PBMC Pellet Lysis, Digestion, and SDS removal

Samples were processed in blinded batches of18 samples plus 2 reference pooled PBMC samples: 1 from aliquoted cell pellets, the other from a pooled lysate of the same PBMC reference samples.

Lysis reagents
0.1% SDS in 50mM Tris, pH 8.5
100mM $MgCl_2$ in 50mM Tris, pH 8.5 (for Benzonase activity)
Simple Stop 1 100x phosphatase Inhibitor cocktail
Simple Stop 4 100x phosphatase Inhibitor cocktail
Benzonase nuclease 25units/ul
1M DTT in 50mM Tris, pH 8.5 (stock), 175mM working solution (fresh)

- 15 ul stock aliquot + 70.7 ul 50mM Tris, pH 8.5 (1:5.714 dil) **5mM final conc. in lysate**

350mM IAA (iodoacetamide) in 50mM Tris, pH 8.5 (fresh) **10mM final conc. in lysate**

Make lysis buffer:
1.8 ml 0.1% SDS/50mM Tris pH 8.5
18 ul each phosphatase inhibitor
9 ul 100mM $MgCl_2$ (final 0.5 mM MgCl2)
3 ul Benzonase

Lysis procedure for 19 samples – 18 patient and 1 Pellet (P) sample (U01 pooled cells control – Poland lab)
Add 80 ul lysis buffer to each frozen pellet, vortex 5 sec, 2 at a time
Starting 1 to 19, vortex 2 sec, 2 at a time
On ice, 5 min.
Repeat vortex
On ice, 5 min
Repeat vortex
Heat at 95°C, 1400rpm for 10 min

- Cool briefly (~3 min) on ice; spin 500xg for 30 sec
- Pipette up and down twice, transfer 70 ul supernatant to new 1.5 ml microfuge tube for tryptic digest
    - leave remainder in tube for protein assay

Total protein was determined using a BCA assay against a bovine serum albumin (BSA) standard. Each sample was diluted 1:5 and run in triplicate in the assay. To determine inter-assay variation, an aliquot of a lysate pool was run in each protein assay for each batch.

Reduction/Alkylation for 20 samples
Retrieve one 70 ul pooled lysate sample, **Lysate (L)**

- To all samples, add 2 ul 175mM DTT 30 min at 60°C, 1000rpm (use heated shaker)
- Add 2 ul 350mM IAA 30 min RT, in the dark, 1000rpm (use vortex shaker)
- Add 1 ul 175 mM DTT scavenger
    - Total vol = ~75 ul

Tryptic digest of 20 samples

Reconstitute four 20ug Trypsin vials with 20 ul 50mM Tris, pH 8.5 = 1 ug/ul; pool all tubes into one vial

Add 1-6 ul (1-6 ug of trypsin) to all tubes

- ~1:20-30 ratio of trypsin to protein

Incubate overnight at 37°C (14h) on shaker, 1000rpm, covered

SDS Detergent removal column procedure:

- Equilibrate twenty 0.5 ml Pierce detergent removal columns in 50 mM Tris, pH 8.5 according to product instructions.
- Load all of each digested sample on column and collect detergent-depleted peptide sample in 1.5 ml tube.

Desalting and fractionation by Strong Anion Exchange (SAX) chromatography

Acidification, desalting, and SAX fractionation of each batch of 20 samples was split into 2 parts with 10 samples in each: 3 subjects with their 3 time points plus one of the PBMC QA samples.

Due to varying numbers of cells in each sample, the total protein amount available after digestion varied from 30ug to > 150 ug. As a result we set a target amount of 80ug to go through the peptide fractionation and on to LC-MS/MS analysis. Some samples had less than 80ug, in those cases the total volume of digest was used and the protein amount was recorded and subsequently compensated for when reconstituting samples for LC-MS/MS analysis.

Desalting was performed on a 3mm x 8mm cartridge Peptide trap from Michrom BioResources, now available through Optimize Technologies. The cartridge was manually loaded, washed, and eluted using 250 uL syringe volumes and collected into microcentrifuge tubes via tubing on the exit end of the cartridge.

The sample syringe was first filled with 50uL of Desalt A as a half-sandwich backing buffer to aid transfer of sample into trap (50uL Desalt A, then 150 uL of sample). Samples were then washed with 2 X 250 uL syringes of 2% acetonitrile in 0.1% TFA. The loading volume and the 2 wash volumes were combined and saved.

Peptides were eluted from the cartridge in reversed direction using 250 uL of 2 elution solvents:

1. ACN/H20/HCOOH (60/40/0.2 by volume)
2. ACN/IPA/H20/HCOOH (50/30/20/0.2 by volume).

Both elution volumes were combined and evaporated to dryness for SAX fractionation.

SAX fractionation was performed with stepped elutions of decreasing pH volatile buffers (volatile buffer kit from Column Technology, Inc., Fremont, CA) on disposable pipette tips packed with Poros strong anion exchange phase (TT2PSA from Glygen). Samples were processed in parallel (10) using a microcentrifuge for 2 min. at 1000 x g to load sample and elute fractions from the tips into 2mL microcentrifuge tubes containing 2 uL of 0.1%.

The SAX tips were:

1. cleaned with 2 x 150 uL of 0.1% TFA/0.2% formic acid (pH ~2.5)
2. Equilibrated with 3 x 150 uL of 0.1% di-isopropyldiethylamine (DIEA) in water/ACN/IPA (95/5/5 by volume)
3. Samples were reconstituted in 90 uL of 0.1% DIEA to which another 10 uL of methanol was added, loaded onto SAX tips, and eluted into microcentrifuge tubes as SAX fraction 1.
4. 5 subsequent fractions were eluted into respective tubes with 100 uL volumes of decreasing pH:

SAX2	0.1% N-ethyl-morpholine in water/ACN (95/5)
SAX3-6	pH buffers 8.0, 7.0, 6.0, 2.5 (from Column Technology)

5. 2 uL of 0.1% (1mg/mL) Zwittergent 3-16 (CalBiochem) was added to each SAX fraction.
6. 50 uL (50%) of each fraction was transferred into 100 uL polypropylene autosampler vials, frozen, and evaporated to dryness on a Savant. Autosampler vials were stored dry at -80°C until reconstitution for LC-MS/MS analysis.

LC-MS/MS Analyses

1. Reconstitution of samples and Promega 6x5 peptide standards
2. LC-MS/MS Instrument
3. Column/trap, mobile phases, and gradient
4. DDA conditions
5. LC-MS/MS run order

1. Dried samples in autosampler vials were reconstituted in an aqueous solution of 0.2% formic acid, 0.1% TFA, and 0.002% Zwittergent 3-16. They were reconstituted in a volume proportional to the protein content of each sample as determined by BCA assay of each sample lysate, so that 10 ug of digest from each sample was distributed among the 6 SAX fractions. The same reconstitution volume was used in each SAX fraction from that sample. This reconstitution volume ranged from 30uL to 100 uL. A fixed volume of 20 uL was loaded for each SAX fraction of each sample for pre-concentration onto a reversed trap prior to elution from the trap through the nanoLC column. The reconstitution buffer also contained 5 fm/uL of the Promega 6x5 stable isotope-labeled peptide internal standard mix.

2. nLC-MS/MS analyses using were performed on a nanoLC Ultra-2D and AS400 autosampler from Eksigent interfaced to a Q-Exactive (first generation) mass spectrometer (ThermoFisher, Bremen, Germany).

3. Samples were loaded from the autosampler onto a reversed phase trap mounted in a 10-port valve. The trap was a 0.25uL bed OptiPak format trap from Optimize Technologies (Orgegon) that was custom-packed with 5um, 200A Magic C8 stationary phase. Sample was transferred to the trap and washed for 4 minutes at 10 uL/min using an aqueous loading buffer of 0.2% formic acid and 0.05% TFA. Peptides were eluted from the trap by the gradient of the nLC mobile phase in a reversed direction relative to loading. Peptide separations were performed at 400 nL/min and 40°C on a 40cm x 100 um i.d. PicoFrit column self-packed with Agilent Poroshell 120S, 2.7um EC-C18 stationary phase. A gradient of 2-30% B in 110 min, then 30%-50%B in 15 min., 5 min. to 95%B, held at 95%B for 10 min, re-equilibrated at 2%B, 150 minutes total. Mobile phase A was 2% acetonitrile in water, with 0.2% formic acid overall; mobile phase B was acetonitrile/isopropanol/water (80/10/10 by volume) with 0.2% formic acid overall.

4. Mass spectrometry data was collected in a data-dependent manner with the following parameters:
    - MS1 data was collected at 70,000 resolving power (measured at m/z 200) with an AGC value of 3E6 over a m/z range of 360-2000, using lock masses from background polysiloxanes at m/z 371.10123 and 446.12002.
    - MS2 spectra were collected on the top 20 precursor masses with charges of 2,3, or 4, using an AGC value of 2E5, a max ion fill time of 100 ms, and an isolation window of 2 for batches 1-6, and an isolation window of 4 for sample batches 6-9. Precursors were fragmented at a normalized collision energy (NCE) value of 26, with fragments measured at 17,500 resolving power and a fixed first mass of 140.
    - Precursors selected for MS2 were placed on the dynamic exclusion list for 80 s
5. LC-MS/MS data was acquired by sample processing batches (Batches 1-9) in a randomized block order as prescribed by our biostats group. Each batch contained18 samples (3 time points from 6 subjects) plus 2 PBMC QA samples (described above in Lysis/digestion methods). Each of these 20 samples had been fractionated into 6 SAX fractions that were run in the order of Fraction 6 to Fraction 1 for each sample. An instrument blank was run after SAX fraction 1 from each sample, with additional instrument performance standards being run at the conclusion of SAX fractions from every 3rd sample.