Module 7: The O-GlcNAc modification

Faculty Leader: *Natasha Zachara Ph.D.*

Prepare Tissue Lysates

Extraction

1. Make buffer 50mM HEPES with inhibitors (Make more buffer than needed)
2. Add to heart, lung, or brain powder in 2ml eppendorf tube, thaw on ice for 5min
    a. Use 10ml per 100mg
3. Grind with Polytron 2x30s (Leave on ice in-between)
4. Spin in microfuge 20minutes at full spend
5. Save supernatant
6. Estimate protein using the Pierce 660nm kit
7. Make lysates equal concentration

OGT Assays

Reagents

- CK2 Peptide 10mM (PGGSTPVSSANMM, MW 1235, 10mM = 12.35mg/ml)
- c-MYC Peptide 10mM (LLFELLPTPPLSPSRR, MW 1836, 10mM = 18.36mg/ml)
- α-Crystallin (AIPVSREEK, MW 1028, 10mM = 10.28mg/ml)
- 0.5 μCi UDP-GlcNAc/reaction (~$1.1x10^6$ DPM)
    - Specific Activity: 60ci/mMol
- 10X OGT Assay buffer: 500mM Sodium Cacodylate pH6.5, 10mg/mL BSA
    - Supplement with Thiamet G
- 25mM 5'AMP (inhibits pyrophosphorylases)
- Alkaline Phosphatase (converts ADP (an OGT inhibitor) to AMP)
- Sealing film
- Positive: OGT wild-type His 6
- Negative: OGT Desalting buffer (20mM Tris-Hcl pH 7.8, 20% glycerol)
- 50mM Formic Acid
- 50mM Formic Acid, 1M NaCl
- MilliQ Water
    - Methanol

- C_{18} Reversed Phase 96 well plate
- Scintillation Vials and Fluid

Protocol:

1) Set your samples (20µL) up as follows in a **round bottomed 96well plates:**

	Group 1 **CK2**			Group 2 **α-Crystallin**			Group 3 **c-Myc**			**No Peptide Control**		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart
B	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart
C	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung
D	Lung	Lung	Lung	Liver	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung
E	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain
F	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain
G	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
H	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

1. Make up the following master mixes (you will add 30µL to each sample to start the reaction)

Reagent	**Per Assay (µL)**	**CK2 (µL)**	**α-Crystallin (µL)**	**c-Myc (µL)**	**No Peptide (µL)**
10X OGT Assay Buffer	5	140	140	140	140
Alkaline Phosphatase	0.25	7	7	7	7
UDP-GlcNAc	0.5	14	14	14	14
25mM 5'AMP	0.5	14	14	14	14
10mM Peptide	5	140	140	70	0
Water	19.25	539	539	539	679
Total (28 reactions)	**30**	**840**	**840**	**420**	**420**

Note, the UDP-GlcNAc is dried down to remove the ethanol, which would inhibit the reaction.

2. Add 30µL to each reaction, as follows (Use the color guide).

	Group 1 CK2			**Group 2 α-Crystallin**			**Group 3 c-Myc**			**No Peptide Control**		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart
B	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart	Heart
C	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung
D	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung	Lung
E	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain
F	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain	Brain
G	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
H	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

3. Use the repeating pipet for this job. Cover the plate in sealing film. Spin down and incubate at room temperature for 30minutes.
4. Stop the reaction by adding 150µL of 50mM Formic Acid, 1M NaCl. Replace sealing film and respin plate. Note. The reactions can be stored at -20°C at this point for several months.
5. Activate the C_{18} plate with 2mL of Acetone. Apply the vacuum.
6. Add 2mL of methanol. Flow under gravity, each wash will take ~7-10 minutes. At the end of each wash, lift the C_{18} plate and apply the vacuum to draw away the excess buffer.
    a. *Note: Vacuum should not be applied to the plate until the last step. You get better and more reproducible chromatography under gravity. The columns will not run dry.*
    b. *Repeat this step once.*
7. Equilibrate the plate with 3x 2mL 50mM Formate, 1M NaCl. Let at least 90% of the buffer flow-through before applying the next wash. (Again, lift up the plate and apply the vacuum to remove excess buffer)
8. Load sample.
9. Wash assay tubes/wells with 200µL 50mM formate, 1M NaCl. Apply to column.
10. Wash with 2x2mL 50mM Formate, 1M NaCl.
11. Wash with 2x2mL MilliQ.
12. Wash with 2x2mL 50mM formic acid.
13. You can now apply a vacuum to remove the excess aqueous buffer from the plate.

14. Plate a collection plate under the 96 well plate.
15. Elute with 1.5mL 100% methanol (this should be added with the repeating pipette, which is more accurate than the repipette). Allow the sample to elute under gravity, and apply the vacuum to elute the last of the methanol.
16. Transfer 750µL (50%) of the eluent into scintillation vials:
17. Add 5-10mLs of scintillation fluid and count on the 1mL program. The ration of scintillation fluid to methanol should be 3:1. ***This step should be performed in the hood to avoid breathing in the liquid that aerosolizes during this step.***
18. Count on the 1 minute program
19. Calculate your activity per minute, per mg.
20. Make sure you dispose of all radiation waste appropriately, and that this has been documented.

O-GlcNAcase assays

There are three substrates you can use to detect O-GlcNAcase activity. *p*NP-GlcNAc is the least sensitive and depending on the cell type you will need 20-50µg of cell extract minimum and a 2-24h incubation time. We are using 4-methylumberlipherol βGlcNAc. As a negative control we are using 4-methylumberlipherol βGalNAc, we do this as it allows us to assess the activity of contaminating lysosomal hexosaminidases. Thiamet G should inhibit OGA (Hexosaminidase C), whereas GalNAc should inhibit the lysosomal hexosaminidases.

Reagents and Equipment

- 1M GalNAc, inhibits lysosomal hexosaminidases
- 10X OGT Assay buffer: 500mM Sodium Cacodylate pH6.5, 10mg/mL BSA
- Positive Control Protein (β-N-acetylhexosaminidase, NEB P0721)
    - HexA/C: Dilute 5µl in OGT Desalting buffer
- 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (Dissolved in DMSO, Sigma # M2133), protect from light (4MU-GlcNAc)
- 4-Methylumbelliferyl N-acetyl-β-D-galactosaminide (Dissolved in DMSO, Sigma # M9659), protect from light (4MU-GalNAc)
- 4-methylumberlipherol standard: 50µM
- 100mM TMG
- Quench Reagent Buffer: 200mM Glycine pH 10.75
- Black Flat-bottomed 96 well plates

- Plate reader

Protocols

1) Set your samples up as follows in black flat bottomed plates as follows:

	4MU-GlcNAc			4MU-GalNAc			4MU-GlcNAc/GalNAc			4MU-GlcNAc		
	1	2	**3**	4	5	**6**	7	8	**9**	10	11	**12**
A	Heart	Heart	**Heart**	Heart	Heart	**Heart**	Heart	Heart	**Heart**	-ve	-ve	-ve
B	Lung	Lung	**Lung**	Lung	Lung	**Lung**	Lung	Lung	**Lung**	-ve	-ve	-ve
C	Brain	Brain	**Brain**	Brain	Brain	**Brain**	Brain	Brain	**Brain**	-ve	-ve	-ve
D	+ve	+ve	+ve	+ve	+ve	**+ve**	+ve	+ve	**+ve**	STD1	STD1	**STD1**
E	Heart	Heart	**Heart**	Heart	Heart	**Heart**	Heart	Heart	**Heart**	STD2	STD2	**STD2**
F	Lung	Lung	**Lung**	Lung	Lung	**Lung**	Lung	Lung	**Lung**	STD3	STD3	**STD3**
G	Brain	Brain	**Heart**	Brain	Brain	**Brain**	Brain	Brain	**Brain**	STD4	STD4	**STD4**
H	+ve	+ve	+ve	+ve	+ve	**+ve**	+ve	+ve	**+ve**	STD5	STD5	**STD5**

2) The blank should contain 30µL of desalting buffer, and HEX should contain NEB beta-hexosaminidase (5µL in 1000µL). Using a multichannel pipette transfer 20µL of the desalted protein from the desalting plate to the assay plate.

21. Set up the following Master Mixes

Component	**Per Assay (µL)**	**4MU-GlcNAc (µL)**	**4MU-GalNAc (µL)**	**4MU-GlcNAc (µL)**	**4MU-GalNAc (µL)**	**4MU-GlcNAC (µL)**	**4MU-GalNAc (µL)**
10X Buffer	5	100	100	100	100	100	100
1M Gal	5	100	100	-	-	-	
TMG 1mM	0.5	-	-	-	-	5	5
4MU-GlcNAc	0.5	10	-	10	-	10	-
4MU-GalNAc	0.5	-	10	-	10	-	10
Water	-	390	390	490	490	485	485
Total	30	600	600	600	600	600	600

22. Add 30µL to each well (as indicated in the color key above)

  a. Stop rows 3, 6, 9, and 12 with quenching reagent

  b. This is your ZERO time-point control

c. This control is most important when using *p*NP-GlcNAc, as some proteins adsorb at A400. It may also be important is using cells containing fluorescent proteins.

23. Seal the plate with foil sealing film
24. Spin the plate down
25. Incubate for 30min at 37°C
26. Stop the reaction by adding 150µL of Quenching Buffer
27. Set up a free methylumberliferol standard curve.
28. Read your samples
    a. excitation 368nM and emission 450nm