**Glycomic Analysis of N-glycans and O-glycans by Mass Spectrometry (MS)**

**For Cells or Tissue**

EGMIC provides services in the preparation of glycolipids, N-glycans and O-glycans from cells or tissue. Products can be used for glycomics profiling by Mass Spectrometry (MS) or further used for shotgun glycan microarray development. The starting material for cell samples is 2-10 E10 cells for mass profiling. Glycoproteins such as 100ug Ribonuclease B, human transferrin or fetuin can be used as controls for the procedure. Use 100ug fetuin or mucin as process control for O-glycan release.

**General Protocol**

1. *Homogenization and Lipid Extraction*

Cells are thawed on ice for 30 min and suspended in Milli-Q water to bring the volume to 3 ml. Cell suspension is homogenized using Micro-tip sonication at low intensity with 3 pulses at 10 sec each separated by 30-60 sec pause intervals. Methanol and chloroform are added to adjust mixture volume to a ratio of chloroform/methanol/water=4/8/3 (v/v/v). Suspension is centrifuged at 10,000rpm for 15 min, keep the supernatant. Lipids are extracted using different chloroform/methanol mixtures with ratios (1/2), (1/1), (2:1), and ethanol. Repeat the centrifuge step between each wash. The combined supernatants of lipid extracts are reduced volume by rotary-vac and dried by freeze-drying. Lipid extract is further separated into two phases by Folch’s Partition, profiled by TLC chromatography, or used for further experiments. The pellet is dried under an air stream or nitrogen stream to remove any trace of organic solvent.

1. *Reduction and Alkylation*

The dry pellet sample is re-suspended in 2 ml of 0.2 M Tris –HCl, pH8.2. Add 2 ml of 8.0 M Guanidine hydrochloride (in 0.2 M Tris-HCl, pH8.2), then add 2 ml of 0.18 M DTT (in 0.2 M Tris-HCl, 8M Guanidine hydrochloride, pH8.2), and incubated at RT (Room Temperature) for 1 hour on the shaker. 7.75 ml of 0.18 M iodoacetamide (in 0.2 M Tris-HCl, 8 M guanidine hydrochloride, pH8.2) is added and incubated at RT for 30 min on the shaker in the dark. Sample is dialyzed (3,500 MW cutoff) against running tap-water overnight at 4 °C and lyophilized to dry.

1. *Trypsin and PNGaseF Digestion*

Dry sample is re-suspended in 3ml 50 mM phosphate buffer (pH8.2). 30 µl of 10 mg/ml TPCK-treated Trypsin in 50mM phosphate buffer (pH8.2) is added to the sample and incubate at 37°C overnight. The reaction is stopped by boiling for 5 min. The pH of the sample is adjusted to pH 7.5 using droplet of NaOH, then sodium azide is added to the final conc. of 0.02%. 10µl PNGase F (NEB, 50,000 units/ml) is added to sample and incubated at 37°C for 72 hours. Additional 10µl PNGase F is added at the 24 hours. The reaction is stopped by boiling for 5 min.

1. *Purification and desalting*

PNGase F digest is loaded to a pre-conditioned 1 ml C18 SepPak column. Flow through and 6 ml of Milli-Q water washes from C18 SepPak columns are further loaded to a pre-conditioned 1 ml carbogrpahy column. Peptides are eluted from C18 SepPak column sequentially with 2ml 60% methanol, 80% methanol and 100% methanol. Eluents from C18 SepPak columns are pooled, reduced volume on a speed-vac and lyophilized to dry. N-glycans are eluted from carbography column with 2ml 50% acetonitrile with 0.1% Triflouroacetic acid (TFA). Eluent containing N-glycans released by PNGase F is reduced volume on a speed-vac and lyophilized to dry.

1. *PNGase A digestion (optional)*

Dry sample from C18 SepPak columns eluent is re-suspended in 0.9 ml Miili-Q water, mixed with 0.1 ml of 10X glycobuffer 3 (provide in the kit) and 20 ul of PNGase A (5,000 units/ml). The reaction mixture is incubated at 37°C overnight and stopped by boiling 5 min. The reaction mixture is treated with a purification procedure in step IV. Eluent from C18 Sepak column is tryptic peptide mixture and eluent from Carbogrpahy column is N-glycan released by PNGase A.

1. *Chemical removal of O-glycans by reductive β -elimination*

Dry peptide sample is re-suspended in 400 ul freshly 2 M sodium borahydride (in 0.1 M sodium hydroxide) and incubated at 45oC for 16 hours. The reaction is stopped by dropwise addition of ~ 2 ml 0.25 M acetic acid-methanol solution until the fizzling stops. O-glycans are desalted by a 1 ml ion-exchange AG 50W-X8 resin 50 -100 mesh (BioRad) column. Flow through and 5 ml 5% acetic acid washes are collected, pooled, and lyophilized. Borate in the reaction product is removed by re-suspending dry sample with 1 ml methanol and drying the sample under N2 stream or on a Speed Vac. This step is repeated twice. The dry samples are O-glycans.

1. *Permethylation for MALDI MS analysis*

Permethylation is a commonly utilized technique to derivatize glycan for MALDI-MS detection. During the permethylation process, all reactive glycan hydrogens are replaced with methyl groups which hence ionization efficiency and stabilizes the sialic acids for better MALDI detection. The common protocol of permethylation is carried out by adding 0.5 ml 50% slurry of NaOH in Dimethyl Sulfoxide (DMSO) and 0.2ml Iodomethane into dry glycan. The mixture is shaken for 30 min at room temperature, and the reaction is quenched by dropwise addition of 1 ml Mili-Q water. Permethlyated samples are extracted into 1 ml dichloromethane. After a quick centrifuge, upper phase is discarded, and the lower phase is washed with three 1 ml 0.5 M sodium chloride washes. Dry the lower phase fraction on a Speed Vac.

Reconstitute the dry sample in 10 µl 50% Methanol. Spot 1 µl of sample and 1 µl of Matrix (20 mg/ml DHB in 50% acetonitrile) on the plate and procced to MALDI analysis with positive mode, range (500 – 5000 D). If no clear MS peaks are observed, better resolution can be obtained by cleaning the sample with a 1ml C18 SepPak column. Re-suspend the sample in 200 µl of 50% methanol solution, load into a C18 SepPak column and wash the column with 2 ml of 15% acetonitrile solution. Eluents from 3 ml 50% acetonitrile is collected, reduced volume on a Speed Vac and lyophilized. Reconstitute the sample in 10 µl of 50% methanol and repeat MALDI analysis.

1. *MALDI-TOF- MS*

Data acquisition is conducted either by manual operation or by Automated data acquisition using the AutoXecute feature on the AutoFlex Speed MALDI-TOF-MS instrument from Bruker with a Smartbeam-II laser and system speed of 2 kHz for MS and 200 Hz for MS/MS. FlexControl 3.4 software from Bruker Daltonics is used for acquiring data for mass spectral measurements of permethylated glycans in reflectron positive (RP) mode. A window of *m/z* 300-2000 are used for O-glycan profiles and *m/z* 500-5000 is used for N-glycan profiling. Peptide calibration standards are used to calibrate the instrument. Bruker Daltonics flexAnalysis software version 3.4 is used for data representation of [M+Na]+ permethylated glycans. EGMIC provides glycan composition for selected glycan related mass. MZXML files are exported and can be used by other analysis software.