Module 5: Metabolic Engineering/Glycan Labeling: Release & Detection

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Detection of glycoproteins

In this laboratory we will utilize click chemistry, galactosyltransferase labeling, and lectin blotting to detect changes in the glycans of glycoproteins. The techniques chosen are accessible in most laboratories, but have significant caveats that are highlighted by the samples. **Each group will receive 2 mystery samples in which N- or O-linked glycans have been manipulated.** We'll discuss the data at the end of class. *It's critical that you label your samples well (so someone else knows who they are).*

We have run this class 3 times before and this has been a SIGNIFICANT issue.

Step	**Description**	**Day**	
	Protein Lysates	Wednesday	
1	Precipitate proteins	Wednesday	am
2	PNGase F digestion	Wednesday	am
3	Precipitate proteins	Wednesday	pm
4	Gal Transferase	Wednesday	pm
5	Precipitate proteins	Thursday	am
6	Click Chemistry	Thursday	am
7	Precipitate proteins	Thursday	am
8	Protein Estimation	Thursday	am
9	Gels/Blots – Cathrine will run blots, scholars will apply antibodies and lectins	Thursday	*middle of day*
10	Finish gels and blots	Friday	pm

Protein Extraction – This has been completed for you.

Materials and equipment:

1. Ice bucket/Ice
2. Dry ice in a foam container
3. Ice Cold PBS, Scraper
4. Cells
5. Centrifuge at 4^{o}C
6. NETN extraction buffer: 50mM Tris HCl, 250mM NaCl, pH7.5, 0.5% v/v NP-40, 5mM EDTA, pH8
7. Protease, glycosidase, and phosphatase inhibitors

Procedure:

1. Wash cells in PBS;
2. Harvest, and pellet.
3. Add inhibitors to extraction buffer*, for 5mls:
    a. 5µl PMSF (Stock 100mM in ETOH, final concentration 0.1mM) **;
    b. 5µl PIC3 and PIC2 (Stock 1000x, final concentration = 1x);
    c. 5µl ThiametG (Stock 2mM, final concentration = 2µM);
    d. 5µl of β-hexosaminidase inhibitor
    e. 500µl KF or NaF (Stock 1M, final concentration 1mM)
    f. 500µl α-glycerophosphate (Stock 1M, final concentration 1mM);

    The inhibitors "expire". So we add inhibitors to the volume of buffer that we are going to use for our experiment. PMSF is inactivated within 30minutes of adding it to solutions. Add PMSF right before adding extraction buffer to the samples. PMSF is VERY toxic, especially as a powder. Take appropriate precautions.
4. Add extraction buffer to each sample: 400µl
5. RSS the cells by vortexing.
6. Incubate the cells on ice for 20minutes. Vortex the cells for 5-10s every 5minutes.
7. Sonicate cells for ~5s on setting 2.5 (Hart Lab)
8. Pellet at full speed in a microcentrifuge or 14,000RPM for 20minutes at 4^{o}C.
9. Remove tubes to an ice bucket, and transfer the supernatant to a new labeled tube.
10. Discard the pellet.
11. Determine the protein concentration of the samples.

Protein Estimation – This has been completed for you.

Materials and equipment:

1. Pierce 660 Reagent
2. BSA protein standard
3. Cuvettes
4. Microcentrifuge tubes
5. Vortex
6. Spectrophotometer

Procedure

1. Mix 10µL of standard or sample with 1ml of Pierce 660nm

2. Vortex samples
3. Incubate at room temperature for 5min
4. Measure absorbance at 660nm.
    a. When assessing the concentration of samples
5. Make samples 1mg/ml, volume 350µL. Dilute samples using excess extraction buffer.

Step *1 - Methanol Chloroform Precipitation*

This step desalts and de-lipidates protein extracts. Each person will precipitate one mystery sample in duplicate.

Reagents and equipment:

1. Centrifuge at room temperature
2. Screw capped eppendorf tubes
3. Methanol
4. Chloroform
5. Water
6. Speed-vac

Procedure:

1) Cells: To 2 x 100µL (100µg of protein) of cell lysate in a **screw capped eppendorf** tube add:
   *The maximum volume of the sample and water should be 600*µL.
    i. 600µL of methanol, mix well by vortexing;
    ii. 150µL of chloroform, mix well by vortexing;
    iii. 500µL of water to cell lysates, mix well by vortexing.
2) Centrifuge at >12,000xg for 5min at room temperature.
3) The protein pellet forms at the interface – it will look like a white disc;
4) Remove the aqueous layer (upper);
   (Sometimes it's possible to remove the lower layer as well and to skip steps 7-9. This is more likely if you have a lot of protein and you can flip the disk of protein onto the side of the tube.)
5) Add 600µL methanol, mix well by vortexing;
6) Centrifuge at >12,000xg for 5min at room temperature;
7) The pellet should now be at the bottom of the tube. Remove the remaining liquid
8) Dry samples briefly, **do not overdry** of the protein will be difficult to resuspend.

Step 2 - PNGase F release of glycans

To differentiate between changes in O-GlcNAc, O-linked glycosylation, and N-linked glycosylation, we will remove N-linked glycans from half of your samples using PNGase F.

Reagents and equipment

- PNGase F (Glycerol Free)
- 10X glycoprotein denaturing buffer (5% w/v SDS, 0.4M DTT)
- 10X G7 Buffer (0.5M Sodium phosphate pH7.5)
- 10% NP-40

Procedure

1) For the PNGase F reactions, set up the following reactions:

Trainee	Tube #	Sample	Water (µL)	10X Denaturing buffer (µL)
1	1	Mystery sample 1	45	5
1	2	Mystery sample 1	45	5
2	3	Mystery sample 2	45	5
2	4	Mystery sample 2	45	5
3	5	Mystery sample 3	45	5
3	6	Mystery sample 3	45	5
4	7	Mystery sample 4	45	5
4	8	Mystery sample 4	45	5
5	9	Mystery sample 5	45	5
5	10	Mystery sample 5	45	5
6	11	Mystery sample 6	45	5
6	12	Mystery sample 6	45	5
7	13	Ovalbumin 1mg/ml	45	5
7	14	Ovalbumin 1mg/ml	45	5

Only prepare the reactions assigned to you!

2) Vortex well

3) Heat to 100^{o}C for 10minutes

Person	Tube #	Sample	10% NP-40	10X G5 buffer	Water	PNGase F
1	1	Mystery sample 1a	10µL	10µL	25µL	5µL
1	2	Mystery sample 1b	10µL	10µL	30µL	-

2	3	Mystery sample 2a	10µL	10µL	25µL	5µL
2	4	Mystery sample 2b	10µL	10µL	30µL	-
3	5	Mystery sample 3a	10µL	10µL	25µL	5µL
3	6	Mystery sample 3b	10µL	10µL	30µL	-
4	7	Mystery sample 4a	10µL	10µL	25µL	5µL
4	8	Mystery sample 4b	10µL	10µL	30µL	-
5	9	Mystery sample 5a	10µL	10µL	25µL	5µL
5	10	Mystery sample 5b	10µL	10µL	30µL	-
6	11	Mystery sample 6a	10µL	10µL	25µL	5µL
6	12	Mystery sample 6b	10µL	10µL	30µL	-
7	13	Ovalbumin 7a	10µL	10µL	25µL	5µL
7	14	Ovalbumin 7b	10µL	10µL	30µL	-

4) Vortex; spin briefly; **incubate at 37°C for 4h**.
   a. The volume of the reaction should be 100µL

Step 3 - Methanol Chloroform Precipitation

This step desalts proteins for the galactosyltransferase reactions. Notably, this step will also remove free N-linked glycans that could be labeled in the "Galactosyltransferase" reaction.

Reagents and equipment:

1. Centrifuge at room temperature
2. Screw capped eppendorf tubes
3. Methanol
4. Chloroform
5. Water
6. Speed-Vac

Procedure:

1) Cells: To 100µL (100µg of protein) of cell lysate in a **screw capped eppendorf** tube add:
   i. 600µL of methanol, mix well by vortexing;
   ii. 150µL of chloroform, mix well by vortexing;
   iii. 500µL of water to cell lysates, mix well by vortexing.
2) Centrifuge at >12,000xg for 5min at room temperature.
3) The protein pellet forms at the interface – it will look like a white disc;
4) Remove the aqueous layer (upper);

Sometimes it's possible to remove the lower layer as well and to skip steps 7-9. This is more likely if you have a lot of protein and you can flip the disk of protein onto the side of the tube.

5) Add 600µL methanol, mix well by vortexing;
6) Centrifuge at >12,000xg for 5min at room temperature;
7) The pellet should now be at the bottom of the tube. Remove the remaining liquid
8) Dry samples briefly, do not overdry of the protein will be difficult to resuspend.
9) Resuspend proteins at **2mg/ml (50µL**) in 1% w/v SDS in Tris-HCl pH8.0
    i. Place these on the vortex for 10min
    ii. Heat to 100^{o}C for 5 min
    iii. Place these on the vortex for 10min
    iv. Check to ensure the proteins have dissolved. IF NOT, repeat step B and C again.
    v. Spin proteins at 14,000xg at room temperature for 5min to pellet any debris.

Step 4 – Galactosyltransferase Reaction

O-GlcNAc was detected serendipitously when Torres and Hart (1984) were using β-1,4-galactosyltransferase (Gal-T) and ^{3}H-UDP-Gal to label lymphocyte glycoproteins. Gal-T will label any beta-linked terminal GlcNAc residue and will thus react with O-GlcNAc, eO-GlcNAc, and many N-linked glycans. Thus, It's critical to include appropriate controls to differentiate between O-GlcNAc (Thiamet-G, OGT deletion, OSMI-1) and N-linked glycans (PNGase F). Recently, Gal-transferase labeling has been expanded to include the transfer of unnatural sugars that contain groups (azido and keytone groups) that can be further derivatized (Clicked) with useful chemical groups such as TAMRA, Dapoxyl® alkyne, or biotin alkyne. To utilize an azido GalNAc (GalNAz), we will utilize a permissive mutant of β-1,4-galactosyltransferase (Y289L). Once labeled glycoproteins can be detected by western blot with Neutravidin-HRP (as in this class) or enriched by affinity chromatography.

Reagents and equipment:

O-GlcNAc Enzymatic Labeling System (MP33368)

1. **Component A** - UDP-GalNAz, (To make a 0.5mM solution of UDP-GalNAz:, dissolve in 144µL of 10mM HEPES buffer, pH7.9, Store aliquots at -80^{o}C for up to one year)
2. **Component B** - Gal-T1 (Y289L)
3. **Component C** - Click-iT - *O*-GlcNAc enzymatic labeling buffer (2.5X in a solution containing 125 mM NaCl, 50 mM HEPES, 5% NP-40, pH 7.9)
4. **Component D** - $MnCl_2$

Procedure:

Typically, we would run these experiments +/-GalNAz or +/-Gal-T to provide a negative control, as there are off target reactions with the protein backbone. Today, we wont do this to simplify the experiment… and as you have some excellent controls.

It is best to make up a master mix and then add 150µl to each tube (The enzyme should be added independently):

*Water (20 x 49) – 980µ*L

Buffer C (20 x 80) - 1600µL

McCl2 (20 x 11) - 220µL

UDP-GalNAz (20 x 10) – 200µL

Person	Tube #	Sample	Water (µL)	Buffer C (µL)	MnCl2 (µL)	UDP-GalNAz (µL)	Enzyme (µL)
1	1	Mystery sample 1a – 40µL	49	79	11	10	1
1	2	Mystery sample 1b – 40µL	49	79	11	10	1
2	3	Mystery sample 2a – 40µL	49	79	11	10	1
2	4	Mystery sample 2b – 40µL	49	79	11	10	1
3	5	Mystery sample 3a – 40µL	49	79	11	10	1
3	6	Mystery sample 3b – 40µL	49	79	11	10	1
4	7	Mystery sample 4a – 40µL	49	79	11	10	1
4	8	Mystery sample 4b – 40µL	49	79	11	10	1
5	9	Mystery sample 5a – 40µL	49	79	11	10	1
5	10	Mystery sample 5b – 40µL	49	79	11	10	1
6	11	Mystery sample 6a – 40µL	49	79	11	10	1
6	12	Mystery sample 6b – 40µL	49	79	11	10	1
7	13	Ovalbumin 7a – 40µL	49	79	11	10	1
7	14	Ovalbumin 7b – 40µL	49	79	11	10	1

1. Mix, Add enzyme, Spin Briefly.

   The final reaction volume should be ~200µL

2. Incubate reactions overnight at 4^{o}C

Step 5 - Methanol Chloroform Precipitation (Yes, another one)

This step removes unused UDP-GalNAz.

Reagents and equipment:

1. Centrifuge at room temperature
2. Screw capped eppendorf tubes
3. Methanol
4. Chloroform
5. Water
6. Speed-vac
    - ⊙ Each group should precipitate 2x 200µL

Procedure:

1) To 200µL (80µg of protein) of cell lysate in a **screw capped eppendorf** tube add:
    i. 600µL of methanol, mix well by vortexing;
    ii. 150µL of chloroform, mix well by vortexing;
    iii. 400µL of water to cell lysates, mix well by vortexing.
2) Centrifuge at >12,000xg for 5min at room temperature.
3) The protein pellet forms at the interface – it will look like a white disc;
4) Remove the aqueous layer (upper);
    i. *Sometimes it's possible to remove the lower layer as well and to skip steps 7-9. This is more likely if you have a lot of protein and you can flip the disk of protein onto the side of the tube.*
5) Add 600µL methanol, mix well by vortexing;
6) Centrifuge at >12,000xg for 5min at room temperature;
7) The pellet should now be at the bottom of the tube. Remove the remaining liquid
8) Dry samples briefly, do not overdry of the protein will be difficult to resuspend.
9) Resuspend proteins at 2mg/ml (40µL) in 1% w/v SDS in Tris-HCl pH8.0
    i. Place these on the vortex for 10min
    ii. Heat to 100^{o}C for 5 min
    iii. Place these on the vortex for 10min
    iv. Check to ensure the proteins have dissolved. *Note: repeat step B and C again.*
    v. Spin proteins at 14,000xg at room temperature for 5min to pellet any debris.

Step 6 – Click Reaction

Reagents and equipment:

1) Biotin alkyne (Component A) (in C33372), in 70µL of DMSO
2) Click-iT reaction buffer, (Component B)
3) $CuSO_4$ (Component C)
4) Click-iT reaction buffer additive 1 (Component D)
5) Click-iT reaction buffer additive 2 (Component E)

Procedure:

Prepare reagents (This will be performed for you)

1) Add 60 µL of the alkyne solution **(Component A)** to the Click-iT Reaction Buffer **(Component B)**. This solution may be stored at ≤–20℃ for up to 1 year.
2) Add 500 µL of water to the Click-iT Reaction Buffer Additive 2 **(Component E)**. This solution may be stored at 2–6℃ for up to 6 months.
3) Prepare Click-iT Reaction Buffer Additive 1 **(Component D)…… FRESH** on the day of use. Add 100 µL of water to 1 vial. Store the solution at 2–6℃ for up to 1 week.

Click Reactions

To each of your tubes (which should contain 40µL/ 80µg):

1) 100 µL of 2X Click-iT Reaction Buffer containing the alkyne detection reagent prepared above (1)
2) Add 20 µL of water - bringing the volume to 160 µL, vortex.
3) Add 10 µL of $CuSO_4$ (Component C) and vortex for 5 seconds.
4) Add10µL of Click-iT Reaction Buffer additive1 (prepared above, 3) and vortex for 5 seconds.
   a. Wait for 2–3 minutes before proceeding to the next step.
5) Add 20µL of reconstituted Click-iT Reaction Buffer Additive 2 (prepared above, 2) and vortex for 5 seconds. The solution should will turn bright orange.
6) Mix end-over-end for 20minutes in the dark.
7) Depending on the concentration of your proteins, you can mix you samples with sample buffer or concentrate them by precipitation. We will precipitate proteins (Methanol Chloroform) and resuspend them in 50µL of 0.5% SDS in Tris-HCl pH7.5.
8) Perform protein estimation and make samples 1mg/ml.
9) Mix 60µL of sample @ 1mg/ml with 20 µL of sample buffer

Cathrine will run 2 gels out for each group.

Lane	Sample	Load	Protein

		(µL)	(µg)
1	Pre-stained Markers	5	N/A
2	Mystery Samples #1 - Prelabeling	13.3	10
3	Mystery Samples #2 - Prelabeling	13.3	10
4	Mystery Samples #3 - Prelabeling	13.3	10
5	Mystery Samples #4 - Prelabeling	13.3	10
6	Mystery Samples #5 - Prelabeling	13.3	10
7	Mystery Samples #6 - Prelabeling	13.3	10
8	Mystery Samples #1a – Post Click	13.3	10
9	Mystery Samples #1b – Post Click	13.3	10
10	Mystery Samples # 2a – Post Click	13.3	10
11	Mystery Samples # 2b – Post Click	13.3	10
12	Mystery Samples # 3a – Post Click	13.3	10
13	Mystery Samples # 3b – Post Click	13.3	10
14	Mystery Samples # 4a – Post Click	13.3	10
15	Mystery Samples # 4b – Post Click	13.3	10
16	Mystery Samples # 5a – Post Click	13.3	10
17	Mystery Samples # 5b – Post Click	13.3	10
18	Mystery Samples # 6a – Post Click	13.3	10
19	Mystery Samples # 6b – Post Click	13.3	10
20	Sample buffer	5	N/A

Blot 1/2: ConA – Blocked in BSA

Procedure

1. Blot gels to nitrocellulose membrane
2. Block membranes in 3% BSA in TBS (**Be careful, Con A binds tween-20**)
3. Wash membranes in 2x in TBS (5min)
4. Apply lectin O/N at 4^{o}C
    a. Con A at 1µg/mL in TBS with 1mM $CaCl_2$, $MgCl_2$, and $MnCl_2$
    b. Con A at 1µg/mL in 100mM mannose dissolved in TBS with 1mM $CaCl_2$, $MgCl_2$, and $MnCl_2$
5. Wash blots 3x10min in TBS with 1mM $CaCl_2$, $MgCl_2$, and $MnCl_2$
6. Wash blots 1x10min in TBS
7. ECL

Blot 3/4: CTD 110.6 – Blocked in Milk

This antibody recognizes O-GlcNAc and during glucose deprivation an N-linked di-acetyl-chitobiose residue. Typically, this latter signal can be suppressed by incubating membranes in milk. Nonetheless, if

strong signals are observed below 50kDa it's advisable to treat samples with PNGase F.

Procedure

1. Blot gels to nitrocellulose
2. Block membranes in 3% Milk in TBST
3. Wash membranes in 2x in TBST (5min)
4. Apply Antibody O/N at 4^{o}C
    a. CTD110.6 at 1μg/mL in 3% BSA in TBST
    b. CTD110.6 at 1μg/mL in 3% BSA in TBST with 100mM GlcNAc
5. Wash blots 3x10min in TBST
6. Apply anti-Mouse IgM 1/5000 in 3% BSA in TBST
7. Wash blots 4x10min in TBST
8. Wash blots 1x10min in TBS
9. ECL

Blot 5: Neutravidin – Blocked in Milk

Neutravidin is a mutant of Streptavidin in which the lectin binding domains have been mutated.

Procedure

1. Blot gels to nitrocellulose
2. Block membranes in 3% Milk in TBST
3. Wash membranes in 2x in TBST (5min)
4. Apply Antibody O/N at 4^{o}C
    a. Neutravidin-HRP 1/1000 in 3% BSA, 0.3% Milk in TBST
5. Wash blots 4x10min in TBST
6. Wash blots 1x10min in TBS
7. ECL

Gel a: Stain gel using Commassie G250

1. Add 10mls of methanol to the coomassie G250 stock
2. Incubate the gel in the resulting mixture overnight
3. De-stain in water