Sample preparation methods for targeted single-cell proteomics

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Supporting Information. A listing of the contents of each file supplied as Supporting Information is included.

Supplemental Figures S1-S13 and Supplemental Document 1: Methods and results communicating; selective peptide standard N-terminal carboxymethylation, LOD and LOQ measurements, stability of CM-NAT and CM-SIL peptides in one-pot rapid digestion, selectivity and matrix effect. Detailed instrumentation parameters used for data acquisition, Figure S1, S2, S3 and S4. Single RBC isolation via limiting dilution Figure S5, CellenONE automated single RBC isolation Figure S6, nano-LC pressure profiles for samples prepared via one-pot rapid digestion, Figure S7, MALDI-TOF mass spectra of synthetic peptide standards Figure S8, MRM

chromatograms fo synthetic peptide standards Figure S9, UV-HPLC chromatograms and MALDI-TOF mass spectra of purified peptide standards Figure S10, PRM assay chromatograms and LOD and LOQ measurements Figure S11, MRM chromatograms and peak areas of peptide standards subjected to the one-pot rapid digestion workflow Figure S12., assessment of selective measurement of endogenous CM-VHLTPEEK Figure S13. Details of the blood specimen collection, chronology, erythrocyte separation from whole blood and erythrocyte spotting in OP384 wells. Supplemental_Document_1.

Supplemental Document 2: MSFragger_log_file_for _DDA_search_log_2021-05-15_22-20-41(Text File)

Supplemental Document 3: MSFragger_log_file_for _DIA_search_log_2021-05-15_22-20-41(Text File)

Supplemental Document 4: MSFragger_log_file_for _DIA_speclibrary_log_2021-05-15_22-20-41 (Text File)

Supplemental Document 5: MSFragger_parameters_for_searching_DDA_data (Text File)

Supplemental Document 6: MSFragger_parameters_for_searching_DIA_wide_window_data (Text File)

S3. 1 Selective Peptide Standard N-Terminal Carboxymethylation

With the aim of developing a quantitative and reproducible assay for measuring heterogeneity in RBCs using single and/or near-single numbers of cells, peptide standards were synthesized to measure the N-terminal proteolytic peptide of HBB; VHLTPEEK and the carboxymethyl (CM) valine advanced glycation end (AGE) product; CM-VHLTPEEK. Corresponding NAT and SIL peptides were synthesized and results of the MALDI analysis to assess yield, MRM to confirm N-terminal carboxymethylation and UV-HPLC and MALDI

analyses to assess purity, after preparative HPLC purification, are provided in Supplemental **Figures S8-S10**. The MALDI-TOF analysis of synthetic peptides before preparative HPLC purification showed a mixture of parent NAT and SIL m/z along with their counterpart carboxymethyl adducts which displayed a mass shift of 58+ m/z (Supplemental **Figure S8**). An approximate yield of 16-22% for carboxymethylated peptide standards (CM-NAT and CM-SIL) was observed, a notably low yield. It should be noted here, measurement of endogenous proteolytic peptide targets in single and low cell numbers was in the attomole concentration range while synthesis was on the nanomole scale. Peptide standards were further characterized by MRM which revealed a 58+ Dalton mass shift for a, b and c product ions but not y ions, when comparing fragment ion chromatograms of NAT and SIL peptides to those of CM-NAT and CM-SIL peptides (Supplemental **Figure S9**), suggestive of N-terminal carboxymethylation. Purity analysis via MALDI-TOF and UV-HPLC demonstrated a purity greater than 95% (Supplemental **Figure S10**). Lastly, amino acid analysis of purified peptide standards quantified a final yield of 1180 nmol SIL, 730 nmol NAT, 62 nmol CM-NAT and 40 nmol CM-SIS.

S3.2 Isotope-Dilution PRM LOD and LOQ Assessment

Peptide standards were used to develop a 4-plex PRM assay modifying various acquisition parameters (Supplemental **Figure S1**) to maximize sensitivity while maintaining quantitative reproducibility (**Figure S11**). PRM chromatograms, displayed in **Figure S11**, showed consistent peak shape, product ions and isotopic envelopes. An initial limit of detection was performed in a background matrix composed of a proteolytic digest of RBCs diluted to a protein concentration equivalent to one RBC per microliter (~32 pg/uL) (**Figure S11** left graph). Isotope dilution with CM-SIL and SIL peptides revealed LODs of 9.8 and 62.5 amol of peptide injected on column for CM-SIL and SIL peptides, respectively (**Figure S11** left graph). The

intraassay LOQ was assessed in our collaborator's laboratory at Brigham Young University using a slightly different experimental and instrument setup (**Figure S11** middle and right figures). A sub-attomole LOQ was observed at an imprecision that may find application as a Tier 2 level assay [1].

S3.3 Stability of CM-NAT and CM-SIL Peptides in One-Pot Rapid Digestion

To test whether the high temperature used in the one-pot rapid digestion protocol would alter the stability of CM modification and thereby reduce assay sensitivity for measuring endogenous CM modified peptide, peptide standards were subjected to the entire workflow described in detail in the methods section **2.8**. Control samples were treated in a similar manner, but high temperature sonication was omitted and control samples were instead incubated at 4˚C. Samples were analyzed using MRM, the results of which are presented in **Figure S12**. Compared to the control, MRM chromatograms of peptides prepared using one-pot rapid digestion displayed higher peak intensities and higher peak areas (**Figure S12**). One-pot rapid digestion also improved peak shape qualitatively (**Figure S12**) and the calculated imprecision across the three replicates showed improved peak area CVs, a median of 9.1% for one-pot rapid digestion treated peptides versus a median of 23% for the control. These results showed high temperature sample preparation did not alter carboxymethylated residues suggesting the one-pot rapid digest workflow could be used to measure target peptides.

Regarding the improved MRM chromatograms, it was not determined whether the observed improvements were a result of complete emulsification of peptides in solution, achieved via high temperature sonication treatment, or due to some other variable such as

potential solvent evaporation that may have resulted in more peptide being injected at the same injection volume.

S3.4 Assessment of Assay Selectivity in Replicate Biological Matrix

Endogenous CM-VHLTPEEK has previously been identified, quantified and associated with poorly managed diabetes [2], however, that study did not utilize peptide standard(s). Having access to peptide standards in the present study the selectivity of the assay was assessed as described in detail in methods section **2.10**. Utilizing MRM on a triple quadrupole mass spectrometer, endogenous CM-VHLTPEEK was measured in packed blood cells obtained from six different individuals. Three-point response curves were generated by measuring endogenous signal, and signal from spike-in of CM-NAT at 25X the LOQ and subsequently at 50X the LOQ, CM-SIL being used as a normalizer across all injections (**Figure S13A**). Response curves across individual samples were comparable qualitatively, and quantitative analyses of the slope of each response curve against the averaged slope across all six response curves showed differences not exceeding 10%. The relatively lower resolution of the triple quadrupole mass spectrometer revealed an analyte at RT 4 min which shared many of the same product and precursor ions with endogenous CM-VHLTPEEK (**Figure S13B**), however the peptide of interest had an RT of 4.3 min.

(1) Carr, S. A.; Abbatiello, S. E.; Ackermann, B. L.; Borchers, C.; Domon, B.; Deutsch, E. W.; Grant, R. P.; Hoofnagle, A. N.; Hüttenhain, R.; Koomen, J. M.; Liebler, D. C.; Liu, T.; MacLean, B.; Mani, D. R.; Mansfield, E.; Neubert, H.; Paulovich, A. G.; Reiter, L.; Vitek, O.; Aebersold, R.; Anderson, L.; Bethem, R.; Blonder, J.; Boja, E.; Botelho, J.; Boyne, M.; Bradshaw, R. A.; Burlingame, A. L.; Chan, D.; Keshishian, H.; Kuhn, E.; Kinsinger, C.;

Lee, J. S. H.; Lee, S.-W.; Moritz, R.; Oses-Prieto, J.; Rifai, N.; Ritchie, J.; Rodriguez, H.; Srinivas, P. R.; Townsend, R. R.; Van Eyk, J.; Whiteley, G.; Wiita, A.; Weintraub, S. Targeted Peptide Measurements in Biology and Medicine: Best Practices for Mass Spectrometry-Based Assay Development Using a Fit-for-Purpose Approach. *Mol. Cell. Proteomics MCP* **2014**, *13* (3), 907–917. https://doi.org/10.1074/mcp.M113.036095.

(2) Jagadeeshaprasad, M. G.; Batkulwar, K. B.; Meshram, N. N.; Tiwari, S.; Korwar, A. M.; Unnikrishnan, A. G.; Kulkarni, M. J. Targeted Quantification of N-1-(Carboxymethyl) Valine and N-1-(Carboxyethyl) Valine Peptides of β-Hemoglobin for Better Diagnostics in Diabetes. *Clin Proteomics* **2016**, *13*, 7. https://doi.org/10.1186/s12014-016-9108-y.

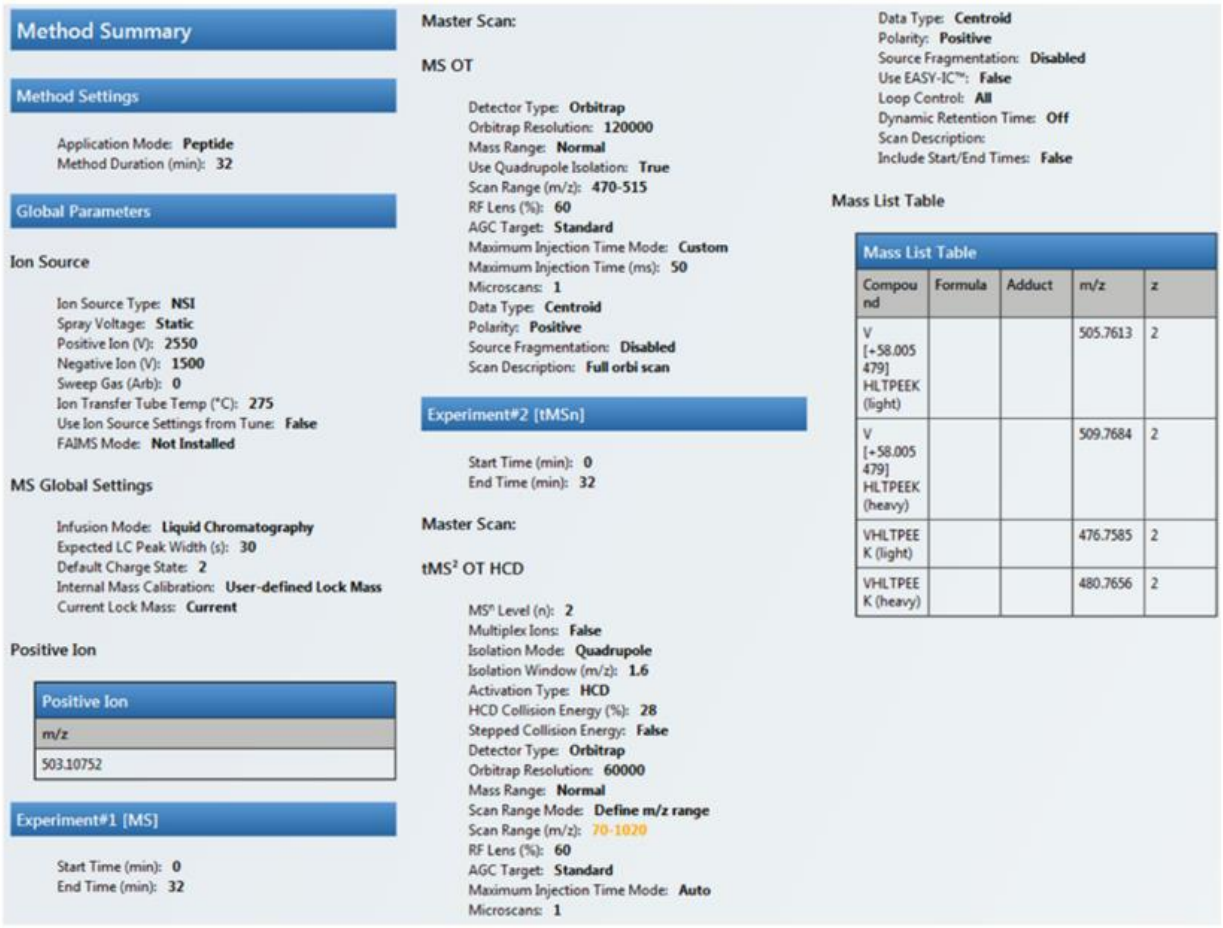


Figure S1. Thermo Orbitrap Fusion ETD settings used to perform PRM.

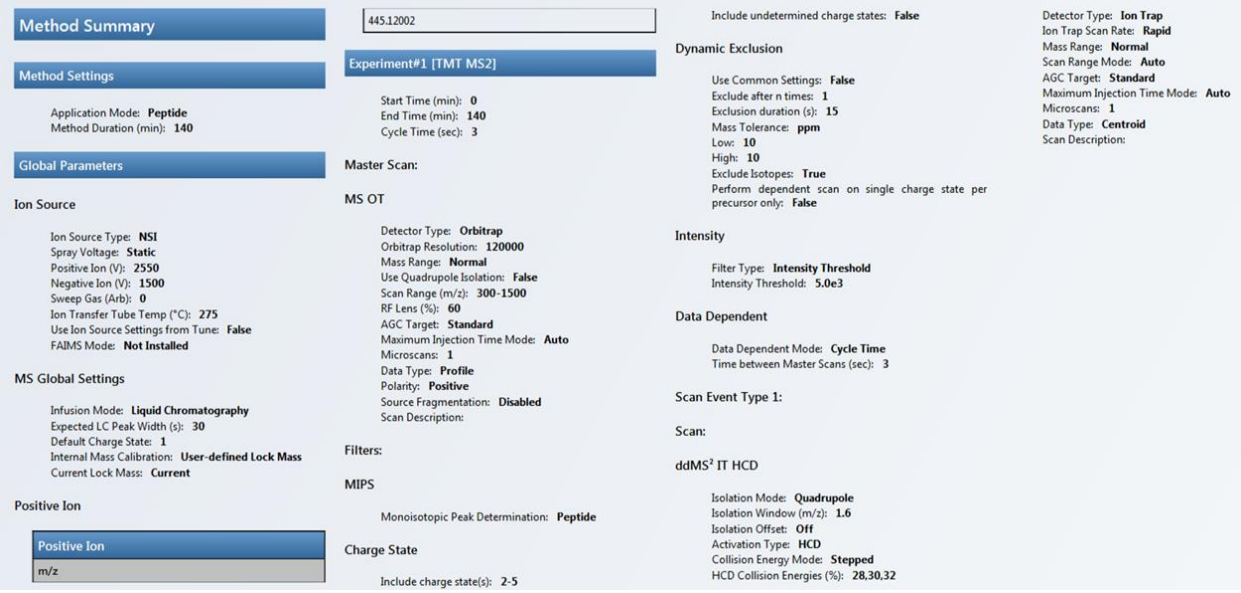


Figure S2. Thermo Orbitrap Fusion ETD settings used to perform DDA.

Method Summary

Method Settings

Application Mode: **Peptide**
Method Duration (min): **140**

Global Parameters

Ion Source

Ion Source Type: **NSI**
Spray Voltage: **Static**
Positive Ion (V): **2550**
Negative Ion (V): **1500**
Sweep Gas (Arb): **0**
Ion Transfer Tube Temp (°C): **275**
Use Ion Source Settings from Tune: **False**
FAIMS Mode: **Not Installed**

MS Global Settings

Infusion Mode: **Liquid Chromatography**
Expected LC Peak Width (s): **30**
Default Charge State: **3**
Internal Mass Calibration: **Off**

Experiment#1 [MS]

Start Time (min): **0**
End Time (min): **140**

Master Scan:

MS OT

Detector Type: **Orbitrap**
Orbitrap Resolution: **60000**
Mass Range: **Normal**
Use Quadrupole Isolation: **False**
Scan Range (m/z): **395-505**

RF Lens (%): **60**
AGC Target: **Standard**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Centroid**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Scan Description:

Experiment#2 [tMSn]

Start Time (min): **0**
End Time (min): **140**

Master Scan:

tMS² OT HCD

MSⁿ Level (n): **2**
Multiplex Ions: **False**
Isolation Mode: **Quadrupole**
Isolation Window (m/z): **4**
Activation Type: **HCD**
HCD Collision Energy (%): **33**
Stepped Collision Energy: **False**
Detector Type: **Orbitrap**
Orbitrap Resolution: **30000**
Mass Range: **Normal**
Scan Range Mode: **Define m/z range**
Scan Range (m/z): **145-1450**
RF Lens (%): **60**
AGC Target: **Custom**
Normalized AGC Target (%): **800**
Maximum Injection Time Mode: **Dynamic**
Desired minimum points across the peak: **6**
Microscans: **1**
Data Type: **Centroid**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Use EASY-IC™: **False**
Dynamic Retention Time: **Off**
Scan Description:
Include Start/End Times: **False**

Mass List Table

Mass List Table

Compound	Formula	Adduct	m/z	z
			402.4328	3
			406.4346	3
			410.4365	3
			414.4383	3
			418.4401	3
			422.4419	3
			426.4437	3
			430.4456	3
			434.4474	3
			438.4492	3
			442.451	3
			446.4528	3
			450.4547	3
			454.4565	3
			458.4583	3
			462.4601	3
			466.4619	3
			470.4638	3
			474.4656	3
			478.4674	3
			482.4692	3
			486.471	3
			490.4728	3
			494.4746	3
			498.4765	3

			400.4319	3
			404.4337	3
			408.4356	3
			412.4374	3
			416.4392	3
			420.441	3
			424.4428	3
			428.4446	3
			432.4465	3
			436.4483	3
			440.4501	3
			444.4519	3
			448.4537	3
			452.4556	3
			456.4574	3
			460.4592	3
			464.461	3
			468.4628	3
			472.4647	3
			476.4665	3
			480.4683	3
			484.4701	3
			488.4719	3
			492.4738	3
			496.4756	3
			500.4774	3

Figure S3. Thermo Orbitrap Fusion ETD settings used to perform narrow window DIA.

Only the mass list for m/z values from 400-500 are displayed. Independent methods were created for m/z ranges, 500-600, 600-700, 700-800, 800-900 and 900-1000 m/z. These were used to collect data for building spectral libraries.

Method Summary

Method Settings

Application Mode: **Peptide**
Method Duration (min): **140**

Global Parameters

Ion Source

Ion Source Type: **NSI**
Spray Voltage: **Static**
Positive Ion (V): **2550**
Negative Ion (V): **1500**
Sweep Gas (Arb): **0**
Ion Transfer Tube Temp (°C): **275**
Use Ion Source Settings from Tune: **False**
FAIMS Mode: **Not Installed**

MS Global Settings

Infusion Mode: **Liquid Chromatography**
Expected LC Peak Width (s): **30**
Default Charge State: **3**
Internal Mass Calibration: **Off**

Experiment#1 [MS]

Start Time (min): **0**
End Time (min): **140**

Master Scan:

MS OT

Detector Type: **Orbitrap**
Orbitrap Resolution: **60000**
Mass Range: **Normal**
Use Quadrupole Isolation: **True**
Scan Range (m/z): **385-1015**
RF Lens (%): **60**
AGC Target: **Standard**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Centroid**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Scan Description:

Experiment#2 [tMSn]

Start Time (min): **0**
End Time (min): **140**

Master Scan:

tMS^2 OT HCD

MS^n Level (n): **2**
Multiplex Ions: **False**
Isolation Mode: **Quadrupole**
Isolation Window (m/z): **16**
Activation Type: **HCD**
HCD Collision Energy (%): **33**
Stepped Collision Energy: **False**
Detector Type: **Orbitrap**
Orbitrap Resolution: **30000**
Mass Range: **Normal**
Scan Range Mode: **Define m/z range**
Scan Range (m/z): **145-1450**
RF Lens (%): **60**
AGC Target: **Custom**
Normalized AGC Target (%): **800**
Maximum Injection Time Mode: **Dynamic**
Desired minimum points across the peak: **6**
Microscans: **1**
Data Type: **Centroid**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Use EASY-IC™: **False**
Dynamic Retention Time: **Off**
Scan Description:
Include Start/End Times: **False**

Mass List Table

Mass List Table

Compound	Formula	Adduct	m/z	z
			408.4356	3
			424.4428	3
			440.4501	3
			456.4574	3
			472.4647	3
			488.4719	3
			504.4792	3
			520.4865	3
			536.4938	3
			552.501	3
			568.5083	3
			584.5156	3
			600.5228	3
			616.5302	3
			632.5374	3
			648.5447	3
			664.552	3
			680.5592	3
			696.5665	3
			712.5737	3
			728.5811	3
			744.5884	3
			760.5956	3
			776.6029	3
			792.6101	3
			808.6174	3
			824.6248	3
			840.632	3
			856.6393	3
			872.6466	3
			888.6538	3
			904.6611	3
			920.6683	3
			936.6757	3
			952.6829	3
			968.6902	3
			984.6975	3
			1000.7047	3
			400.4319	3
			416.4392	3
			432.4465	3
			448.4537	3
			464.461	3
			480.4683	3
			496.4756	3
			512.4828	3
			528.4901	3
			544.4974	3
			560.5046	3
			576.512	3
			592.5192	3
			608.5265	3
			624.5338	3
			640.541	3
			656.5483	3
			672.5555	3
			688.5629	3
			704.5702	3
			720.5774	3
			736.5847	3
			752.5919	3
			768.5992	3
			784.6066	3
			800.6138	3
			816.6211	3
			832.6284	3
			848.6356	3
			864.6429	3
			880.6502	3
			896.6575	3
			912.6647	3
			928.672	3
			944.6793	3
			960.6865	3
			976.6939	3
			992.7011	3

Figure S4. Thermo Orbitrap Fusion ETD settings used to perform wide window DIA.

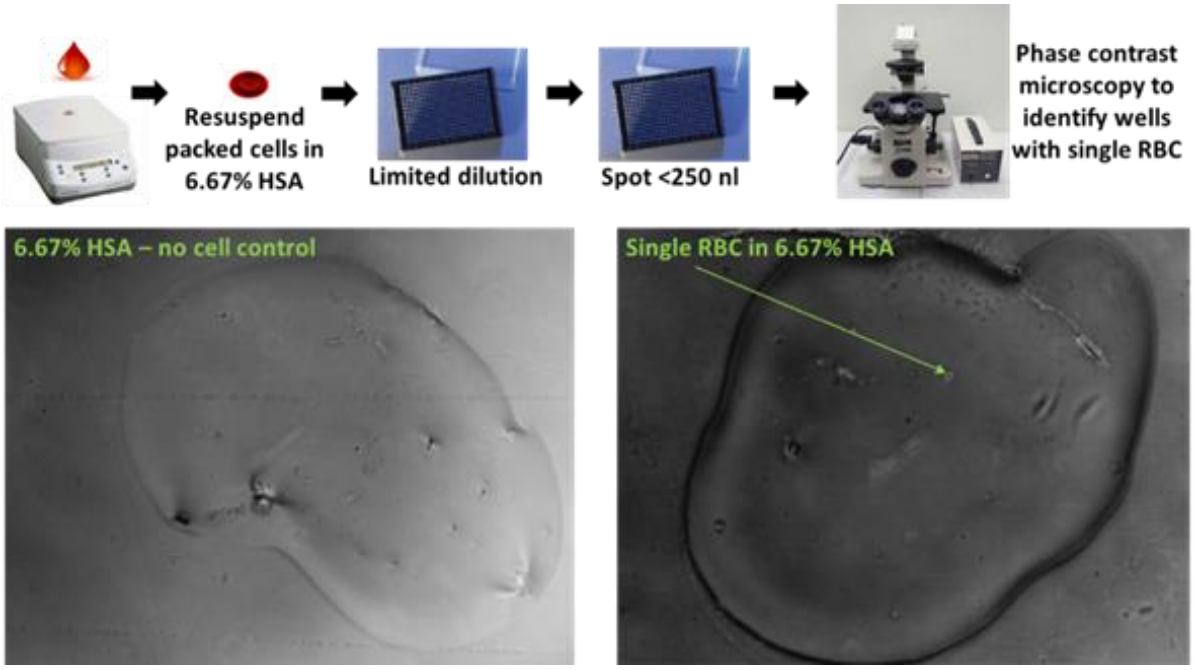


Figure S5. **Single RBC isolation via limiting dilution into OP384**. Capillary blood was collected via TAP® and packed cells separated via low-speed centrifugation. To retain RBC integrity during spotting while retaining compatible optical clarity for downstream microscopy, cells were resuspended in a solution of 6.67% human serum albumin (HSA). Limiting dilution (in HSA) was used to identify a dilution factor that could be used to isolate single RBCs when spotting 80 nl in OP384. The HSA droplets can be distinguished by their irregular shape and elevated edges which appear to cast a shadow along the outer edge/perimeter of the droplet. Single RBCs within the droplet were identified using phase contrast microscopy and a single RBC is shown by the green arrow in the lower right image.

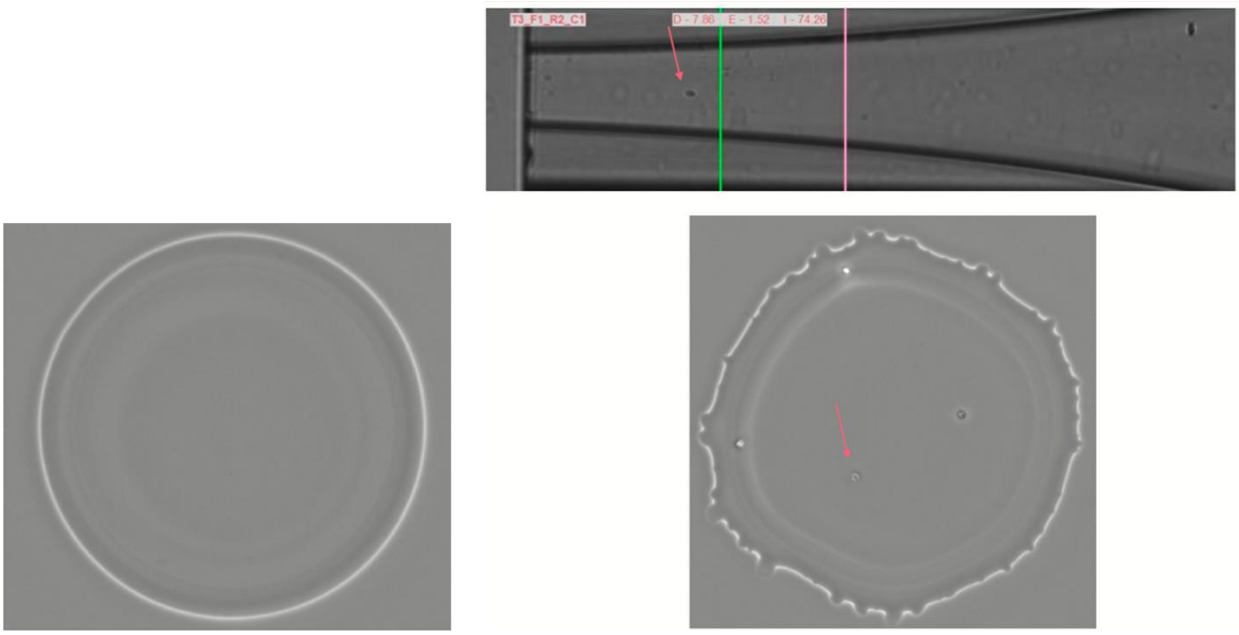


Figure S6. CellenONE automated single RBC isolation. RBCs were diluted in 2.22% HSA and the CellenONE instrument was used to deposit RBCs onto a microscopy slide in a final volume of 700 pl. The left lower image shows a 2.22% HSA droplet deposited without RBC. The upper image on the right shows an RBC in the CellenONE instrument under active flow prior to being dispensed on the microscopy slide. The lower right image shows an HSA droplet with a single RBC. Both the HSA control no RBC droplet and the RBC containing droplet were viewed at 40X magnification using brightfield microscopy.

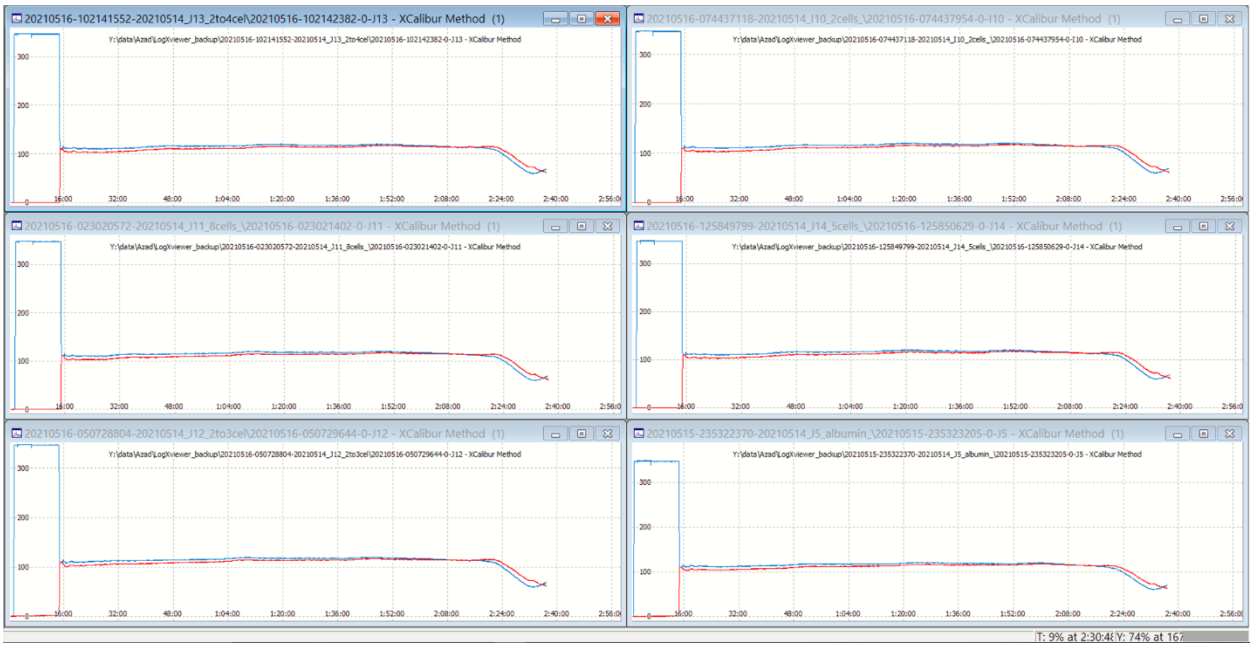


Figure S7. Nano-LC pressure profiles for samples prepared via one-pot rapid digestion. The nano-LC log files generated when acquiring raw data collected during DIA analysis (**Figure 1** in the manuscript) were viewed (in LogViewer 2.18) to assess whether pressure profiles remained consistent over the course of the mobile phase gradient and across injections. The Y-axis unit is in bar and the X-axis unit is time in h:min. The blue line is the pressure profile of pump A (mobile phase A), and the red line is the pressure profile of pump B (mobile phase B). The pressure profiled remained consistently below 125 bar for the full length of the run across all six injections. The ~350 bar pressure observed for pump A between minutes 0-16 reflected the pressure controlled sample loading onto the trap column.

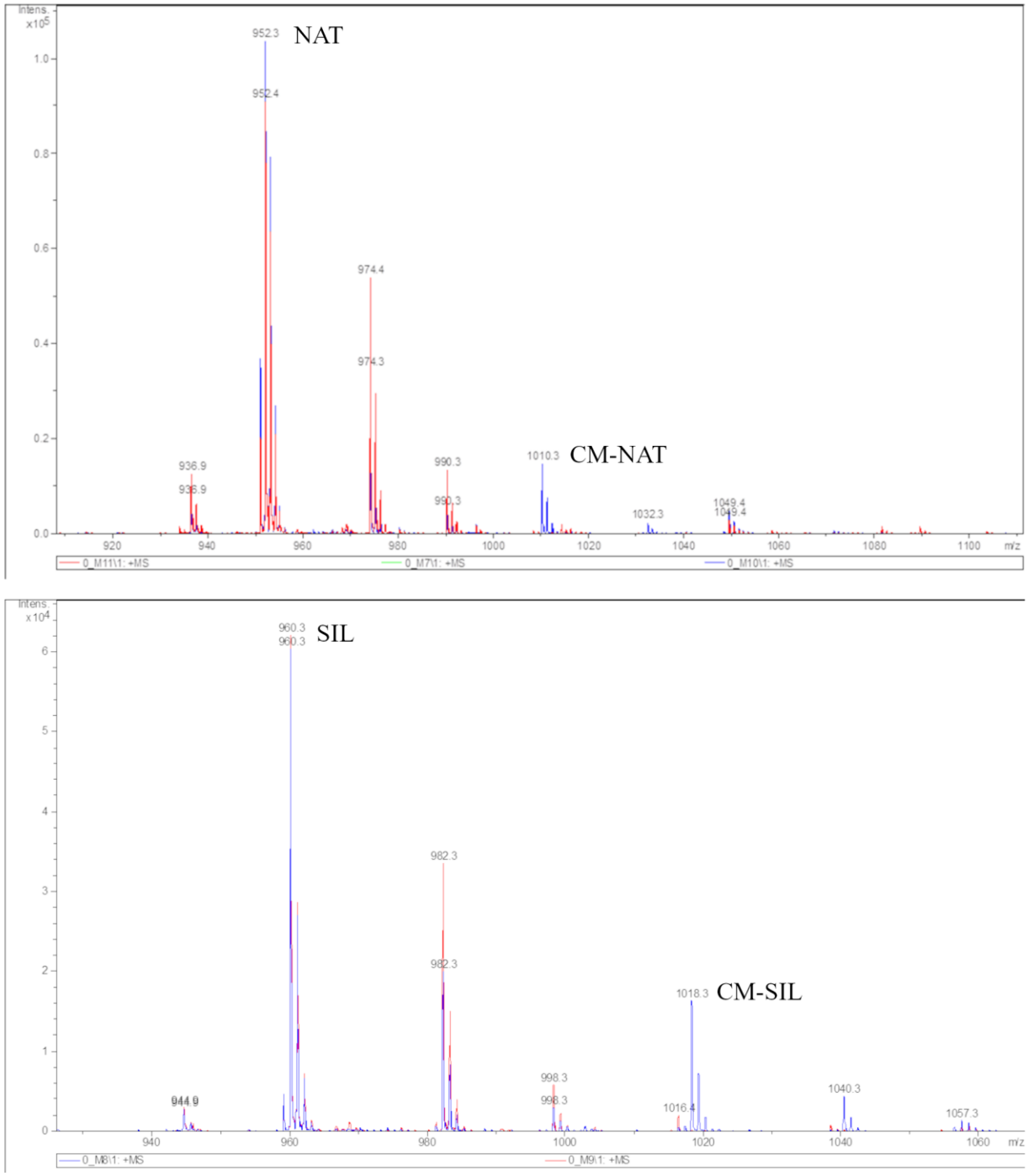


Figure S8. MALDI-TOF mass spectra of unpurified natural (NAT) and heavy isotope labeled (SIL) peptide standards subjected to carboxymethylation. The mass spectra show a 58+ Dalton shift in addition to the parent m/z indicative of carboxymethylation. The expected

NAT and SIL m/z of 952.3 and 960.3 were observed, respectively, along with their Na+ adducts (974.3 and 982.3 m/z) and K+ adducts (990.3 and 998.3 m/z). The expected m/z for the carboxymethyl peptides were 1010.3 for CM-NAT and 1018.3 for CM-SIL were observed as well including Na+ and K+ adducts of. The relative yield of the N-terminal carboxymethylation was estimated as 17% for the CM-NAT and 22.5% for CM-SIL peptide standards.

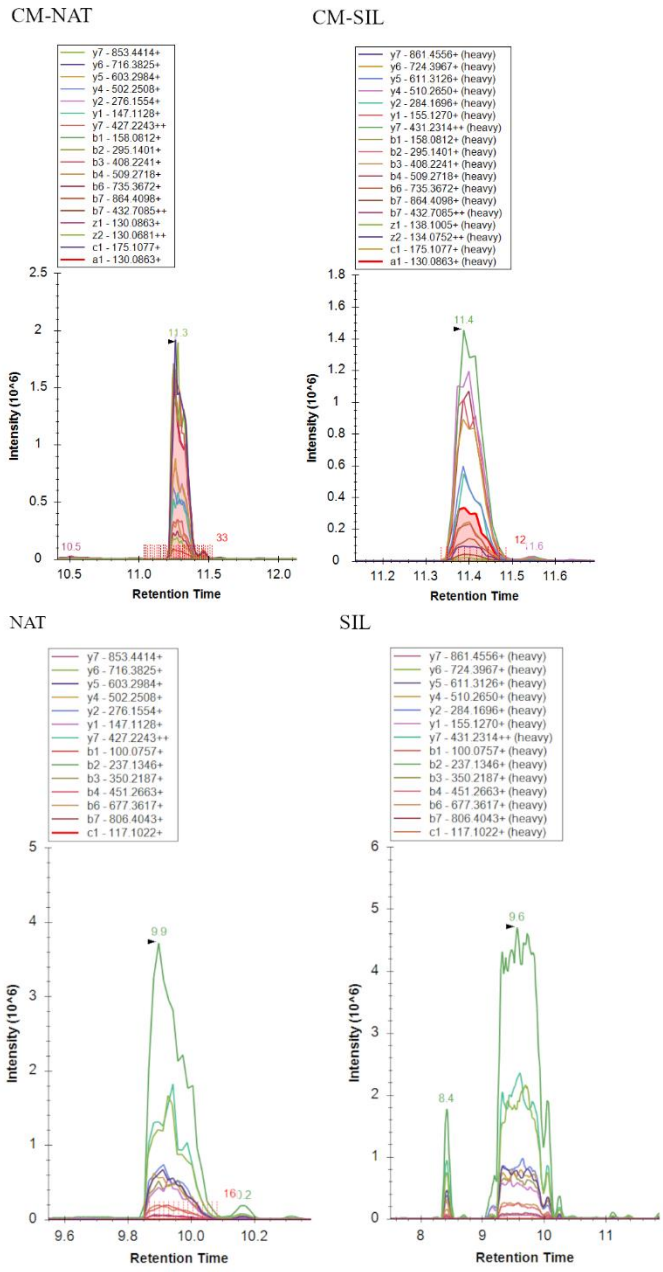


Figure S9. Dynamic multiple reaction monitoring to confirm selective N-terminal carboxymethylation of CM-NAT and CM-SIL peptide standards. The a1, c1 and b ions displayed a 58+ Da shift while y ions displayed identical m/z when comparing product ions

between native and carboxymethylated peptide standards, further evidence carboxymethylation was on the N-terminal valine only.

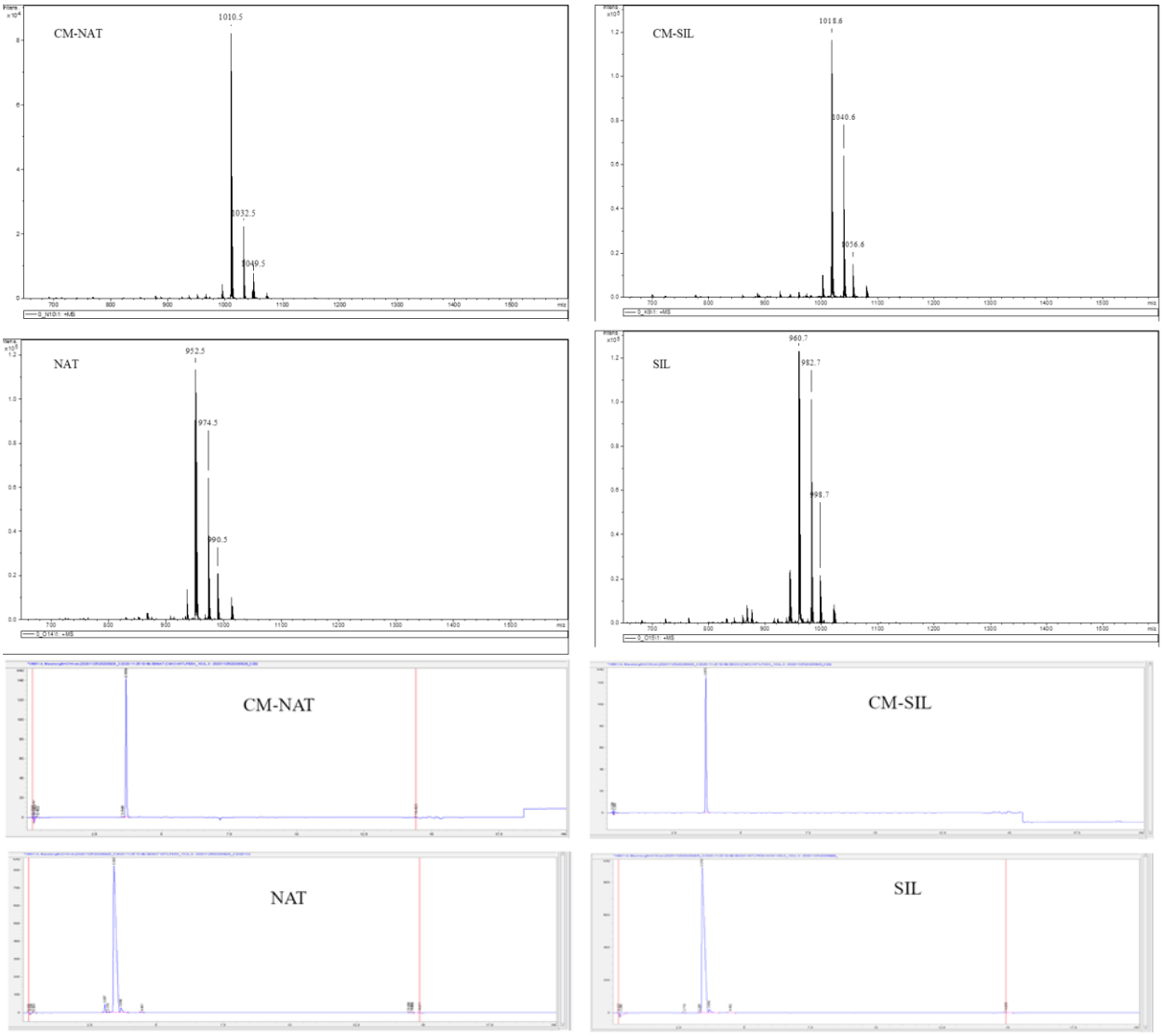


Figure S10. Purity analysis of peptide standards after preparative HPLC via MALDI-TOF and UV-HPLC. The top four panels display MALDI-TOF spectra. The spectra show the parent monoisotopic masses of 1010.5, 1018.6, 952.5 and 960.7 for CM-NAT, CM-SIL, NAT and SIL peptide standards, respectively. The Na+ and K+ adducts were also present at 22+ and 39+ Da mass shifts, respectively. The lower four panels display UV-HPLC analysis for the four peptide standards indicative of 95-99% purity.

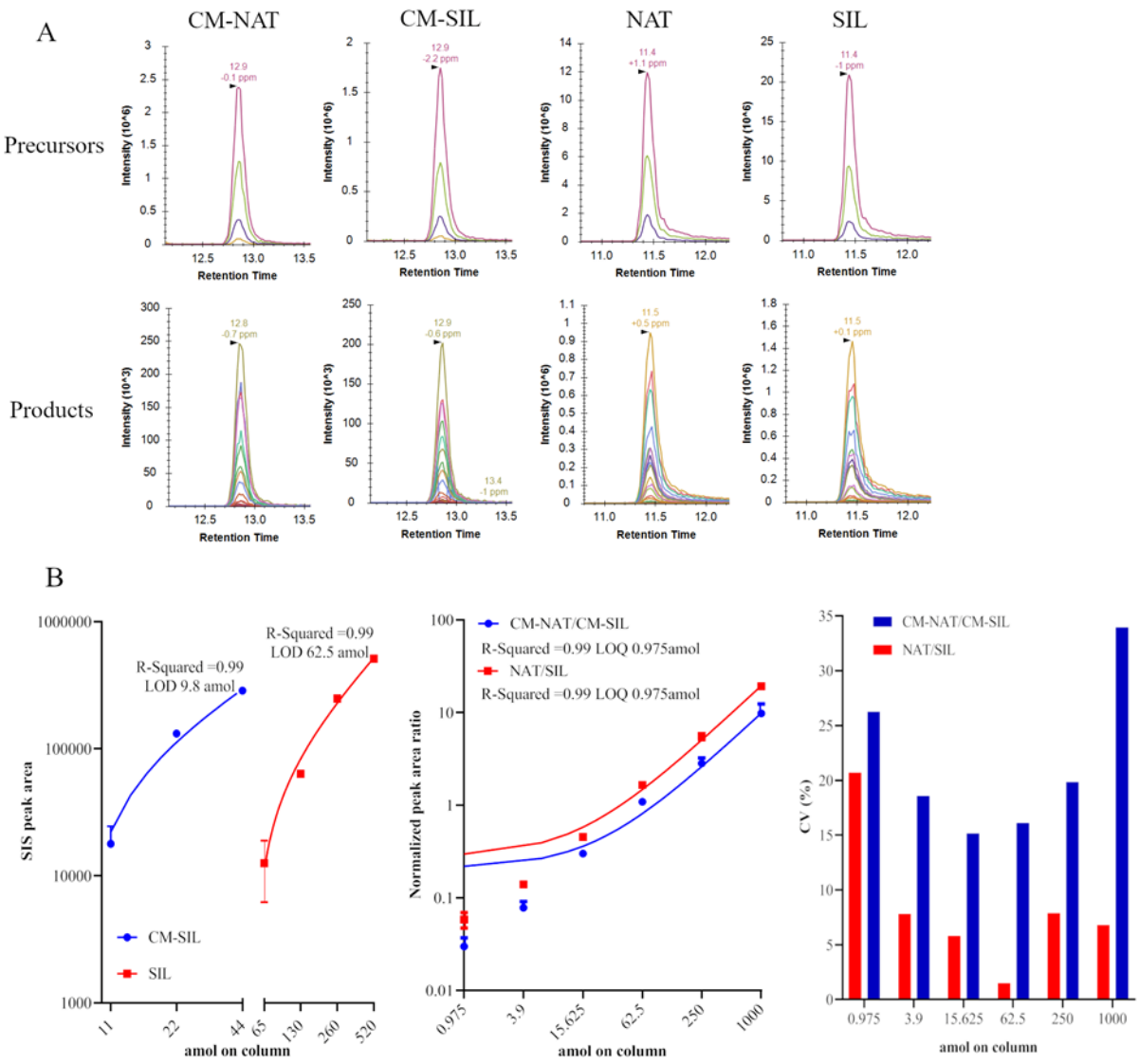


Figure S11. PRM assay chromatograms and LOD and LOQ measurements. PRM chromatograms of peptide standards are depicted in panel A where the top chromatograms show isotopic envelops and the lower chromatograms show product ions generated from higher-energy C-trap dissociation (HCD). Panel B, left most graph displays LOD measurements using the aforementioned instrument setup using isotope dilution in a background matrix composed of a proteolytic digest of RBCs diluted to a protein concentration equivalent to one RBC per microliter (~32 pg/uL). These initial LODs were measured without normalization. The X-axis

shows the amount of (amol) peptide injected on column and the Y-axis shows the peak area of the resulting chromatograms. The R-squared and measured LODs are shown. The middle graph in panel B displays LOQs measured in another laboratory (site 2). For these experiments isotope dilution was performed using CM-SIL and SIL peptides as normalizers and varying the concentrations of CM-NAT and NAT peptides spiked in a background of mobile phase A. The X-axis displays the amount of peptide injected on column and the Y-axis displays the normalized peak area ratio. The R-squared values and LOQs are provided in the graph. The right-most graph in panel B displays the intra-assay CV measured over three replicate injections at each concentration. The X-axis display the amount of peptide injected on column and the Y-axis displays the calculated CV.

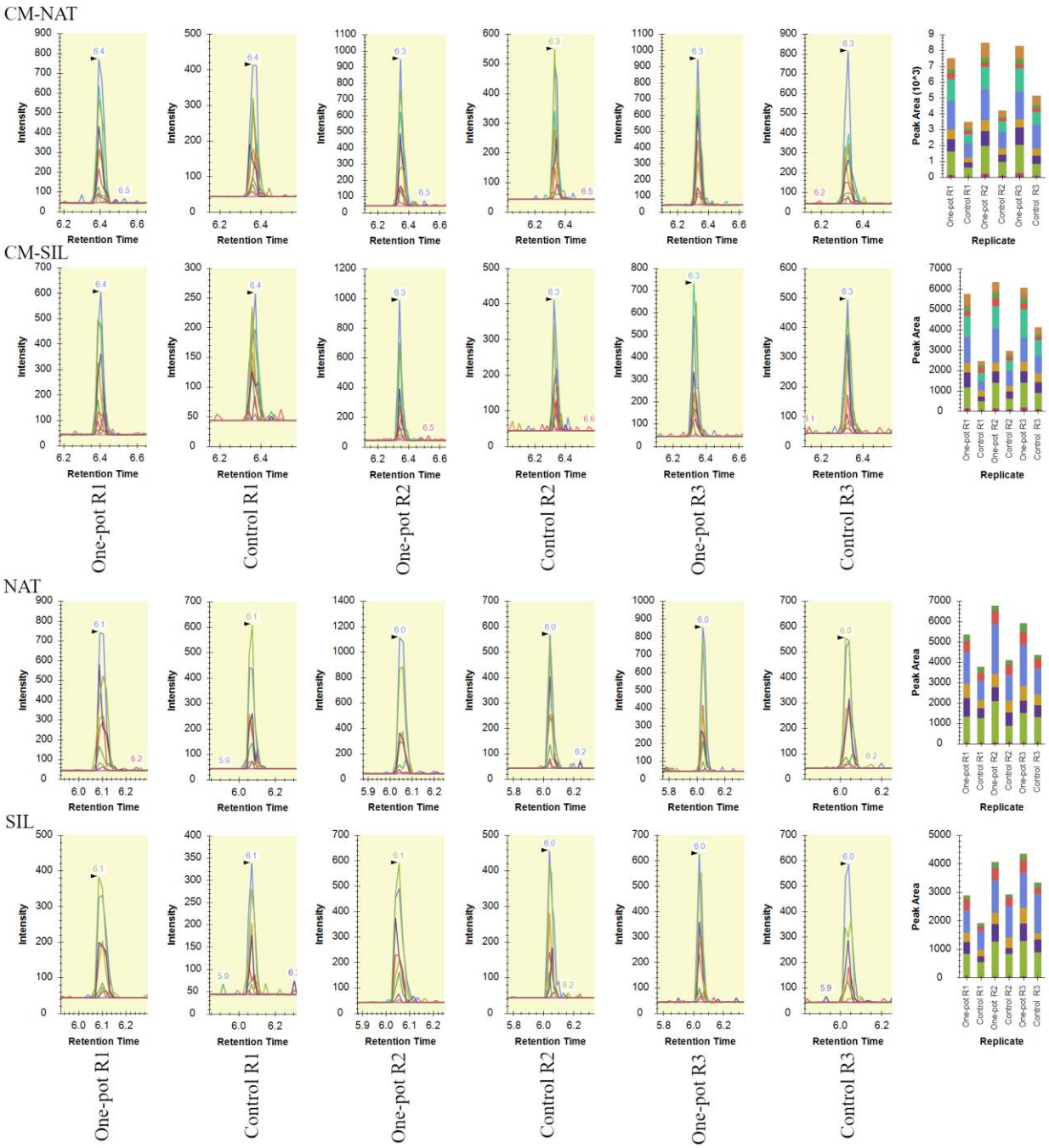


Figure S12. MRM chromatograms and peak areas of peptide standards subjected to the one-pot rapid digestion workflow. Each of the four rows is representative of a single peptide and is accordingly labeled sequentially from top to bottom; CM-NAT, CM-SIL, NAT and SIL. Each column is representative of a single treatment and accordingly labeled from left to right;

One-pot R1, Control R1, One-pot R2, Control R2, One-pot R3 and Control R3. MRM chromatograms consist of the monitored product ions and cumulative peak areas for each transition and for each treatment is presented as bar graphs to the right of the MRM chromatograms. MRM chromatograms from one-pot rapid digest treated samples displayed higher peak areas than controls and qualitative assessment showed improved peak shape.

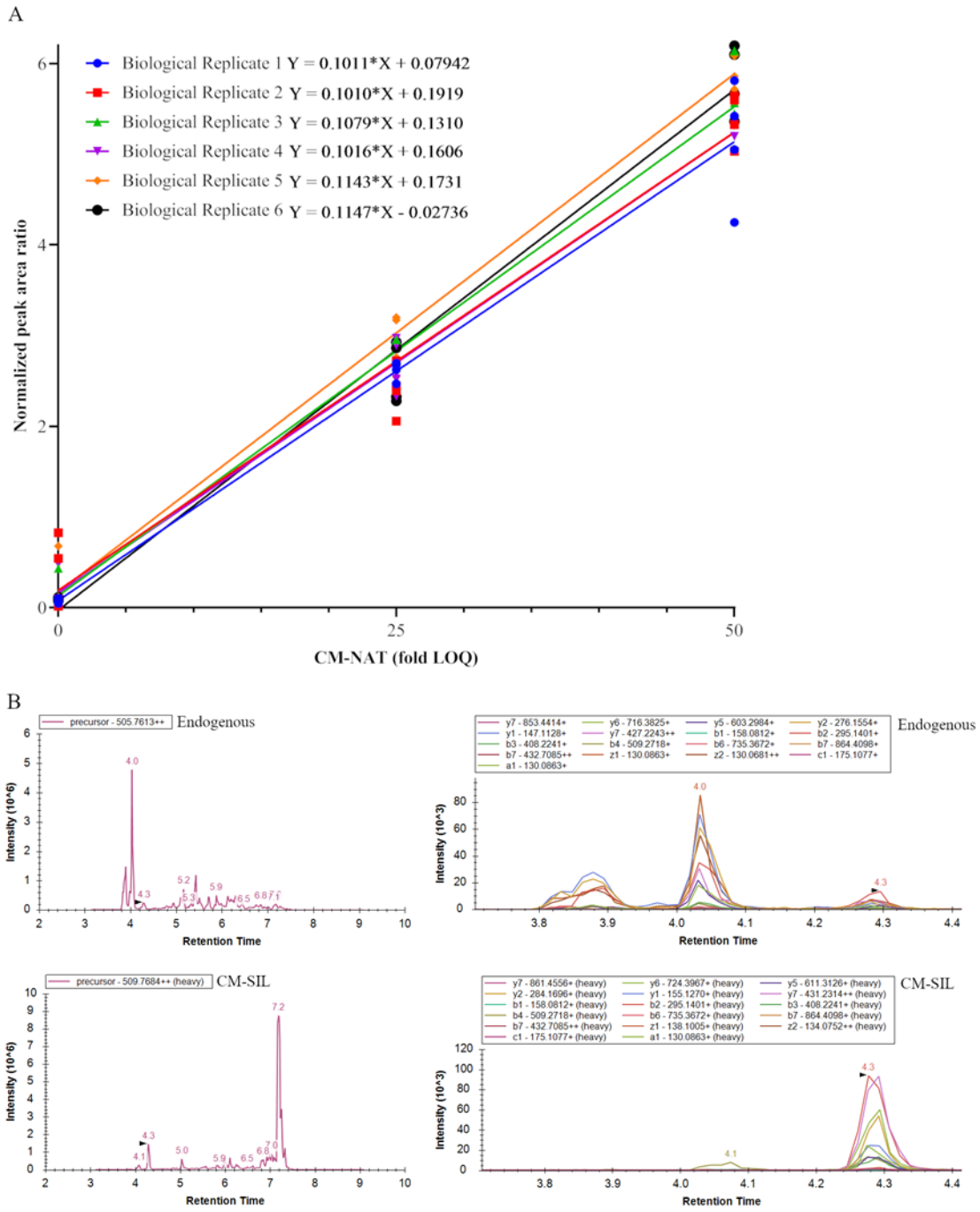


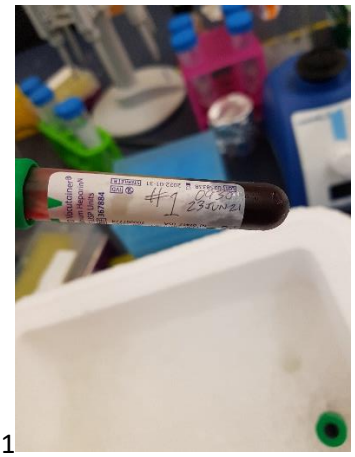
Figure S13. Assessment of selective measurement of endogenous CM-VHLTPEEK. Panel A displays response curves (generated from MRM chromatograms) across six biological replicates (N=6 blood samples from 6 different individuals) where endogenous CM-VHLTPEEK was

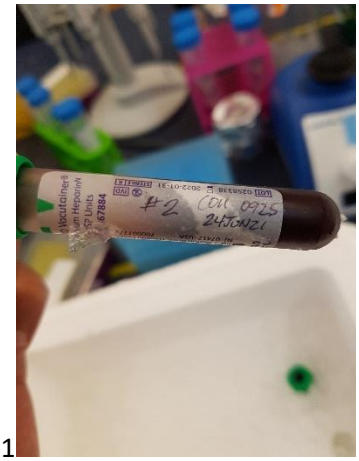
measured without CM-NAT spiked in and subsequently measured with CM-NAT spiked in at 25 fold the concentration of the LOQ and again at 50 fold the concentration of the LOQ. For all measurements, CM-SIL was spiked in at a fixed concentration and used for normalization. Linear regression was performed for each curve and the resulting equation of the line is displayed for each curve. Individual slopes were within 10% of the average (across all 6 slopes). Panel B displays a representative MRM chromatogram (an injection from specimen 6) displaying the precursor ion chromatogram on the left and product ions on the right. Top and lower chromatograms represent endogenous and CM-SIL signal, respectively, and are labeled accordingly. The relatively low resolution of the triple quad instruments reveals signal in addition to that of the endogenous target for both the precursor and product ion transitions. Of note was significantly higher peak intensity and peak areas at 4 min retention time. This unknown endogenous signal shared the majority of a, b, c and y ions with the endogenous CM-VHLTPEEK target but was not the peptide of interest.

Supplemental Document 1

June 24 2021

1. The following whole blood samples (N=2), collected via venipuncture, were picked up from the Victoria Lipid Clinic and transported back to the University of Victoria Genome BC Proteomics Centre (PC) on ice:

-#1 coll 0930 23Jun21 

-#2 coll 0925 24Jun21 

2. From each sample, 100 µl was aliquoted into 1.5 ml microcentrifuge tubes and centrifuged at 300x*g* for 5 min. The supernatant was pipetted out and discarded.

3. To the cell pellet 100 µl of 6.67% HSA was added and all samples were stored at 4 ˚C until spotting in 384 well microplates.

June 25, 2021

Sample spotting in 384 wells, aiming for 5-25 erythrocytes per well. Two-fold dilution series performed in Greiner low volume 384 well plate. The dilution which provided ~5-25 erythrocytes per 0.08 µl spot was used to spot 10 replicates in Corning COC film low volume 384 well plate.

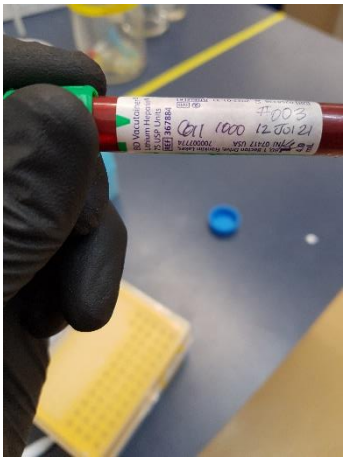
Dilutions were performed by first adding 10 µl HSA to column 1 rows A to K. To A1 was added 10 µl of blood which was already diluted in HSA as described above. A Two-fold serial dilution was performed through to row K.

The dilution well that would give the ideal number of cells, 5-25, was empirically determined by spotting 0.08 µl and subsequently used for spotting 10 replicates for each sample.

In practice the spotting was performed as follows. 0.25 µl was sampled from the well and the tip was kept in contact with the well bottom for 10 sec. Sample exited into well by capillary action. The same tip was used for the subsequent two wells. Therefore, each 0.25 µl sample was used to spot 3 wells without depressing the pipett. Sample exited via capillary action. It is estimated the droplet volume would be less than 0.08 µl per well, since liquid can be visualized in the pipett tip after spotting of 3 wells.

July 13 2021

1. On July 12 2021, the following whole blood samples (N=2), collected via venipuncture, were picked up from the Victoria Lipid Clinic and transported back to the PC on ice:



-#003 coll 1000 12Jul21

-#004 coll 1050 12Jul21

2. Samples were stored in the 4 ˚C fridge until the next day. July 13 2021.

3. From each sample 100 µl was aliquoted into 1.5 ml microcentrifuge tubes and centrifuged at 300x*g* for 5 min. The supernatant was pipetted out and discarded.

4. To the cell pellet 100 µl of 6.67% HSA was added.

Sample spotting in 384 wells, aiming for 5-25 erythrocytes per well. Two-fold dilution series performed in Greiner low volume 384 well plate. The dilution which provided ~5-25 erythrocytes per 0.08 µl spot was used to spot 10 replicates in Corning COC film low volume 384 well plate.

Dilutions were performed by first adding 10 µl HSA to column 1 rows A to K. To A1 was added 10 µl of blood which was already diluted in HSA as described above. A Two-fold serial dilution was performed through to row K.

The dilution well that would give the ideal number of cells, 5-25, was empirically determined by spotting 0.08 µl and subsequently used for spotting 10 replicates for each sample.

In practice the spotting was performed as follows. 0.25 µl was sampled from the well and the tip was kept in contact with the well bottom for 10 sec. Sample exited into well by capillary action. The same tip was used for the subsequent two wells. Therefore, each 0.25 µl sample was used to spot 3 wells without depressing the pipett. Sample exited via capillary action. It is estimated the droplet volume would be less than 0.08 µl per well, since liquid can be visualized in the pipett tip after spotting of 3 wells.

July 20 2021

1. On July 20 2021, the following whole blood sample (N=1), collected via venipuncture at 9:20 am, was picked up from the Victoria Lipid Clinic and transported back to the PC on ice:

-#005

2. On the same day (July 20) 100 µl was aliquoted into a 1.5 ml microcentrifuge tube and centrifuged at 300x*g* for 5 min. The supernatant was pipetted out and discarded.

3. To the cell pellet 100 µl of 6.67% HSA was added.

Sample spotting in 384 wells, aiming for 5-25 erythrocytes per well. Two-fold dilution series performed in Greiner low volume 384 well plate. The dilution which provided ~5-25 erythrocytes per 0.08 µl spot was used to spot 10 replicates in Corning COC film low volume 384 well plate.

Dilutions were performed by first adding 10 µl HSA to column 1 rows A to K. To A1 was added 10 µl of blood which was already diluted in HSA as described above. A two-fold serial dilution was performed through to row K.

The dilution well that would give the ideal number of cells, 5-25, was empirically determined by spotting 0.08 µl and subsequently used for spotting 10 replicates for each sample.

In practice the spotting was performed as follows. 0.25 µl was sampled from the well and the tip was kept in contact with the well bottom for 10 sec. Sample exited into well by capillary action. The same tip was used for the subsequent two wells. Therefore, each 0.25 µl sample was used to spot 3 wells without depressing the pipett. Sample exited via capillary action. It is estimated the droplet volume would be less than 0.08 µl per well, since liquid can be visualized in the pipett tip after spotting of 3 wells..

<u>July 21 2021</u>

1. On July 21 2021, the following whole blood sample (N=1), collected via venipuncture at 12:10 pm, was picked up from the Victoria Lipid Clinic and transported back to the PC on ice:

-#006 coll 1210 21Jul2021

2. On the same day (July 21) 100 µl was aliquoted into a 1.5 ml microcentrifuge tube and centrifuged at 300x*g* for 5 min. The supernatant was pipetted out and discarded.

3. To the cell pellet 100 µl of 6.67% HSA was added.

Sample spotting in 384 wells, aiming for 5-25 erythrocytes per well. Two-fold dilution series performed in Greiner low volume 384 well plate. The dilution which provided ~5-25 erythrocytes per 0.08 µl spot was used to spot 10 replicates in Corning COC film low volume 384 well plate.

Dilutions were performed by first adding 10 µl HSA to column 1 rows A to K. To A1 was added 10 µl of blood which was already diluted in HSA as described above. A two-fold serial dilution was performed through to row K.

The dilution well that would give the ideal number of cells, 5-25, was empirically determined by spotting 0.08 µl and subsequently used for spotting 10 replicates for each sample.

In practice the spotting was performed as follows. 0.25 µl was sampled from the well and the tip was kept in contact with the well bottom for 10 sec. Sample exited into well by capillary action. The same tip was used for the subsequent two wells. Therefore, each 0.25 µl sample was used to spot 3 wells without depressing the pipett. Sample exited via capillary action. It is estimated the droplet volume would be less than 0.08 µl per well, since liquid can be visualized in the pipett tip after spotting of 3 wells..

August 12 2021

1. On August 11 2021, the following whole blood sample (N=1), collected via venipuncture at 0930am, was picked up from the Victoria Lipid Clinic on August 12 2021 and transported back to the PC on ice:

-#7 coll 0930 11Aug21

2. On the same day (Aug 12) 100 µl was aliquoted into a 1.5 ml microcentrifuge tube and centrifuged at 300x*g* for 5 min. The supernatant was pipetted out and discarded.

3. To the cell pellet 100 µl of 6.67% HSA was added.

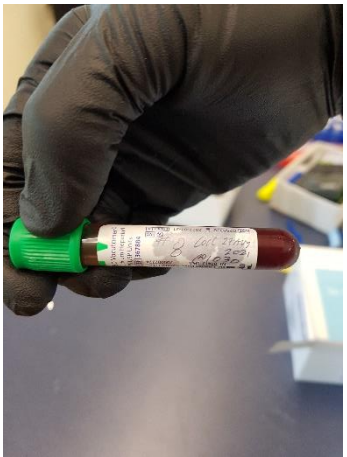
Sample spotting in 384 wells, aiming for 5-25 erythrocytes per well. Two-fold dilution series performed in Greiner low volume 384 well plate. The dilution which provided ~5-25 erythrocytes per 0.08 µl spot was used to spot 10 replicates in Corning COC film low volume 384 well plate.

Dilutions were performed by first adding 10 µl HSA to column 1 rows A to K. To A1 was added 10 µl of blood which was already diluted in HSA as described above. A two-fold serial dilution was performed through to row K.

The dilution well that would give the ideal number of cells, 5-25, was empirically determined by spotting 0.08 µl and subsequently used for spotting 10 replicates for each sample.

In practice the spotting was performed as follows. 0.25 µl was sampled from the well and the tip was kept in contact with the well bottom for 10 sec. Sample exited into well by capillary action. The same tip was used for the subsequent two wells. Therefore, each 0.25 µl sample was used to spot 3 wells without depressing the pipett. Sample exited via capillary action. It is estimated the droplet volume would be less than 0.08 µl per well, since liquid can be visualized in the pipett tip after spotting of 3 wells.

August 27 2021



1. On August 27 2021, the following whole blood sample (N=1), collected via venipuncture at 1030am, was picked up from the Victoria Lipid Clinic on August 27 2021 at 1pm and transported back to the PC on ice:

-#8 cpll 27Aug2021 @1030

2. On the same day (Aug 27) 100 µl was aliquoted into a 1.5 ml microcentrifuge tube and centrifuged at 300x*g* for 5 min. The supernatant was pipetted out and discarded.

3. To the cell pellet 100 µl of 6.67% HSA was added.

Sample spotting in 384 wells, aiming for 5-25 erythrocytes per well. Two-fold dilution series performed in Greiner low volume 384 well plate. The dilution which provided ~5-25 erythrocytes per 0.08 µl spot was used to spot 10 replicates in Corning COC film low volume 384 well plate.

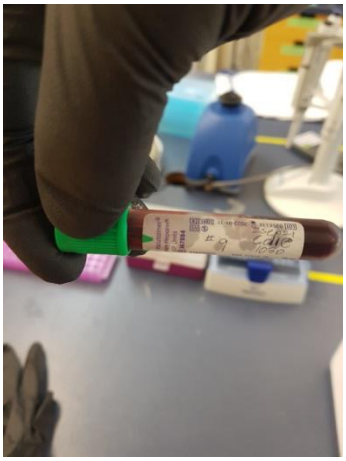
Dilutions were performed by first adding 10 µl HSA to column 1 rows A to K. To A1 was added 10 µl of blood which was already diluted in HSA as described above. A two-fold serial dilution was performed through to row K.

The dilution well that would give the ideal number of cells, 5-25, was empirically determined by spotting 0.08 µl and subsequently used for spotting 10 replicates for each sample.

In practice the spotting was performed as follows. 0.25 µl was sampled from the well and the tip was kept in contact with the well bottom for 10 sec. Sample exited into well by capillary action. The same tip was used for the subsequent two wells. Therefore, each 0.25 µl sample was used to spot 3 wells without depressing the pipett. Sample exited via capillary action. It is estimated the droplet volume would be less than 0.08 µl per well, since liquid can be visualized in the pipett tip after spotting of 3 wells.

September 2 2021

1. On September 2 2021, the following whole blood samples (N=2), collected via venipuncture at 1030am and 1130am, were picked up from the Victoria Lipid Clinic on September 22021 at 230pm and transported back to the PC on ice:



-#9 2Sep21 cdie 1030

-#10 coll 2Sep21 @1130

2. Both tubes were placed into a 50 ml conical tube and stored in the refrigerator overnight.

3. On September 3 2021 100 µl was aliquoted into a 1.5 ml microcentrifuge tube and centrifuged at 300x*g* for 5 min. The supernatant was pipetted out and discarded.

4. To the cell pellet 100 µl of 6.67% HSA was added.

Sample spotting in 384 wells, aiming for 5-25 erythrocytes per well. Two-fold dilution series performed in Greiner low volume 384 well plate. The dilution which provided ~5-25 erythrocytes per 0.08 µl spot was used to spot 10 replicates in Corning COC film low volume 384 well plate.

Dilutions were performed by first adding 10 µl HSA to column 1 rows A to K. To A1 was added 10 µl of blood which was already diluted in HSA as described above. A two-fold serial dilution was performed through to row K.

The dilution well that would give the ideal number of cells, 5-25, was empirically determined by spotting 0.08 µl and subsequently used for spotting 10 replicates for each sample.

In practice the spotting was performed as follows. 0.25 µl was sampled from the well and the tip was kept in contact with the well bottom for 10 sec. Sample exited into well by capillary action. The same tip was used for the subsequent two wells. Therefore, each 0.25 µl sample was used to spot 3 wells without depressing the pipett. Sample exited via capillary action. It is estimated the droplet volume would be less than 0.08 µl per well, since liquid can be visualized in the pipett tip after spotting of 3 wells.