Targeted N-glycoproteomics of human plasma samples.

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Abstract

This protocol describes the complete protocol for performing N-Glycoproteomics from plasma samples. Albumin removal is performed prior to protein digestion with trypsin. Tryptic peptide mixture is used for enriching N-glycopeptides by lectin-affinity chromatography using a lectin-mix. Enriched N-glycopeptides are cleaned up by reverse phase chromatogrraphy and analyzed by LC-MS and LC-MS/MS. resulting MS/MS spectra is deconvouted and saved as peak lits file which can be searched with GlycopeptideId software to identify the peptide sequence, glycan composition and proposed glycan struccture.

Citation: Sakari Joenvaara,Mayank Saraswat,Pentti Kuusela,Shruti Saraswat,Rahul Agarwal,Johanna Kaartinen,Asko Jarvinen,Risto Renkonen Targeted N-glycoproteomics of human plasma samples.. **protocols.io**
dx.doi.org/10.17504/protocols.io.ncadase
Published: 25 Feb 2018

Materials

Pierce Albumin removal kit 85160 by Thermo Fisher Scientific

Pierce™ C18 Spin Columns 89870 by Thermo Fisher Scientific

Protocol

Albumin removal

Step 1.

Transfer 400µL of Pierce albumin removal resin to microspin columns. Centrifuge at 12,000 × g for 1 minute to remove excess liquid. Discard flow-through and place spin column back into the same collection tube.

Albumin removal

Step 2.

Add 200µL of Binding/Wash Buffer to the spin column. Centrifuge at 12,000 × g for 1 minute. Repeat this step twice.

Albumin removal

Step 3.

Add 50µL of human plasma to resin and incubate for 1-2 minutes at room temperature. Centrifuge at 12,000 × g for 1 minute. Re-apply flow-through to spin column and incubate for 1-2 minutes at room temperature. Centrifuge at 12,000 × g for 1 minute. Retain flow-through.

Note: Sample must have < 100mM salt for proper albumin binding. Dilute accordingly, if needed.

Albumin removal

Step 4.

Wash 3 times with 50µL binding/wash buffer and combine the washes with step 3 flow through.

This is albumin depleted plasma proteins. Determine the protein cocentration using BCA or Bradford assay at this step.

Trypsin digestion

Step 5.

350µg equivalent of total protein is aliquoted from albumin-depleted plasma and 7µg of bovine fetuin protein is added and mixture is dried in speed vacuum system.

Dissolve the dried proteins in 35µL, 6M urea (made in 50mM Tris pH 7.8).

Trypsin digestion

Step 6.

Add 1.8µL of Dithiothreitol (DTT, stock solution 200mM) to the tube and shake the tube at room temeparrature (RT) for 1 hour.

Trypsin digestion

Step 7.

Add 7µL of iodoacetamide (stock solution 200mM) to the tube and shake the tubes for 1 hour at RT in dark.

Trypsin digestion

Step 8.

Dilute the solution with 50mM Tris buffer (pH 7.8) to bring urea concentration to 0.6M.

Add bovine pancreatic trypsin to the ratio of 1:50 (trypsin: total protein) and incubate overnight at 37ºC.

Note: Any kind of trypsin can be used.

Lectin affinity chromatography (LAC)

Step 9.

Dilute 60 µL of tryptic peptides mixture from previous step by 540 µL of 10mM HEPES buffer pH 7.4 containing 1mM CaCl2 and 1mM MnCl2 (Buffer HB).

This is the starting material for LAC.

Lectin affinity chromatography (LAC)

Step 10.

Four lectins were added to ratio of Con-A: SNA: LCA: AAL, 5:3:3:1 for a final volume of lectin resin slurry of 150 µL in microspin column. Add 400µL buffer HB and centrifuge the column at 1000xg and discard the flow through. Repeat this step 2 times.

Note: Add appropriate amount of lectin-agarose slurry to the microspin columns. Users can determine their own ratio or mix equal amounts of these lectins depending on their objectives and downstream applications.

Lectin affinity chromatography (LAC)

Step 11.

Add starting material from step 9 to lectin spin column and cap the column top and bottom. incubated at 4°C on rotation overnight.

Lectin affinity chromatography (LAC)

Step 12.

Wash 3x with buffer HB and elute N-glycopeptides with 200µL of sugar-mix solution containing fucose (100mM), α-methyl mannoside (200mM), α-methyl glucoside (200mM) and lactose (400mM) followed by second elution with 200µL of 1% formic acid.

Note: These eluted N-glycopeptides contain many impurities including high concentration of free sugars. In next steps, it will be cleaned with C18 reverse phase chromatography.

Reverse-phase chromatography (C18)

Step 13.

C18 Spin Columns are used for cleaning the N-glycopeptide free of impurities.

Condition C18 spin column with 200µL of 50% acetonitrile twice and discard the flow through by centrifuging at 1500 x g

Reverse-phase chromatography (C18)

Step 14.

Equilibrate the C18 spin columns with 200µL of 0.5% formic acid (FA) in 5% acetonitrile (ACN). Repeat the step twice. Remove the liquid by centrifuging at 1500 × g for 1 minute.

Reverse-phase chromatography (C18)

Step 15.

Add sample buffer (2% FA in 20% ACN; 1µL for every 3µL of sample) to N-glycopeptide elution solution from step 12 and vortex briefly to mix.

Load sample on top of resin bed. Place column into a receiver tube. Centrifuge at 1500 × g for 1 minute. To ensure complete binding, recover flow-through and repeat the loading.

Reverse-phase chromatography (C18)

Step 16.

Add 200µL Wash Solution (0.5% FA in 5% ACN) to column and centrifuge at 1500 × g for 1 minute. Discard flow-through. Repeat twice additionally.

Reverse-phase chromatography (C18)

Step 17.

Place column in a new receiver tube. Add 20µL of Elution Buffer (70% ACN) to top of the resin bed. Centrifuge at 1500 × g for 1 minute. Repeat once more and combine both elutions (40µL total volume).

Dry the elution in speed vacuum system and reconstitue in appropriate volume of 0.1% FA (20µL typically with these concentrations of starting material). Transfer to mass spectrometry vials to be analyzed.

Mass Spectrometry

Step 18.

A Waters SYNAPT G2 High Definition MS connected to a Waters nanoACQUITY UPLC was used for the analysis. Positive mode with sensitivity mode was used for MSE (100-2000 Da mass range) and FAST DDA mode for N-glycopeptide fragmentation (50-2500 Da mass range). The Collision-induced dissociation (CID) collision energy ramp was 20–60 V. Calibration was performed with sodium formate. The trapping column was a nanoACQUITY UPLC Trap, 180 µm x 20 mm (5 µm), Symmetry®C18, and the analytical column was a nanoACQUITY UPLC, 75 µm x 100 mm (1.8 µm), HSS T3. Samples were loaded, trapped and washed for two minutes with 8.0 µL/min with 1% B.

Mass Spectrometry (Gradient)

Step 19.

The analytical gradient used is as follows: 0-1 minutes 1% B, at 2 minutes 5% B, at 45 minutes 30% B, at 48 minutes 50% B, at 50 minutes 85% B, at 53 minutes 85% B, at 54 minutes 1% B and at 60 minutes 1% B with 450nL/min for MSE while 300 nL/min for N-glycopeptide fragmentation.

Note: Any other suitable gradient depending on sample complexity can also be used.

Mass Spectrometry (Data Analysis)

Step 20.

The raw files are imported to Progenesis QI for proteomics software (Version V2, Nonlinear Dynamics, Newcastle, UK) using lock mass correction with 785.8426 m/z, corresponding to doubly charged Glu1-Fibrinopeptide B. Default parameters in the software for peak picking and alignment algorithm are used. The software facilitates the label-free quantification. Known amount of bovine Fetuin added to the samples before trypsin digestion allowed the normalization of N-glycopeptides by known bovine Fetuin N-glycopeptides (This is optional and normalization with all ions is also possible).

Mass Spectrometry (Data Analysis)

Step 21.

Run alignment

The run with the most features (ions) is used as reference and all other runs are aligned to the reference.

Peak picking

An aggregate data set is created from the aligned runs which contains all peak information from all sample files. This aggregate peak list is then matched to each sample.

Ion abundance quantification

Peptide ion abundance is a sum of *areas* which is calculated using intensities of the peaks of a peptide ion's and peaks' width.

Normalization

Thes ion aubndances are then normalized to be able to compare the aubndances across separate muultiple runs. Normalization to known ions such as fetuin N-glycopeptides (recall fetuin was added in step 5) or to all ions can be performed easily in the software.

Mass Spectrometry (Data Analysis)

Step 22.

After the analysis of ion abundances between case and controls and establishing the differentially abundant ions by t-test, FAST-DDA mode is run (MS/MS) to fragment selected N-glycopeptides. The MS/MS spectra are deconvoluted in MaxEnt3 module of Waters MassLynx 4.1 software and saved as peak lists (.pkl). Identification of N-glycopeptides was performed on the publicly available software GlycopeptideID, which can perform automated CID MS/MS spectrum analysis.

Note: If available, other N-glycopeptide search engines capable of identifying the N-glycopeptides form CID-MS/MS spectra, can also be used. GlycopeptideID is available from Appliednumerics OY.

Deconvoluted combined pkl files can be uploaded to GlycopeptideId software and precursor and fragment tolerances can be specified. It gives an output of identified N-glycopeptides, the peptide sequence, glycan compositions, proposed glycan structures. The peptide and glycan compositions are scored. Together, the sum of these 2 scores generates glycopeptide score. False discovery rate at the peptide level can be applied.

Warnings

Wear labcoat and gloves at every step of the protocol. Formic acid and acetonitrile can be harmful, handle with care.