspired inhibitors. J Biol Chem 280(27):25313-25322.

STEP 1: EXTRACT PROTEIN FROM CELL PELLETS (FROZEN AT -80 DEGREES C)

Reagents and Equipment:

a. Ice-cold extraction buffer: 1% NP-40 in TBS (20 mM Tris HCl, 150 mM NaCl), 2mM EDTA, pH 7.4

b. Inhibitors:

PIC2 (protease inhibitor) (Calbiochem #539132)

PIC3 (protease inhibitor) (Calbiochem #539134)

10 mM β-hex in DMSO (hexosaminidase inhibitor) (Calbiochem #376820)

0.5 mM Thiamet-G in 1 M Hepes pH 7.5 (O-GlcNAcase inhibitor)

3 mM TSA1 in DMSO (optional; deacetylase inhibitor)

100 mM PMSF in 100% ethanol (serine protease inhibitor; very toxic)

1 M NaF in milliQ water (phosphatase inhibitor)

1 M β-glycerophosphate in milliQ water (phosphatase inhibitor)

c. Bucket with ice

d. Vortex

e. Sonicator

f. Centrifuge at 4 degrees C

g. Eppendorf tubes

Procedure:

a. Add the following inhibitors at 1/1000 to ice cold extraction buffer:

PIC2

PIC3

β-hex (10 μM final)

Thiamet-G (0.5 μM final)

TSA1 (3 μM final; optional)

PMSF (0.1 mM final; this inhibitor is inactivated within 30 minutes of being added to solutions, so it is essential to add this inhibitor immediately before adding the extraction buffer to cell pellets)

b. Add the following inhibitors at 1/100 to the extraction buffer:

NaF (10 mM final)

β-glycerophosphate (10 mM final)

Note: If you are only doing O-GlcNAcase assays and not OGT assays, you can perform a total nuclear and cytoplasmic protein extraction and then omit the desalting step (**step 3**). Leave out the Thiamet-G and β-hex inhibitors from the extraction buffer.

c. Add 220 μL of extraction buffer with inhibitors to each cell pellet (~8 million U2OS cells). Note that the volume of extraction buffer added depends on the cell type and cell number.

d. Incubate on ice for 30 minutes, vortexing every 5 minutes.

e. Sonicate for 5 seconds on setting 3. For larger cell pellets it may be necessary to sonicate longer.

f. Centrifuge at 17,110 x g for 30 min at 4 degrees C.

g. Collect the supernatant and place in a new Eppendorf tube. Determine the protein concentration. We typically use the Pierce 660 assay.

STEP 2: PIERCE 660 PROTEIN ESTIMATION

Reagents and Equipment:

a. Pierce 660 protein assay reagent (Pierce #22660)

b. Eppendorf tubes

c. BSA at 2 mg/mL (Pierce #23209)

d. Plastic cuvettes

e. UV/visible spectrophotometer

f. Vortex

Procedure:

a. Add 1mL of Pierce 660 assay reagent to Eppendorf tubes.

b. Make a BSA standard curve and vortex. (For example, add 0, 2.5, 5, 7.5, or 10 μL of 2 mg/mL BSA to standard tubes)

c. Add 5 μL of each sample to tubes and vortex.

d. Incubate at room temperature for 5 minutes.

e. Transfer to a plastic cuvette. Read the absorbance at 660 nm in a UV/visible spectrophotometer.

f. Generate a BSA standard curve and determine the protein concentration for each sample.

g. Make each sample equal concentration, typically 1mg/mL. Dilute the samples with extraction buffer containing inhibitors.

h. Save a small aliquot for SDS-PAGE analysis.

STEP 3: DESALT SAMPLES

There are several methods that can be used to desalt protein samples, which include G50 sephadex (home poured), PD10 columns (GE Healthcare), G50 spin columns (Pierce), and Zeba spin desalting plates (Pierce). For large numbers of samples, we prefer to use Zeba spin desalting plates.

Reagents and Equipment:

a. Zeba Spin Desalting Plates (Pierce #89807); includes wash and elution plates
b. Desalting buffer: 20mM Tris-HCl pH 7.8, 20% glycerol
c. Centrifuge at 4 degrees C

Procedure:

a. Equilibrate a Zeba spin desalting plate to room temperature.

b. Remove the sealing material from the bottom of the plate. Only uncover the number of wells that you will use.

c. Place the desalting plate on top of the wash plate.

d. Remove the sealing material from the top of the plate. Only uncover the number of wells that you will use.

e. Spin at 1,000 x g for 2 min at 4 degrees C, discard the flowthrough. You will need an old plate as a balance. Typically we weigh the new plate and add water to the old plate to balance.

f. Carefully apply 250 μL of desalting buffer, spin at 1,000 x g for 2 min, repeat x4.

g. Stack desalting plate on top of blue elution plate.

h. Apply 20-100 μL of sample to resin bed, spin at 1,000 x g for 2 min (see below for an example layout). For 20 μL samples (> 0.3 μg/μL), apply 20 μL of desalting buffer on top of the resin bed after the sample has fully absorbed to ensure maximal protein recovery.

i. Reassess the protein concentration on 10 uL of sample in 96-well format. We typically use the Pierce 660 assay (**step 4**).

An example desalting plate is shown below. In this particular example, a maximum of 3x100 uL can be loaded onto the resin in total for each sample. It is important to load enough of each sample to perform a minimum of 6 assays and protein estimation. Depending on the cell type, >10 μg will be required for each replicate. Thus at least 70 μg of protein for each sample is required to complete the protein estimation and assay.

	1	2	3
A	Sample 1		
B	Sample 2		
C	Sample 3		
D	Sample 4		
E	Sample 5		
F	Sample 6		
G	Sample 7		
H	Sample 8		

Note: The samples obtained from this desalting procedure can also be used to perform OGT activity assays. OGT activity assays typically require less protein.

STEP 4: PIERCE 660 PROTEIN ESTIMATION IN 96-WELL FORMAT

Reagents and Equipment:

a. Pierce 660 protein assay reagent (Pierce #22660)

b. Desalted samples from step 3

c. BSA standards

d. Desalting buffer: 20mM Tris-HCl pH 7.8, 20% glycerol

e. Clear 96-well plate and sealing tape (Corning #6570)

f. Vortex with 96-well plate attachment

g. Centrifuge at 4 degrees C

h. Microplate reader

Procedure:

Note: It is important that the final volume in each well is equal!

a. Add 100 μL of Pierce 660 assay reagent to each well using a repeating pipette.

b. Add 10 μL of each sample.

c. Add 10 μL of extra Pierce 660 only wells.

d. Make up a BSA standard curve in desalting buffer (example is shown below). Add 10 μL to each well in duplicate.

0 μg/μL, 0.2 μg/μL, 0.4 μg/μL, 0.6 μg/μL, 0.8 μg/μL, 1.0 μg/μL, 1.5 μg/μL, 2 μg/μL

e. Agitate on the vortexer to mix.

f. Spin down at 1,000 x g for 15 seconds.

g. Read the absorbance at 660nm in a microplate reader.

h. Do not adjust the protein concentration, as each sample should have a relatively even concentration.

An example protein estimation 96-well plate is shown below:

	1	2	3	4	5	6
A	Sample 1			Pierce 660 only	Desalting buffer	Desalting buffer
B	Sample 2				2 μg BSA	2 μg BSA
C	Sample 3				4 μg BSA	4 μg BSA
D	Sample 4				6 μg BSA	6 μg BSA
E	Sample 5				8 μg BSA	8 μg BSA
F	Sample 6				10 μg BSA	10 μg BSA
G	Sample 7				15 μg BSA	15 μg BSA
H	Sample 8				20 μg BSA	20 μg BSA

STEP 5: SET UP O-GLCNACASE ACTIVITY ASSAYS

Reagents and Equipment:

a. Black flat-bottomed 96-well plate (Greiner Bio-One #655086) and sealing tape (Corning #6570)

b. 10x O-GlcNAcase assay buffer: 500 mM sodium cacylodate, pH 6.4, 3% BSA

c. 1M GalNAc

d. 100mM 4MU-GlcNAc in DMSO (Sigma #M2133)

e. 100mM 4MU-GalNAc in DMSO (Sigma #M9659)

f. MilliQ water

g. Desalted samples from step 3

h. Desalting buffer: 20mM Tris-HCl pH 7.8, 20% glycerol

i. Free 4MU (4-methylumbelliferone) at 100 mM in 100% ethanol (Sigma #M1381)

j. β-N-acetylhexosaminidase$_f$ (NEB #P0721S)

k. Quenching buffer: 200 mM glycine pH 10.75

l. Centrifuge at 4 degrees C

m. Microplate reader

Procedure:

To a black flat-bottomed 96-well plate:

a. Add 30 μL of desalting buffer (blank) to A7-12 (see template, next page).

b. Dilute 5 μL of HexC positive control (β-acetylhexosaminidase$_f$) in 1mL of desalting buffer. Add 30 μL to B7-12 (see template).

c. Add 30 μL of each sample to the appropriate rows/columns (see template).

d. Prepare a 4MU standard curve in desalting buffer (see template). Add 50 μL to each well and perform this in triplicate. The standard curve typically saturates at ~75 nmoles.

0 mM, 0.1 mM (5 nmoles/50 μL), 0.2 mM (10 nmoles/50 μL), 0.5 mM (25 nmoles/50 μL), 1.0 mM (50 nmoles/50 μL), 1.5 mM (75 nmoles/50 μL)

An example setup for 96-well format O-GlcNAcase assays is shown below. Remember that a minimum of 10 μg of protein is required per assay. We use a multichannel pipette to move protein from the desalting plate to the assay plate. In the template shown below, you can assess 11 samples. It may be possible to assess 12 samples by dropping two points on the 4MU standard curve or by performing the standard curve in duplicate.

	4MU-GlcNAc			4MU-GalNAc			4MU-GlcNAc			4MU-GalNAc		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1						Desalting buffer (blank)					
B	Sample 2						β-acetylhexosaminidase$_f$ (+ control)					
C	Sample 3						0 nmoles 4MU			25 nmoles 4MU		
D	Sample 4						5 nmoles 4MU			50 nmoles 4MU		
E	Sample 5						10 nmoles 4MU			75 nmoles 4MU		
F	Sample 6											
G	Sample 7											
H	Sample 8											

e. Prepare two master mixes:

Component	1 assay*	4MU-GlcNAc master mix for 50 assays	4MU-GalNAc master mix for 50 assays
10x O-GlcNAcase assay buffer	5 μL	250 μL	250 μL
1 M GalNAc	5 μL	250 μL	250 μL
100 mM 4MU-GlcNAc or	0.5 μL	25 μL	-
100 mM 4MU-GalNAc		-	25 μL
MilliQ water	9.5 μL	475 μL	475 μL
Total	20 μL	1000 μL	1000 μL

Note: 1 M GalNAc is added to the reactions to inhibit the lysosomal hexosaminidases. 4MU-GlcNAc is the substrate of O-GlcNAcase. 4MU-GalNAc is a negative control substrate that allows for an assessment of the activity of any contaminating lysosomal hexosaminidases.

*Note: The total master mix volume per assay can vary depending on the volume of the sample used. The minimum volume of master mix used is 10 μL and the maximum sample volume is 40 μL.

f. Add 20 μL of the appropriate master mix to each well, but do not add master mix to the 4MU standard curve. The contents of each master mix are shown above.

g. Immediately quench the reactions in columns 3, 6, 9, and 12 with 150 μL of quenching buffer. This is our zero time-point control.

h. Seal plate with foil and spin down for 15 seconds at 1,000 x g.

i. Incubate for 1-2 hours at 37 degrees C.

After ~1 hour of incubation, read the assays on the microplate reader (excitation 368nm, emission 450nm, sensitivity of 50). If your samples read 3- to 5-fold over background, then the reaction can be quenched.

j. Quench the remaining wells with 150 μL of quenching buffer.

k. Read samples in the microplate reader (excitation 368nm, emission 450nm, sensitivity of 50).

l. Determine activity of O-GlcNAcase. Calculate the nmoles of GlcNAc released per minute per mg of protein.