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Isolating and Quantifying Plasma HDL Proteins by Sequential Density Gradient Ultracentrifugation and Targeted Proteomics

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Summary

The sensitivity and specificity of tandem mass spectrometers have made targeted proteomics the method of choice for the precise simultaneous measurement of many proteins in complex mixtures. Its application to the relative quantification of proteins in high density lipoproteins (HDL) that have been purified from human plasma has revealed potential mechanisms to explain the atheroprotective effects of HDL. We describe a moderate throughput method for isolating HDL from human plasma that uses sequential density gradient ultracentrifugation, the traditional method of HDL purification, and subsequent trypsin digestion and nanoflow liquid chromatography-tandem mass spectrometry to quantify 38 proteins in the HDL fraction of human plasma. To control for the variability associated with digestion, matrix effects, and instrument performance, we normalize the signal from endogenous HDL protein-associated peptides liberated during trypsin digestion to the signal from peptides liberated from stable isotope-labeled apolipoprotein A-I spiked in as an internal standard prior to digestion. The method has good reproducibility and other desirable characteristics for pre-clinical research.

Keywords

Multiple reaction monitoring; tandem mass spectrometry; nanoflow liquid chromatography; stable isotope-labeled internal standard protein; Skyline; high density lipoprotein; protein quantification; quantitative proteomics; density gradient ultracentrifugation

1. Introduction

Elevated plasma concentrations of cholesterol associated with high density lipoproteins (HDL-C) are associated with a reduced risk for developing cardiovascular disease, which has been well-established in large epidemiological studies (1–3). It has been proposed that HDL particles serve several anti-atherosclerotic functions, namely: reverse cholesterol transport from peripheral tissues, including from lipid plaques in the vascular intima of arteries (4, 5); prevention or reduction of inflammation that mitigates endothelial cell activation (6, 7); and prevention or reduction of the oxidation of low density lipoproteins

(LDL) (8, 9). Analysis of the HDL proteome using shotgun mass spectrometry has demonstrated a complex heterogeneous composition of proteins of humoral and cellular origin with a vast array of functions (10–15). The inter-play of these proteins in the development of atherosclerosis and which proteins would serve as effective therapeutic targets is currently an intense area of research (14, 16). Targeted mass spectrometry (MRM/SRM/PRM) has been demonstrated to be an effective method of quantifying apolipoproteins associated with HDL using stable isotope-labeled peptides and protein internal standards (17–19).

The protocol outlined here employs sequential potassium bromide density gradient ultracentrifugation to isolate the high density lipoprotein ($\rho=1.063\text{--}1.210\text{ g/mL}$) fraction of human plasma. Proteins are dialyzed to remove the potassium bromide and the protein concentration is determined using the Bradford method. Isolated HDL proteins are reduced, alkylated and digested with trypsin. The resulting peptides are dried under vacuum and then reconstituted in an acidic slightly organic solvent for analysis using nanoflow liquid chromatography-tandem mass spectrometry. Peptides derived from the proteins of interest are identified by their retention time on the chromatographic column, their precursor mass (detected as the mass-to-charge ratio by the mass spectrometer), and the mass-to-charge ratio of specific fragments generated by collision induced dissociation of the precursor peptide. Stable isotope-labeled internal standard protein (apolipoprotein A-I, apoA-I) spiked prior to digestion is used to control for digestion variability, matrix effects, and fluctuations in instrument performance. The chromatographic peak areas corresponding to the unlabeled endogenous peptides and the ^{15}N -labeled apoA-I peptides are determined in the Skyline software package (20). The relative abundance of each protein in HDL is then calculated as sum of the peak area of two to six of the most intense precursor-fragment pairs (*i.e.*, transitions) of the endogenous peptide normalized by the peak area of one of the stable isotope-labeled internal standard peptides.

2. Materials

All buffers used for HDL isolation should be prepared with ultrapure deionized water and analytical grade reagents. All solvents used in MS analysis should be LC-MS grade purity. All reagents and solvents should be stored at room temperature unless otherwise noted. Waste reagents and solvents should be appropriately disposed of according to local regulations.

2.1 Plasma HDL Isolation

1. Potassium bromide (KBr)-containing microcentrifuge tubes: Add 400 μL of 1.1863 g/mL (@25°C) KBr stock solution to 0.6 mL microcentrifuge tubes and dry down in vacuum centrifuge. Each microcentrifuge tube should contain 108.7 mg of dry KBr. (see Note 1)

¹KBr stock solution at a density of 1.1863 g/mL is prepared by completely dissolving 124 g KBr in 400 mL of H_2O . Intermittent heat may be required to fully dissolve KBr. Allow temperature of KBr solution equilibrate to 25°C, and then adjust density down to 1.1863 g/mL using water at 25°C. Aliquot 400 μL of KBr stock solution into 0.6 mL microcentrifuge tubes. Dry down using vacuum centrifugation with heat at ~80°C for 40 min with a total spin time of 3 h. Cap tubes immediately upon drying. We generally prepare 100 tubes per batch. To determine the variability of the amount of KBr added to the tubes, number and tare 10 of the tubes prior to

2. Saline solution: 0.9% (w/v) NaCl in H₂O with 500 mM EDTA.
3. Potassium phosphate/DTPA (K₂HPO₄/DTPA) buffer: 20 mM potassium phosphate, pH 7.4, 100 μ M diethylene triamine pentaacetic acid (DTPA) in H₂O.
4. Potassium bromide (KBr) solution 1: density=1.21 g/mL or 10.17 M in H₂O (at 25°C).
5. Potassium bromide (KBr) solution 2: density=1.063 g/mL or 8.93 M in H₂O (at 25°C).
6. Dialysis cups: 3.5 kDa molecular weight cut-off (*e.g.*, Thermo P/N 69552)
7. Microcentrifuge tube float, 500 mL beaker, and stir plate at 5°C.
8. Ultracentrifuge with appropriate rotors and tubes (tube dimensions: 8 mm $\times$ 34 mm)
9. Metal (aluminum or stainless steel) ultracentrifuge tube rack.
10. Hamilton syringe with Chaney adapter to set volume at 125 μ L.
11. Microcentrifuge capable of 15k $\times$ g.
12. Rocker table or similar agitation device.

2.2 Bradford Assay

1. Coomassie Plus Protein Assay Reagent (Thermo Pierce). Store at 4°C. Warm to RT before use. The solution is light sensitive and exposure must be kept to a minimum.
2. Potassium phosphate/DTPA (K₂HPO₄/DTPA) buffer: 20 mM potassium phosphate, pH 7.4, 100 μ M diethylene triamine pentaacetic acid (DTPA) in H₂O. Can use solution prepared in 2.1.3.
3. Bovine serum albumin (BSA) standard at 2 mg/mL in K₂HPO₄/DTPA buffer. Store at 4°C. Warm to RT before use.
4. Clear flat bottom 96-well plate.
5. Ultraviolet (UV) plate reader.

2.3 Stable Isotope Labeled Proteins

1. ¹⁵N-labeled apoA-I internal standard protein (Cambridge Isotopes Laboratories, Tewksbury, MA).

2.4 Trypsin Digestion

1. 100 mM Ammonium bicarbonate buffer (AmBic): Dissolve 395.3 mg AmBic in 50 mL H₂O. AmBic buffer should be prepared and used within 12 h.

adding KBr stock solution and weigh each tube again after drying down. Each tube should contain 108.7 mg of KBr. The variability of the amount of KBr added is typically <0.5% CV.

2. 250 mM Dithiothreitol (DTT): Dissolve 50 mg of DTT in 648 mL of 100 mM AmBic. Prepare fresh solution every 12 h.
3. 0.2% *Rap*Gest SF Surfactant (Waters): Dissolve contents of 1 mg vial in 500 μ L of 100 mM AmBic. Solution is stable for 1 week at 4°C.
4. *Rap*Gest/DTT solution: Combine 675 μ L of 0.2% *Rap*Gest and 27 μ L of 250 mM DTT. Sufficient for 24 digestions.
5. 500 mM Iodoacetamide (IAA): Dissolve 50 mg of IAA in 541 μ L of 100 mM AmBic. Note: IAA is extremely light sensitive, prepare just prior to use (2 min). Discard after use.
6. 1 mM Hydrochloric acid (HCl): Add 30 μ L of 500 mM HCl stock to 15 mL of H₂O to obtain a 1 mM HCl solution to dissolve lyophilized trypsin.
7. 0.05 μ g/ μ L Trypsin: Just prior to digestion, add 400 μ L of 1mM HCl to 20 μ g of lyophilized trypsin (*e.g.*, Promega Gold sequencing grade trypsin). Trypsin should be dissolved by gently re-pipetting solution until dissolved. Store on ice until use. Do not vortex.
8. All digestions should be performed in low protein binding microcentrifuge tubes, *e.g.* Eppendorf SafeLock LoBind 1.5 mL, in a Thermomixer that has been calibrated at 37°C and speed of 1400 rpm.
9. 0.25 μ g/ μ L ¹⁵N-labeled apoA-I IS_{prot}: Dissolve 250 μ g of ¹⁵N-labeled apoA-I in 1000 μ L of 100 mM AmBic. Keep on wet ice. Aliquot as necessary and store at –20°C.
10. 10% FA in H₂O (10% FA): Add 455 μ L of 88% FA to a total volume of 4 mL in 15 mL conical tube using H₂O (see ^{Note 2}).
11. 1% FA in Acetonitrile (1% FA/ACN): Add 250 μ L of 88% FA to a total volume of 22 mL in a 50 mL conical tube using acetonitrile (ACN) (see ^{Note 2}).
12. Other equipment necessary for trypsin protein digestion includes: 1.5 mL low protein retention microcentrifuge tubes (*e.g.*, Fisher Scientific P/N02-681-320), microcentrifuge capable of achieving 15k $\times$ g, speedvac, vortexer, phospholipid removal plate (*e.g.*, Phenomenex Phree), and a positive pressure manifold, *e.g.*, Biotage Pressure +96. The methods outlined here have been optimized for Phenomenex Phree 96-well plates.

2.5 Nano-LC-MRM-MS Solvents, Mobile Phases and Columns

1. Sample suspension solvent: 95% H₂O, 5% acetonitrile, 0.1% formic acid (FA) and
2. Sample dilution solvent: 0.1% FA in H₂O.

²Solutions are prepared by adding the total volume of H₂O (4.0 mL) or acetonitrile (22.0 mL) to the tube, then removing the volume of FA, 455 μ L and 250 μ L respectively, from the tube and discarding. Then add back the required volume of 88% FA to H₂O or acetonitrile and mix well. For example, to make the 10% FA solution, add 4.00 mL of H₂O to the tube. Then, using a micropipettor, remove 455 μ L of H₂O and discard. Add back 455 μ L of 88% FA to the tube and mix well.

3. Mobile phase A: 98:1 H₂O to acetonitrile with 0.1% FA. Mobile phase B: 98:1 acetonitrile to H₂O with 0.1% FA.
4. Weak needle wash: H₂O with 0.1% FA. Strong needle wash: 2-propanol with 0.1% FA.
5. Trap column: XBridge BEH C18, 5 µm, 100 Å silica beads (Waters, MA) (or equivalent C18 packing material) are packed into a 0.1 mm i.d. fritted fused silica capillary (New Objective, MA)(or equivalent) to 30 × 0.1 mm column and fused silica capillary is cut to length of ~15 cm.
6. Analytical column: XBridge BEH C18, 3.5 µm, 100 Å silica beads (Waters, MA) (or equivalent C18 packing material) are packed into a pulled tip fused silica capillary (75 µm i.d.) to 120 length.
7. Capillary column oven capable of maintaining temperature of 50°C for analytical column.

3. Methods

All procedures should be performed at room temperature unless otherwise noted. When handling blood products, always take universal precautions such as appropriate PPE and proper use of microcentrifuges to minimize aerosols.

3.1 HDL Isolation from Human Plasma

1. Pre-chill ultracentrifuge and rotors to 5°C.
2. If necessary, rapidly thaw plasma at 37°C, then briefly centrifuge (30 s @ ~2000 × g). Place plasma on wet ice.
3. Add 335 µL of plasma to microcentrifuge tubes containing 108.9 mg KBr, then place on rocker table to dissolve KBr. Briefly centrifuge and place tubes on wet ice.
4. Add 350 µL of plasma/KBr solution from step 3 to ultracentrifuge tube using forward pipetting. Place tubes in metal rack (on wet ice) to keep the tubes chilled when not in the centrifuge.
5. Add 150 µL of KBr solution 1 (ρ=1.21 g/mL @ 25°C) to ultracentrifuge using reverse pipetting.
6. Place ultracentrifuge tubes into chilled rotor. Spin samples at 120k rpm (625,698 × g) for 4.5 h at 5°C.
7. Remove 120 µL from meniscus. Rinse tip 1 time at dispense into the bottom of a new ultracentrifuge tube (see ^{Note 3}).
8. Add 239 µL saline solution to ultracentrifuge tube (see ^{Note 4}).

³Dispense the 120 µL of lipoproteins into a 0.6 mL tube. Re-aspirate and dispense a second time to rinse the walls of the tip. Discard tip after second dispense and use a fresh tip for the next sample.

⁴Dispense 239 µL normal saline near the top inside of the ultracentrifuge tube to rinse the lipoproteins down off the side.

9. Add 140.8 μL KBr solution 2 ($\rho=1.063 \text{ g/mL}$ @ 25°C) to the ultracentrifuge tube.
10. Place ultracentrifuge tube into chilled rotor. Spin samples at 120k rpm ($625,698 \times g$) for 2.5 h at 5°C .
11. Using the Hamilton syringe with Chaney adapter set to 125 μL , carefully remove 125 μL from the bottom of the ultracentrifuge tube, wipe the tip to remove the lipids that became adhered from passing through the meniscus, and dispense into 0.6 mL microcentrifuge tube. Place samples on wet ice ($\sim 5^{\circ}\text{C}$).

3.2 Dialysis of Isolated HDL

1. Test dialysis cups for leaks by placing a tube float with empty dialysis cups in beaker containing $\text{K}_2\text{HPO}_4/\text{DTPA}$ buffer for 10 min at 5°C with gentle stirring. Check for signs of leakage. No liquid should be in the dialysis cup when the membrane is intact, although the membrane will be wet.
2. Remove float with dialysis cups from beaker. Dispense 125 μL of HDL from step 11 above onto the dialysis membrane. Carefully place the float back in the beaker and incubate for 3 h with stirring at 5°C . Buffer should be changed three times during dialysis for a total of three, 3-h incubations.
3. Blot outer portion of dialysis membrane on a clean paper towel to remove excess dialysis buffer. Place the dialysis cup in a labeled 1.5 mL microcentrifuge tube, briefly spin to maximum rpm ($\sim 15k \times g$) to rupture membranes and transfer dialyzed HDL to microcentrifuge tube.
4. Place tubes on wet ice ($\sim 5^{\circ}\text{C}$). Once the protein concentration has been determined, the samples can be aliquoted as necessary.

3.3 Bradford Assay to Determine Protein Concentration of Isolated HDL

1. Dilute standard to make a 7-point calibration curve from 1000 $\mu\text{g/mL}$ to 15 $\mu\text{g/mL}$ by making a 1:1 mixture of 2 mg/mL BSA standard with $\text{K}_2\text{HPO}_4/\text{DTPA}$ buffer (1000 $\mu\text{g/mL}$) and serial dilute (2-fold) to 15.6 $\mu\text{g/mL}$.
2. In 0.6 mL tube containing 27 μL of the $\text{K}_2\text{HPO}_4/\text{DTPA}$ buffer (1:10 dilution), add 3 μL of the dialyzed HDL from step from 3.2.4.
3. In duplicate, pipette 10 μL of standard or sample into the appropriate wells of the clear, flat-bottom 96-well plate. Do not allow bubbles to form in well when pipetting.
4. In each well, add 150 μL of the Coomassie Plus Protein assay reagent to each well. Again, do not introduce bubbles into the well.
5. Gently agitate the plate for 1 min and incubate for a total of ten min. (see ^{Note 5}).
6. Read plate at $\lambda=595 \text{ nm}$.

⁵The Bradford reaction develops at different rates for BSA and for HDL proteins. Maintaining constant incubation times is vital for reproducible results between batches.

7. Once the protein concentrations of the HDL samples have been determined (be sure to account for 1:10 dilution), prepare aliquots containing 5 µg HDL protein in Eppendorf LoBind SafeLock Tubes.

3.4 Digestion of isolated HDL Using Trypsin

1. To each 5 µg HDL protein aliquot, add 4 µL of the 0.25 µg/µL ¹⁵N-labeled apoA-I IS_{prot} working stock solution.
2. Denaturation and reduction of proteins is performed by adding 26 µL of *Rapi*Gest/DTT solution, vortex briefly and incubate for 1 h in Thermomixer at 37°C and 1400 rpm. Then briefly centrifuge to collect condensate.
3. Prepare 500 mM IAA stock solution (see section 2.4.5). Add 1.5 µL of the 500 mM IAA stock solution ($C_{\text{final}} = 15 \text{ mM IAA}$) to each sample. Vortex briefly, then incubate in dark for 15 min. During the last 5 min of the incubation, prepare the trypsin working stock as described above (see section 2.4.7).
4. Add 5 µL of 0.05 µg/µL trypsin solution to each sample and incubate on Thermomixer at 37°C and 1400 rpm for 3 h. Be sure to place trypsin back on ice.
5. Briefly vortex HDL digests to collect condensate. Then add another 5 µL of 0.05 µg/µL trypsin to each digestion and incubate for 17 h on Thermomixer at 37°C and 1400 rpm.
6. After incubation, briefly vortex digests to collect condensate. Then add 62.4 µL of 10% FA solution to cleave *Rapi*Gest and stop digestion. Incubate for 45 min in Thermomixer at 37°C and 1400 rpm.
7. Centrifuge samples at $15k \times g$ for 15 min. Then verify order of samples in regards to orientation on the SPE plate.
8. Add 390 µL of 1% FA/ACN to each well. Then add 110 µL of HDL digest to each well. Seal plate with clear sealing tape and place on plate shaker for 5 min.
9. Orient SPE plate over collection plate, remove plate seal tape and mount plates on positive pressure displacement manifold.
10. To collect eluent, set initial pressure at 4–5 psi for 5 min. Then increase pressure to 7–8 psi and hold for 5 min. Finally, increase pressure to 10–11 psi for 5 min.
11. Transfer eluent to 1.5 mL low protein binding microcentrifuge tubes and speed vac without heat (~RT) to dryness; approximately 12 h.
12. Store dried samples at –80°C.

3.5 Nano-LC-MRM-MS Analysis of Tryptic Peptides

An example configuration of the nano-LC-MRM-MS system is a NanoAquity UPLC (Waters) coupled with TSQ Vantage (Thermo Scientific). However, the method can be adapted to other triple-quadrupole mass spectrometers. Alternatively, the method could also be used in the form of parallel reaction monitoring (PRM) on hybrid instruments (e.g. quadrupole time-of-flight or quadrupole-Orbitrap). Skyline could facilitate the transfer of

methods described here to another instrument in a vendor-blind manner (*see* below). Capillary column and trap column are maintained at the temperature of 50°C by means of a column heater.

3.5.1. Sample suspension for nano-LC-MRM analysis

1. Remove samples from –80°C and allow them to warm to room temperature.
2. Centrifuge samples at $15k \times g$ for 5 min to ensure peptide pellet is in the bottom of the tube.
3. Add 15 μ L of 95:5 H₂O/ACN with 0.1% FA sample suspension solvent to each sample and vortex at 1400 rpm for 2 h at RT.
4. Then add 15 μ L of 0.1% FA dilution solvent to each sample and briefly vortex.
5. Centrifuge samples at $15k \times g$ for 15 min, then transfer to compatible vials for the auto-sampler coupled to the nano-LC unit. Ensure that no bubbles are present in the solution.

3.5.2. Chromatographic conditions

1. Injected sample is trapped and washed on the trapping column for 5 min with Mobile phase A at the flow rate 4 μ L/min.
2. Peptides are eluted from the trap column onto the analytical column and separated by the gradient detailed in Table 1.

3.5.3. Mass spectrometer acquisition conditions

Capillary temp = 325 °C

Spray voltage = 2200 V

Q1 peak width = 0.7

Q3 peak width = 0.7

Collision gas: 1.5 mTorr

Collision energy: Calculated in Skyline

Dwell time: 10 ms (except selected low abundance peptides where dwell set to 30 ms).

3.6 Develop MRM Transition Lists and Perform Data Analysis Using Skyline

Download and install Skyline from MacCoss laboratory Skyline webpage at: <https://skyline.gs.washington.edu/labkey/project/home/software/Skyline/begin.view> (see ^{Note 6}).

⁶Detailed Skyline tutorials that outline MRM method development and other features of the software not included in this section can be downloaded from the MacCoss laboratory website at: <https://skyline.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorials>.

3.6.1. Skyline Settings for MRM Experiment

1. Under the Settings option, open the Peptide Settings window. Choose the Digestion tab and in the Enzyme dropdown menu, select Trypsin [KR|P]. Select 0 in the Max missed cleavages dropdown menu. The Background proteome should be set to none.
2. In the Filter tab, set the minimum length to 6 and maximum length to 25. Auto-select all matching peptides should be selected.
3. Under the Modifications tab, in the Structural modifications box, select Carbamidomethyl Cysteine and Oxidation (M). Then select OK.
4. Open Transition Settings and click on the Full-Scan tab. In the MS1 Filtering box, choose none from the Isotope peaks included dropdown menu. Below, in the MS/MS filtering box, choose none from the Acquisition method dropdown menu.

3.6.2. Preparing the Transition List

1. Copy and paste each peptide from Table 2 Skyline file Targets list on the left side of the window. Alternatively, import FASTA files for proteins being analyzed and cull the list of peptides to those listed in Table 2 by right clicking on the protein name and selecting Pick Children (see ^{Note 7}).
2. For each apoA-I peptide that has a stable isotope-labeled internal standard counterpart, include the modified residues in the peptide by right clicking on the precursor *m/z* and select Modify. In the Edit Modifications window, in the Isotope heavy column, select the dropdown menu that corresponds to each peptide and choose Label: 15N. Then select OK.
3. Select the optimized transitions for each peptide from Table 2. When selecting transitions for peptides that include an internal standard, be sure to check the box that synchronizes selection for the stable isotope-labeled peptide (see ^{Note 8}).

3.6.3. Export Transition List

1. From the menu bar, navigate to Export then choose Transition List. In the Export Transition List window, select the instrument from the Instrument type dropdown menu. Select Single Method with no optimization and a standard method type.
2. Import transition list directly into MS method software. Verify that all precursor ion, transition ion, declustering potentials, collision energy, and any other necessary method parameters required by the instrument were correctly imported into MS method (see ^{Note 9}).

⁷The peptides and transitions in Table 2 represent proteotypic peptides for these 38 proteins. Proteotypic in this context means the peptide sequence has been determined to be unique in the human proteome (*i.e.*, no splice isoforms, polymorphisms or isobaric peptides being reported) and that it is readily detected using tandem MS. Furthermore, these peptide sequences: (1) do not contain methionine and (2) the peak area ratios of peptides from the same protein are highly correlated across a population of human samples.

⁸If spectral libraries obtained from DDA data or another source are being used to aid in MS peak identification, please visit the Skyline software tutorial page (Note 5) to find detailed tutorials that outline how to implement spectral libraries in Skyline.

3.6.4. Analyzing MRM Data in Skyline

1. Open the Skyline file that was used to prepare the transition list and save it as an analysis file specifically for these MS data and this experiment (see Note 10).
2. To import MS data into Skyline, from the menu bar, navigate to Import then choose Results. In the Import Results window, select Add single-injection replicates in files and none from the Optimizing dropdown menu. Then select OK.
3. Navigate to the data file containing the MS data to be analyzed. Select one or more MS spectra files for import into Skyline and select Open. Save the Skyline document after the spectral data has been imported (see Note 11).
4. Verify that the transitions assigned to each peptide by Skyline are correct. Adjust integration limits for the transition peak areas as necessary. Export the results in .csv file format for analysis in Microsoft Excel (or similar software) under File in the menu bar, then navigate to Export and select Report. In the Export Report window, select Transition Results, then Export. Name the file and select Save.

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⁹Retention time scheduling for precursor ions can be added to the transition list .csv file in Microsoft Excel (or similar software) after exporting. Scheduled methods can be exported from Skyline using iRT retention time prediction. See Note 5.

¹⁰Using a detailed file name that includes the acquisition data and pertinent information regarding the samples analyzed will make it easier to locate the Skyline data analysis file in the future.

¹¹If the MS file names have a common prefix, an Import Results pop-up window will appear indicating the common prefix and the option to retain or remove the prefix. Selecting to remove the prefix will not affect the original file name.

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Table 1

Chromatography schedule and flow rate for nanoLC-MRM analysis of HDL peptides

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]	Flow rate [μ L/min]
0	99	1	0.6
2	93	7	0.6
17	75	25	0.6
20	65	35	0.6
22	20	80	0.6
25	20	80	0.6
26	99	1	0.8
37 (end)	99	1	0.0

Table 2

List of HDL proteins, peptides and corresponding transitions

Protein	Peptide	Precursor Ion (<i>m/z</i>)	1	2	3	4	5	6
ALB	FQNALLVR	480.8	685.4	500.4				
ALB	LVNEVTEFAK	575.3	937.5	694.4				
APOA1	DLATVYVDVLK	618.3	936.5	736.4				
APOA1#	DLATVYVDVLK	624.3	945.5	743.4				
APOA1	DYVSQFEGSALGK	700.8	1023.5	808.4				
APOA1#	DYVSQFEGSALGK	708.3	1035.5	817.4				
APOA1	VQPYLDDDFQK	626.8	1025.5	513.3				
APOA1#	VQPYLDDDFQK	633.3	1035.5	518.2				
APOA2	EPC[+57.0]VESLVSYFQTIVTDYK	1175.5	1436.7	583.3				
APOA2	EQLTPLIK	471.3	571.4	470.3				
APOA2	SPELQAEAK	486.8	788.4	443.2				
APOA4	LGEVNTYAGDLQK	704.4	794.4	631.3				
APOA4	LTPYADEFK	542.3	772.4	435.2				
APOA4	SELTQQLNALFQDK	817.9	835.4	537.3				
APOA5	LRPLSGSEAPR	591.8	913.5	816.4	703.3	616.3	559.3	
APOA5	SVAPHAPASPAR	580.8	903.5	669.4	598.3	487.8	452.2	403.7
APOB	IEIPLPFGGK	535.8	828.5	715.4				
APOB	SVSLPSLDPASAK	636.3	1085.6	885.5				
APOC1	EFGNTLEDK	526.7	605.3	504.3				
APOC1	EWFSETFQK	601.3	739.4	523.3				
APOC1	TPDVSSALDK	516.8	834.4	466.2				
APOC2	TAAQNLYEK	519.3	865.4	666.3				
APOC2	TYLPAVDEK	518.3	771.4	658.3				
APOC3	DALSSVQESQVAQQAR	858.9	1144.6	573.3				
APOC3	DYWSIVK	449.7	620.3	434.3				
APOC3	GWVTDGFSSLK	598.8	953.5	854.4				
APOC4	AWFLESK	440.7	623.3	476.3				

Protein	Peptide	Precursor Ion (<i>m/z</i>)						Fragment Ion (<i>m/z</i>)					
		1	2	3	4	5	6	1	2	3	4	5	6
APOC4	ELLETVVNR	536.8	588.3	388.2									
APOD	IPTTFNGR	517.8	824.4	461.2									
APOD	NPNLPPETVDSLK	712.4	1098.6	985.5									
APOD	VLNQELR	436.3	659.3	545.3									
APOE	AATVGSLAGQPLQER	749.4	827.4	642.4									
APOE	SELEEQLTPVAEETR	865.9	902.5	801.4									
APOF	SGVQQLIQYYQDQK	849.4	1085.5	972.4									
APOF	SLPTEDC[+57.0]JENEK	661.3	923.3	561.2									
APOF	SYDLDPGAGSLEI	668.8	743.4	261.1									
APOH	ATVVYQGER	511.8	751.4	652.3									
APOH	TC[+57.0]PKPDDLPESTVVPLK	638.7	743.5	357.2									
APOLI	LNILNNYK	553.3	878.5	765.4									
APOLI	VTEPISAESGEQVER	815.9	1301.6	1091.5	804.4								
APOM	AFLTPR	409.3	599.4	486.3									
APOM	DGLCI[+57.0]VPR	408.7	531.3	371.2									
APOM	SLTSC[+57.0]LDSK	505.7	810.4	709.3									
C3	TGLQEVEVK	501.8	731.4	422.7									
C3	TIYTPGSTVLYR	685.9	1156.6	892.5									
C4A	GSFEFPVGDAVSK	670.3	919.5	772.4									
C4A	VFALDQK	410.7	721.4	574.3	503.3								
CETP	ASYPDITGEK	540.8	759.4	662.3									
CETP	GTSHEAGIVC[+57.0]R	593.8	804.4	675.4									
CETP	VIQTAFQR	481.8	750.4	622.3									
CLU	ASSIIDELFQDR	697.4	1035.5	922.4									
CLU	ELDESLQVAER	644.8	802.4	474.3	375.2								
CLU	LFDSDPITVTPVEVSR	937.5	886.5	686.4									
HBB	LLVVYPWTQR	637.9	850.4	687.4									
HBB	SAVTALWGK	466.8	774.5	675.4									
HP	VTSIQDWVQK	602.3	1003.5	916.5	803.4								
HPR	GSFPWQAK	460.7	629.3	532.3	388.7								

Protein	Peptide	Precursor Ion (<i>m/z</i>)						Fragment Ion (<i>m/z</i>)					
		1	2	3	4	5	6	1	2	3	4	5	6
HPR	LPEC[+57.0]EAVC[+57.0]GKPK	463.2	589.3	244.2				589.3	244.2				
HPR	TEGDGVYTLNDK	656.3	1081.5	909.5	590.3			1081.5	909.5	590.3			
HPR	VGYYSGWGQSDNFK	772.4	1125.5	795.4				1125.5	795.4				
IHH	AFQVIETQDPPR	700.9	955.5	369.2				955.5	369.2				
LCAT	LEPGQEEYYR	706.3	1169.5	585.3				1169.5	585.3				
LCAT	SSGLVSNAPGVQIR	692.9	941.5	669.4				941.5	669.4				
LCAT	STELC[+57.0]GLWQGR	653.8	876.4	716.4				876.4	716.4				
LPA	GTLSITITIGR	503.8	735.4	446.3				735.4	446.3				
LPA	TPAYYPNAGLIK	654.4	875.5	712.4				875.5	712.4				
LpPLA2	ASLAFLLQK	439.3	719.4	606.4	535.3			719.4	606.4	535.3			
LpPLA2	IAVIGHSFGGATVIQTLSEDQR	767.1	634.3	547.2				634.3	547.2				
PCYOX1	LFLSYDYAVK	609.8	958.5	845.4				958.5	845.4				
PCYOX1	LVC[+57.0]SGLLQASK	588.3	963.5	482.3				963.5	482.3				
PLTP	AVEPQLQEEER	664.3	1028.5	514.8				1028.5	514.8				
PLTP	FLEQELETITIPDLR	909	714.4	500.3				714.4	500.3				
PLTP	GAFFPLTER	519.3	762.4	615.3				762.4	615.3				
PON1	IFFYDSENPPASEVLR	942.5	982.5	868.5				982.5	868.5				
PON1	IQNILTEEPK	592.8	943.5	716.4				943.5	716.4				
PON1	STVELFK	412.2	635.4	536.3	294.2			635.4	536.3	294.2			
PON3	AQALEISGGFDK	618.3	1036.5	723.4				1036.5	723.4				
PON3	LLNYPEDPPGSEVLR	907	1195.6	854.5				1195.6	854.5				
PON3	SVNDIVVLGPEQFYATR	954.5	1068.5	1011.5				1068.5	1011.5				
RBP4	LLNLDGTC[+57.0]ADSYSFVFSR	1033	742.4	409.2				742.4	409.2				
RBP4	YWGVASFLQK	599.8	849.5	350.1				849.5	350.1				
SAA1/2	DPNHFRPAGLPEKY	820.9	763.4	536.3				763.4	536.3				
SAA1/2	SFFSFLGEAFDGAR	775.9	935.5	822.4				935.5	822.4				
SAA4	FRPDGLPK	465.3	686.4	244.2				686.4	244.2				
SAA4	GPGGVWAAK	421.7	688.4	475.3				688.4	475.3				
SERPINA1	LSITGTYDLK	555.8	910.5	797.4				910.5	797.4				
SERPINA1	SVLQLGITK	508.3	829.5	716.4				829.5	716.4				

Protein	Peptide	Precursor Ion (<i>m/z</i>)	Fragment Ion (<i>m/z</i>)					
			1	2	3	4	5	6
SERPINA4	IAPANADFAPR	596.8	1008.5	504.7				
SERPINA4	VGSALFLSHNLK	643.4	971.6	711.4				
VDBP	THLPEVFLSK	390.9	494.3	352.2	234.1			
VDBP	VLEPTLK	400.2	700.4	587.3				
VTN	DVWGIEGPDAAFTR	823.9	1076.5	947.5				
VTN	FEDGVLPDYPR	711.8	875.4	647.3				
VTN	GQYC[+57.0]YELDEK	652.8	1119.5	956.4	796.4			

Note - # ¹⁵N labeled peptides originating from ¹⁵N-APOA-1. Protein abbreviation, protein name: ALB, serum albumin; APOA1, apolipoprotein A-I; APOA2, apolipoprotein A-II; APOA4, apolipoprotein A-IV; APOA5, apolipoprotein A-V; APOB, apolipoprotein B; APOC1, apolipoprotein C-I; APOC2, apolipoprotein C-II; APOC3, apolipoprotein C-III; APOC4, apolipoprotein C-IV; APOD, apolipoprotein D; APOE, apolipoprotein E; APOF, apolipoprotein F; APOH, apolipoprotein H; APOLI, apolipoprotein L-1; APOM, apolipoprotein M; C3, complement C3; C4A, complement C4A; CETP, cholesterol ester transfer protein; CLU, clusterin (apolipoprotein J); HBB, hemoglobin beta; HP, haptoglobin; HPR, haptoglobin related protein; IHH, Indian hedgehog protein; LCAT, Phosphatidylcholine-sterol acyltransferase; LPA, lipoprotein a; LpPLA2, Lipoprotein-Associated Phospholipase A2; PCYOX1, prenylcysteine oxidase 1; PLTP, phospholipid transfer protein; PON1, paraoxonase 1; PON3, paraoxonase 3; RBP4, retinol binding protein 4; SAA1/2, serum amyloid A1/2; SAA4, serum amyloid A4; SERPINA1, serpin A1 (alpha-1-antitrypsin); SERPINA4, serpin A4 (kallistatin); VDBP, vitamin D binding protein; VTN, vitronectin.