Biomarkers Consortium Project

Use of Targeted Mass Spectrometry Proteomic Strategies to Identify CSF-Based Biomarkers in Alzheimer's Disease

Data Primer

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How to reference this data set in presentations and publications:

For the purposes of presentations or publications, when referencing this data set, please use the following format: "**Biomarkers Consortium CSF Proteomics MRM data set**". The required ADNI reference should also be used.

1. Background:

The data and analysis plan described within this document represents the work of the Biomarkers Consortium Project "Use of Targeted Mass Spectrometry Proteomic Strategies to Identify CSF-Based Biomarkers in Alzheimer's Disease". This project was submitted to the Biomarkers Consortium Neuroscience Steering Committee by a subgroup of the Industry Private Partner Scientific Board (PPSB) of the Alzheimer's Disease Neuroimaging Initiative (ADNI) for execution, and was managed by a Biomarkers Consortium Project Team that includes members from academia, government and the pharmaceutical industry (See Appendix III). Funding for this project was provided by the Alzheimer's Drug Discovery Foundation, Eisai, Genentech (a member of the Roche Group), Janssen, Lilly, Merck, Pfizer, and Takeda. This project is the second part of a multi-phased effort seeking to utilize samples collected by ADNI to qualify multiple peptides in cerebrospinal fluid (CSF) to diagnose patients with Alzheimer's disease (AD) and monitor disease progression. These efforts were reviewed by the ADNI Resource Allocation Review Committee (RARC) and approved by the National Institute on Aging (NIA). An earlier phase of the program focused on using a multiplexed immuno-based assay (performed by Rules Based Medicine) to characterize potential Alzheimer's disease (AD) biomarkers in CSF.

The aim of the project was to determine the ability of a panel of peptides measured with mass spectrometry to discriminate among disease states. Additional biomarkers with diagnostic and prognostic value are needed for AD drug development, especially in the context of clinical trials aiming to treat patients before the onset of dementia. In addition, biomarkers that can be used to monitor treatment effects in both early and established AD clinical trials could enable more efficient trial designs and facilitate understanding of therapeutic mechanism of action. A full description of the LC/MS-MRM approach to biomarker quantitation is beyond the scope of this Primer but an overview with key references in provided in Section 2.

The CSF multiplex MRM panel was developed by Caprion Proteomics in collaboration with the Biomarker Consortium Project Team. Proteins and peptides were selected based upon their previous detection in CSF, relevance to AD, and previous results from the Rules Based Medicine (RBM) multiplex immunoassay analysis of ADNI CSF. As described in more detail below, CSF samples were depleted of abundant plasma proteins, digested, and analyzed by LC-MS/MS operating in MRM mode at Caprion Proteomics. In a pilot study, 25 ADNI CSF samples were used to evaluate the Caprion Proteomics Platform and to select peptides for use in the full sample set. In this pilot study, for each protein that had not previously been detected at Caprion ~ 5 peptides were tested and 2 were selected for subsequent use. For each peptide, 2 mass transitions were monitored. In the pilot study, 198/510 peptides were detectable in ADNI CSF. Because the Caprion platform was capable of monitoring ~500 peptides, for the final MRM panel, we supplemented the detectable peptides from the pilot study with a large number of additional peptides, not all of which were known to be detectable in CSF. These

additional peptides included a series of peptides representing inflammatory markers and peptides representing particular proteins of interest identified in the RBM study. The final MRM panel consisted of 567 peptides representing 221 proteins, and for each peptide two mass transitions were monitored. As described below, a number of steps were performed in order to QC and combine (or "roll-up") transitions into a peptide quantitation and peptides into a protein quantitation. These results are reported in arbitrary signal intensity units on a natural log scale. Because alternate splicing or post-translational processing could result in biologically significant differences in the levels of two peptides from the same protein, we recommend use of the "log peptide intensity" for further analysis. The raw data and all the intermediate steps leading up to the final dataset are included in the attached Excel document "**CSFMRM Consolidated Data.xlsx**" and spreadsheets from this document are referred to throughout the Primer.

Synthetic peptides were synthesized for all peptides monitored in this study. These peptides were used for method development. As described in Section 10, a pool of these peptides (at 200 pmol/mL each in buffer) was analyzed before and after the CSF samples. Absolute quantitation by an external standard at a single concentration and in a different matrix can only be considered an approximation. The current dataset includes the raw data results for the external standards and an estimate of concentrations in the ADNI CSF samples based on the raw data at the transition level. However no attempt was made to map the reported rolled-up peptide and protein intensities to an absolute quantitation. Future studies may be conducted using stable-isotope-labeled versions of a subset of peptides to allow more accurate quantitation through the use of internal standards.

306 ADNI-1 baseline CSF samples were sent to Caprion, including 16 blinded technical replicates. Thus 290 unique ADNI-1 baseline subjects were represented: 87 healthy control subjects, 66 AD subjects, 136 MCI subjects, and 1 subject with unknown diagnosis at baseline. Due to limited aliquot supply for some subjects, this collection is a subset of the 301 unique subjects analyzed in the earlier Rules Based Medicine CSF project. The 16 blinded technical replicates were distributed throughout the MS analysis runs and used to assess assay reproducibility. As described in more detail in Section 11, there was very high concordance between these "test/re-test" replicates, indicating overall robustness in the detection and data processing pipeline.

The final results indicate that 320 out of 567 peptides in the final MRM panel were detectable in > 10% of ADNI samples and are included in the final results file "**CSFMRM.csv**".

Caprion initially defined and performed the QC and processing steps described below. The project team replicated the Caprion normalization and quantitation algorithms and collaborated with Caprion to adjust the normalization procedure. The final versions of the rolled-up peptide and protein quantitation and the approach for QC based on the Test/Re-test results were generated by the Project Team. All work described in this primer was performed before the sample ID's were un-blinded to reveal the corresponding ADNI subject IDs. The Project Team

also pre-defined a Statistical Analysis Plan for initial analysis of these biomarkers for diagnostic and prognostic utility (**Appendix II**). The Project Team will conduct this analysis once they are un-blinded, which occurs simultaneously with the posting of the un-blinded data to the LONI website. The Project Team intends to report results of the Statistical Analysis Plan through conferences and/or publications.

2. Description of Technology:

MRM allows the specific and sensitive quantification of peptides and proteins in biological samples. It is the most sensitive mass spectrometry-based platform (Lange et al., 2008; Mol. Syst. Biol. 4, 222) and was demonstrated to be highly reproducible within and across laboratories and instrument platforms (Addona et al., Nat Biotechnol. 633-41 (2009); Kennedy et al., Nat Methods (2013) Adv Online doi:10.1038/nmeth.2763). MRM experiments are performed on triple quadrupole (Q) mass spectrometers. The first (Q1) and third (Q3) mass analyzer are used to isolate a peptide ion and a corresponding fragment ion. The fragment ions are generated in Q2 by collision induced dissociation (CID). The signal of the fragment ion is then monitored over the chromatographic elution time and used for quantification. Up to 750 peptides covering a dynamic range of 5 orders of magnitude can be quantified precisely and accurately in a single 30 minute run. In addition, absolute quantification using the AQUA strategy is possible by spiking known amounts of stable labeled peptides into the study samples (Kuhn et al., 2004; Proteomics 4:1175–1186).

3. Sample Processing and MRM Analysis Overview

The flow charts, presented in **Figure 1** and **Figure 2**, provide an overview of the main steps of the sample analysis. Each of these steps is described below, including notes on predefined QC acceptance criteria and the subsequent QC results.

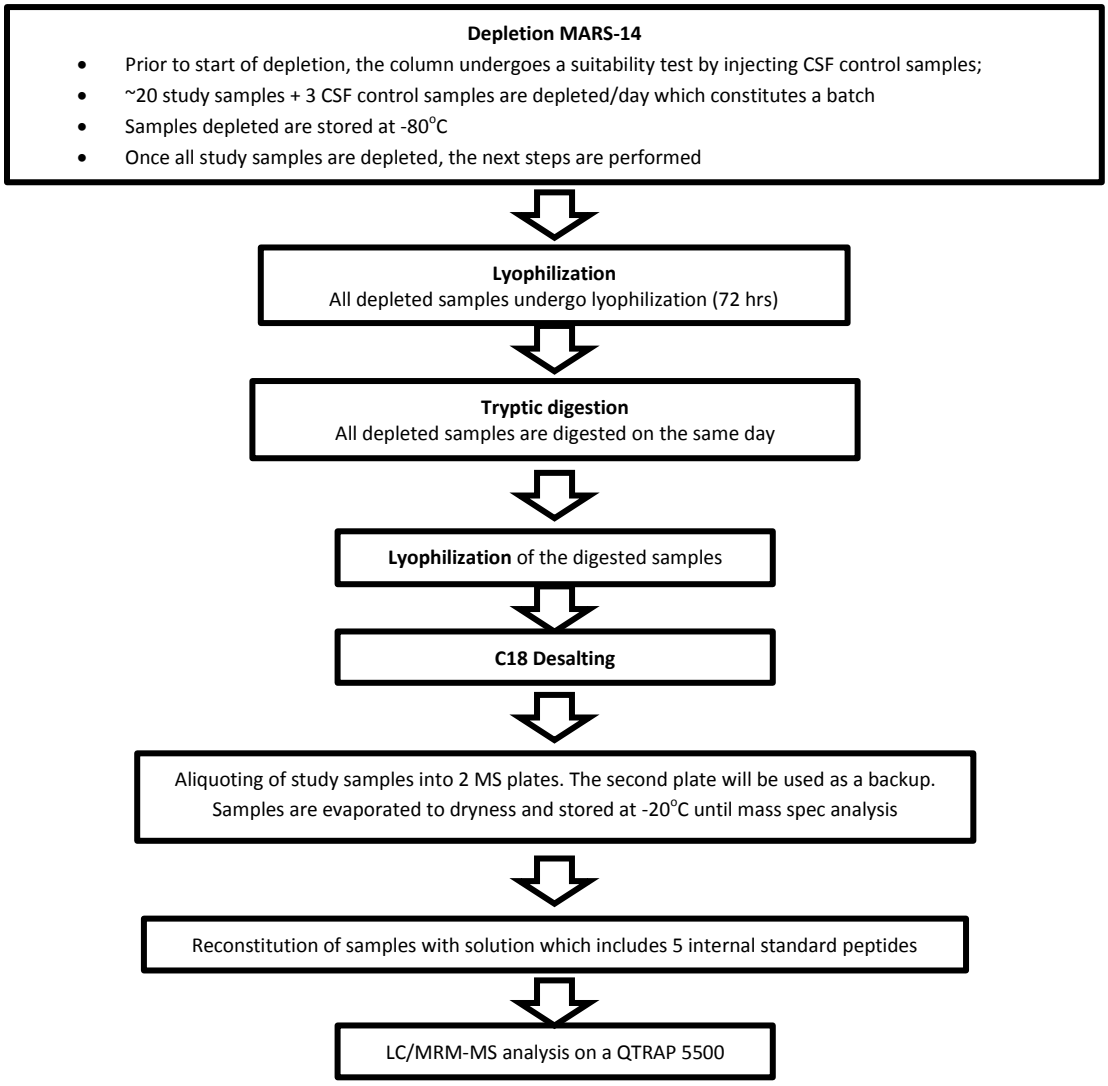

Figure 1. Sample processing and LC-MRM-MS analysis

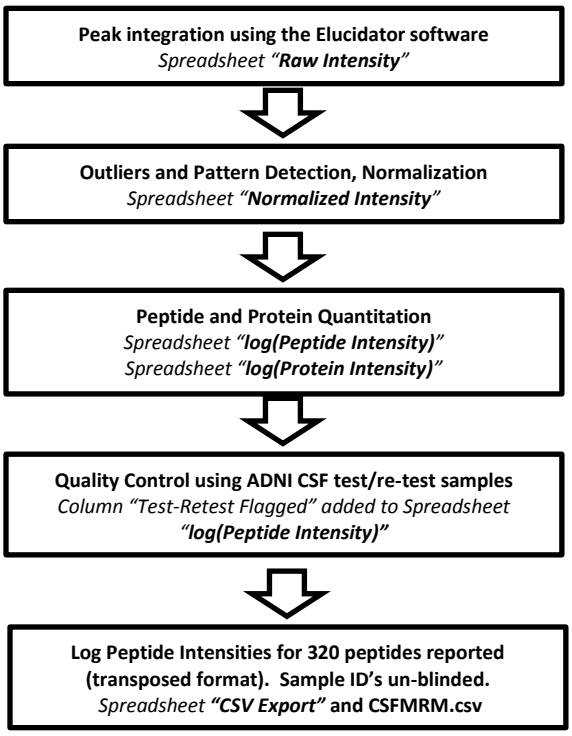

Figure 2. Data processing and QC.

4. CSF Sample Processing:

Three-hundred and six (306) CSF samples were included in this study. CSF sample aliquots of 0.5 mL were shipped to Caprion frozen and stored at -80°C until use.

After thawing, 100 µL of each sample was depleted of high abundance proteins using a MARS-14 immunoaffinity resin (4.6 x 100 mm column, Agilent). The 14 proteins depleted by the column were: Albumin, Haptoglobin, Transferrin, IgG, IgA, a1-Antitrypsin, a2-Marcoglobulin, a1-Acid glycoprotein, Apolipoprotein AI, Apolipoprotein AII, Complement C3, IgM, Transthyretin and Fibrinogen. The depletion gradient used is shown in **Table 1**. Samples were run in batches of 12, 20, or 21 over 15 days, using two separate MARS-14 columns. Details of run order and column usage are included in the spreadsheet **"Sample Info".**

Table 1. LC gradient for sample depletion

TIME (min.)	% 150 mM Ammonium bicarbonate	% Neutralization Buffer (Agilent Buffer A	% Stripping Buffer (Agilent Buffer B)	Flow Rate (mL/min)
0	100	0	0	0.125
18	100	0	0	0.125
18.1	100	0	0	1
20	100	0	0	1
20.1	0	0	100	1
27	0	0	100	1
27.1	0	100	0	1
35	0	100	0	1
35.1	100	0	0	1
43	100	0	0	1

Three (3) in-run QC samples (human gold standard CSF, HGS-CSF, (Bioreclamation)) were included per depletion day (beginning, middle and end). These QC samples were processed at the same time and the same manner as the study samples and were used to assess the reproducibility of the sample processing and mass spectrometry analysis. The HGS-CSF samples are named CSF001- CSF045.

> ***HPLC QC:*** *The coefficient of variation (CV) of the mean area under the curve (AUC) for total protein in the flow-through (FT) fraction of the in-run QCs was calculated per depletion day. The CV of the mean FT AUC should be below 15% for the in-run QC samples depleted on the sample depletion day, below 20% for all QC samples*

depleted on the same depletion column and below 25% across all QC samples. In this study, the CV of the mean FT AUC value was ≤ 2.2 % per depletion day, ≤ 4.7 % per depletion column and 4.9% across all QC CSF samples.

The depleted samples, containing the remaining lower abundance proteins, were stored at -80°C. After all samples were depleted, the frozen samples were lyophilized over 72 hrs. The lyophilized samples were digested overnight with trypsin at an approximate 1:25 protease-to-protein ratio, based on the protein amount determined by BCA for a HGS-CSF sample. The digested samples were lyophilized and desalted using a 3M Empore C18 96-well plate. Two sets of replicate mass spectrometry (MS) plates were prepared for each sample. The plates were dried by vacuum evaporation and stored at -20°C prior to MS analysis.

5. LC/MRM-MS Methods

The CSF multiplex MRM panel was developed by Caprion Proteome Inc. in collaboration with the Biomarkers Consortium and consists of 567 peptides representing 221 proteins. Two transitions per peptide were monitored. The full list of transitions, peptide sequences, and corresponding proteins are listed in the spreadsheet **"Transitions"**. The 640 detectable transitions are listed first, followed by the transitions monitored for the internal standards, followed by the remaining 494 transitions that were not detectable and not carried forward in subsequent analysis.

Sample ZGJ0297 was excluded because peaks shifted outside the MRM detection window. Sample ZGJ0038 was excluded because it aligned poorly with the other samples during the Elucidator peak alignment. This was likely caused by the relative high amount of Hemoglobin present in these 2 samples (samples were pink).

As described below, pre-specified QC performance criteria were set for control samples and peptides. All QC criteria for instrument performance were met.

LC/MRM-MS QC: *Before analyzing the study samples, a system suitability test of the LC/MRM-MS system was performed. The reconstitution solution, which includes 5 internal standard peptides (ISP) at 100 ng/mL was injected in replicates of 5. All of the following criteria for minimum peak intensity must be met:*

Peptide Sequence	*Transition*	*Minimum Peak Intensity*	*Measured Peak Intensity*
FSDISAAK (ISP-1)	*419.7_691.2*	*2.50E+05*	*5.20E+0.5*
ASSILAT (ISP-2)	*662.3_359.2*	*1.00E+05*	*3.00E+05*
NVDQSLLELHK (ISP-3)	*432.6_639.4*	*8.00E+04*	*2.20E+05*
QNNGAFDETLFR (ISP-4)	*706.3_927.4*	*8.00E+04*	*2.20E+05*
ELWFSDDPDVTK (ISP-5)	*726.3_559.3*	*5.00E+04*	*2.50E+05*

- *The CV of the median peak area of the 5 ISP must be below 7.5%. The CV in this study was 2.5%.*
- *The CV of the median retention time of the 5 ISP must be below 0.25%. The CV in the current study was 0.07%.*
- *A synthetic peptide mix containing all synthesized crude peptides (JPT Peptide Technologies), each at a concentration 200 pmol/mL, was injected in replicates of 5. It was verified that the targeted peptides eluted within 30 sec to their predicted retention times and that the median peak area CV over all measured transitions was below 10%. In this study, the CV was 4.8 %.*

Sample analysis was initiated after a successful system suitability test. The processed samples were re-solubilized with 11 µL of a reconstitution solution containing 5 ISP each at 100 ng/mL. These 5 ISP elute at different retention times to cover the gradient run time. In addition, one of the HGS-CSF samples from the backup plate was re-solubilized with 10 µL of the reconstitution solution plus 1 µL of the synthetic peptide mix at 200 pmol/mL. This sample is used later for the retention time alignment in Elucidator. Eight (8) µL of material was injected per sample onto a NanoAcquity UPLC (Waters) coupled to a 5500 QTRAP mass spectrometer (AB Sciex). Peptide separation was achieved using a 320 µm x 150 mm, 5 µm particle size, Thermo Biobasic C18 column. The LC gradient used is shown in **Table 2** below. The flow rate was 10 µL/min.

Table 2. LC gradient of the MRM assay

TIME (min.)	%Water +0.2% FA	%ACN +0.2% FA
Initial	92.5	7.5
0.2	92.5	7.5
23.00	75	25
23.60	40	60
24.60	40	60
24.61	92.5	7.5
30.00	92.5	7.5

QC for internal standard peptide performance in ADNI samples: *The 5 ISP were used to monitor the instrument's performance during sample analysis. The median CV of the 5 ISP should be below 20% across all samples. In the current study, the median CV was 14.8%.*

6. Peak Integration

The raw mass spectrometer data files (WIFF) were converted to mzXML format and loaded into the Elucidator software (Rosetta Biosciences) for chromatogram alignment, noise filtering, data smoothing, peak detection and quantitation. The peak alignment was then manually reviewed. If more than 20% of the peaks of a sample were not well aligned with the others, the sample was excluded. Only one sample (ZGJ0038) was excluded because of misalignment. A report was then created to flag peptides based on the following criteria:

1) Detection threshold
A transition was included if it was observed in at least 10% of the total samples analyzed (HGS-CSF and study samples) with a peak area of 7,500 or more.

2) Wrong intensity ratio
The expected intensity ratios of the two transitions for each peptide were calculated from 10 injections of synthetic peptide standards spiked in buffer (ratio of peak areas summed across 10 samples). The observed transition intensity ratio was then calculated across all CSF samples having Peak Areas >10,000 on both transitions. For each peptide, the distribution of the observed ratios was considered and the 25th, 50th and 75th percentiles of the distribution were extracted. If the expected ratio was not contained within the 25th and 75th percentiles and was not within 1.5 fold of the median ratio, then the peptide was flagged (across all samples) because of suspected matrix interference. Interference may be manually confirmed upon visual inspection of chromatograms.

3) Low intensity correlation of transition pair
A peptide was flagged if the squared Pearson correlation coefficient of the transition pair was lower than 0.25.

4) Imperfect co-elution
A peptide was flagged if the retention time difference of the centroid of the 2 transitions was greater than 0.05 min (3 seconds).

5) Departure from expected RT
A peptide was flagged if the retention time of the pair of transitions was significantly offset (more than 10 seconds) compared to its expected retention time.

Peptides with 1 or more flags were manually reviewed and were either kept or discarded, depending on the overall peak shape, the quality of the alignment and the presence of a neighboring interference.

Once the final set of transitions was validated, the peak area data was then exported to a tab-delimited file and reported in the spreadsheet "**Raw Intensity**".

The number and percentage of detected transitions, peptides and proteins is reported in **Table 3**. The median coefficient of variation of the synthetic peptide mix in buffer was 4.9% in the pre-run samples, 5.9% in the post-run samples and 12.1% overall. **Table 4** lists the median CV of the ISP in the study samples and the median CV of the ISP and all detected non-ISPs in the HGS-CSF for each study day, each column, and for the entire analysis.

Table 3. Detection Summary

		Detected	
	Monitored	**#**	**%**
Proteins	221	142	64.3%
Peptides	567	320	56.4%
Transitions	1,134	640	56.4%

A transition was considered detected if it was observed in at least 10% of the total samples analyzed (HGS-CSF and study samples) with a peak area of 7,500 or more. A peptide was considered detected if both of its transitions were detected. A protein was considered detected if at least one of its peptides was detected.

Table 4. Median Coefficient of Variation (CV) of Internal Standard Peptides (ISP) and Non-IS peptides, per depletion day, column and overall.

	Study Samples	**HGS-CSF**	
Group	**MEDIAN CV (ISP)**	**MEDIAN CV (ISP)**	**MEDIAN CV (Non-IS)**
Day 1	6.0%	8.3%	15.5%
Day 2	8.0%	4.6%	12.5%
Day 3	6.0%	4.9%	7.9%
Day 4	6.0%	3.8%	9.7%
Day 5	5.4%	4.2%	8.6%
Day 6	6.6%	4.8%	7.5%
Day 7	5.7%	3.8%	7.0%
Day 8	4.6%	3.2%	8.0%
Day 9	6.9%	15.4%	15.0%
Day 10	6.2%	4.3%	9.6%
Day 11	8.8%	6.2%	11.2%
Day 12	11.7%	10.8%	10.1%
Day 13	10.4%	6.0%	8.6%
Day 14	6.3%	7.2%	22.7%
Day 15	5.6%	4.5%	13.1%
Column 1	10.2%	10.3%	17.5%

Column 2	14.5%	15.6%	24.6%
Overall	**15.5%**	**14.8%**	**22.6%**

QC for reproducibility of sample processing and MS analysis using the 45 HGS-CSF samples:

The following criteria should be met:

- *The median peak area CV over all transitions detected should be below 30% for the QC CSF samples depleted on the same day. The actual median CV was always ≤22.7%.*
- *The median peak area CV over all transitions detected should be below 35% across all QC CSF samples for the entire study. The actual median CV was 22.6%.*

68 transitions, representing 39 different peptides, with CV > 35% over the 45 HGS-CSF samples were identified (see column **CV(HGS)** in the spreadsheet "**Normalized Intensity**").

Figure 3 displays individual transition CV in HGS-CSF versus average intensity. The average intensity and CV of individual transitions across all HGS-CSF samples are included in the columns **ln(AVE(HGS))** and **CV(HGS)** in the spreadsheet "**Normalized Intensity**". Higher CV across the HGS-CSF samples is associated with lower signal intensity. Rather than select an arbitrary cut-off for exclusion from the roll-up process, all 640 detected transitions were included in the roll-up to peptide quantitation described in Section 9.

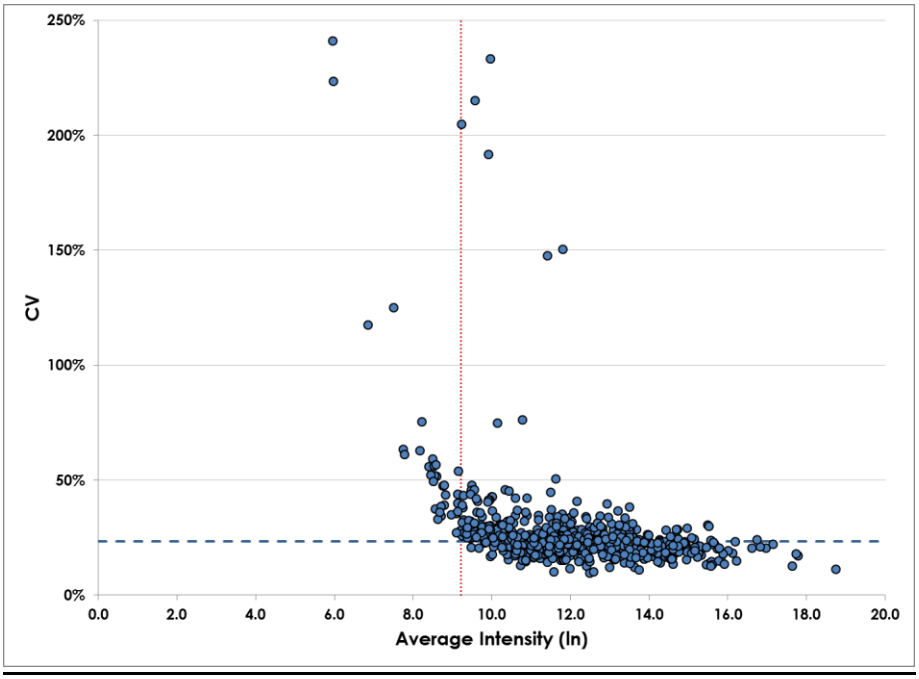

Figure 3. Individual transition CV in HGS-CSF versus average intensity. The dashed blue line represents the median transition CV (23%). The red dotted line is located at the intensity threshold of 10,000.

7. Outlier and Pattern Detection

Data Transformation

All intensity values were transformed on the natural log scale, in order to bring their distribution closer to a Gaussian one. To account for the 0 values, the exact transformation is ln(intensity+1) and is referred to as log-intensity. This transformation was used for all following steps, except when mentioned otherwise.

Outliers and Pattern Detection

The distributions of the number of detected transitions by sample with a peak area greater than 0 (**Figure 4**) or greater than 10,000 (**Figure 5**) were examined. The distribution of the sample average intensity was also evaluated (**Figure 6**). In the case of the averages, the peak areas were first transformed on the log scale ln(peak area + 1). Two samples with average intensity greater than 3 standard deviations from their group mean were flagged as outliers (ZGJ0043 (Batch 13) and ZGJ0005 (Batch 14) but these samples were still included in subsequent peptide and protein quantitation steps.

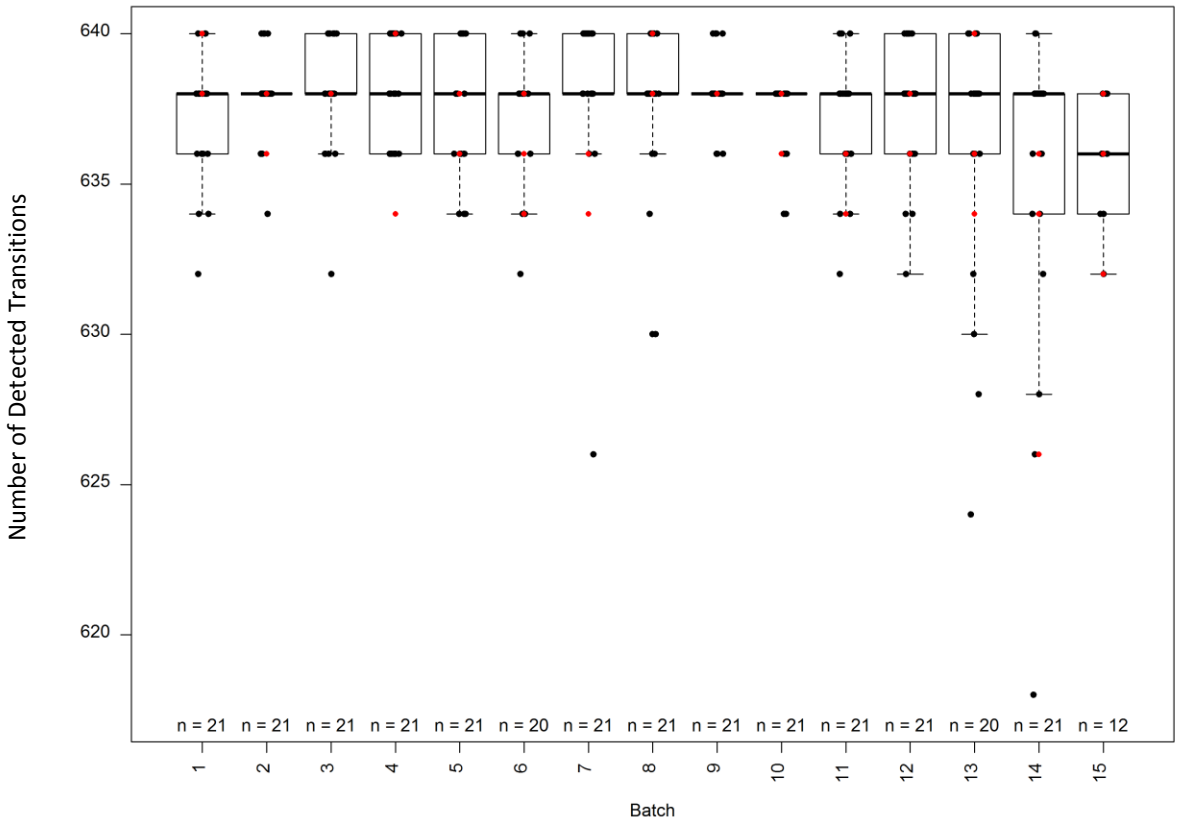

Figure 4. Number of transitions detected by sample with peak area > 0. Samples are grouped by depletion batch. Black: Study samples, Red: HGS-CSF QC samples. Boxes are delimited at the top by the third quartile (Q3) and at the bottom by the first quartile (Q1). The thick black line

within a box represents the median (Q2). Whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range (Q3-Q1) from the box. Several points are superimposed due to samples having the same number of detected transitions.

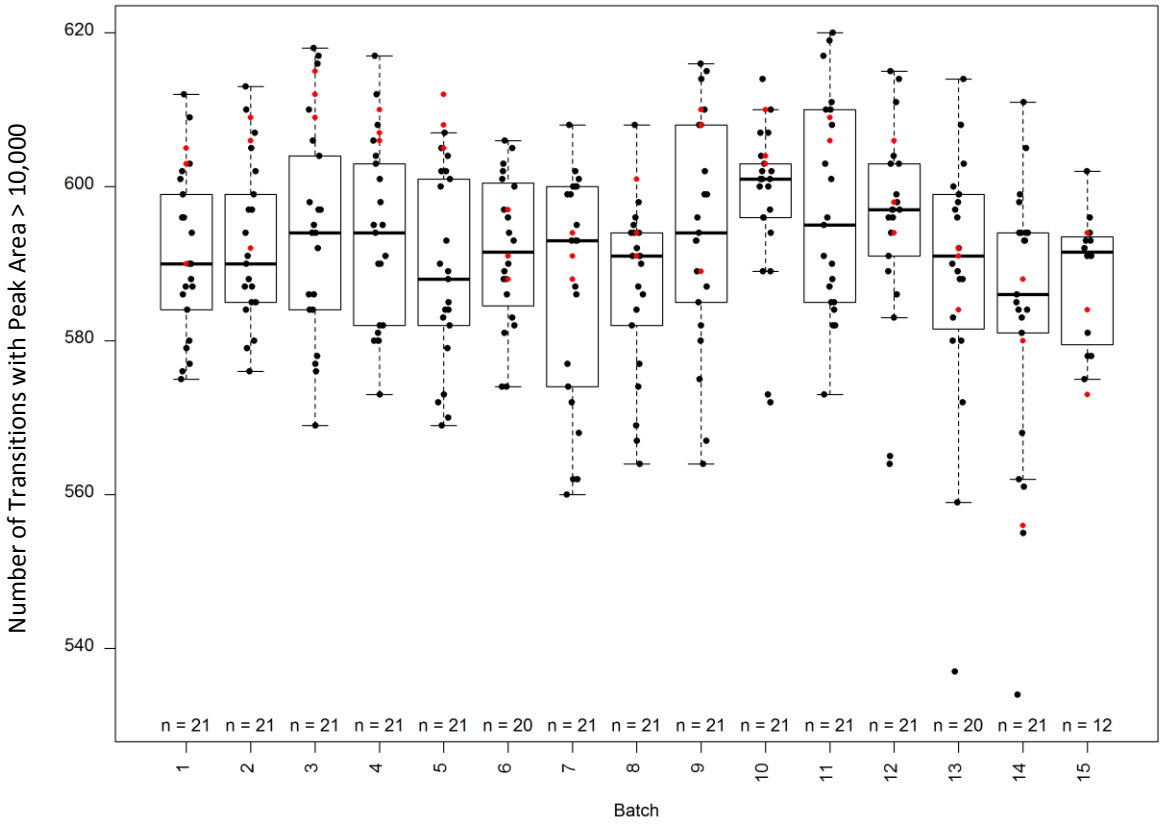

Figure 5. Number of transitions detected by sample with peak area > 10,000. Samples are grouped by depletion batch. Black: Study samples, Red: HGS-CSF QC samples. Boxes are delimited at the top by the third quartile (Q3) and at the bottom by the first quartile (Q1). The thick black line within a box represents the median (Q2). Whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range (Q3-Q1) from the box.

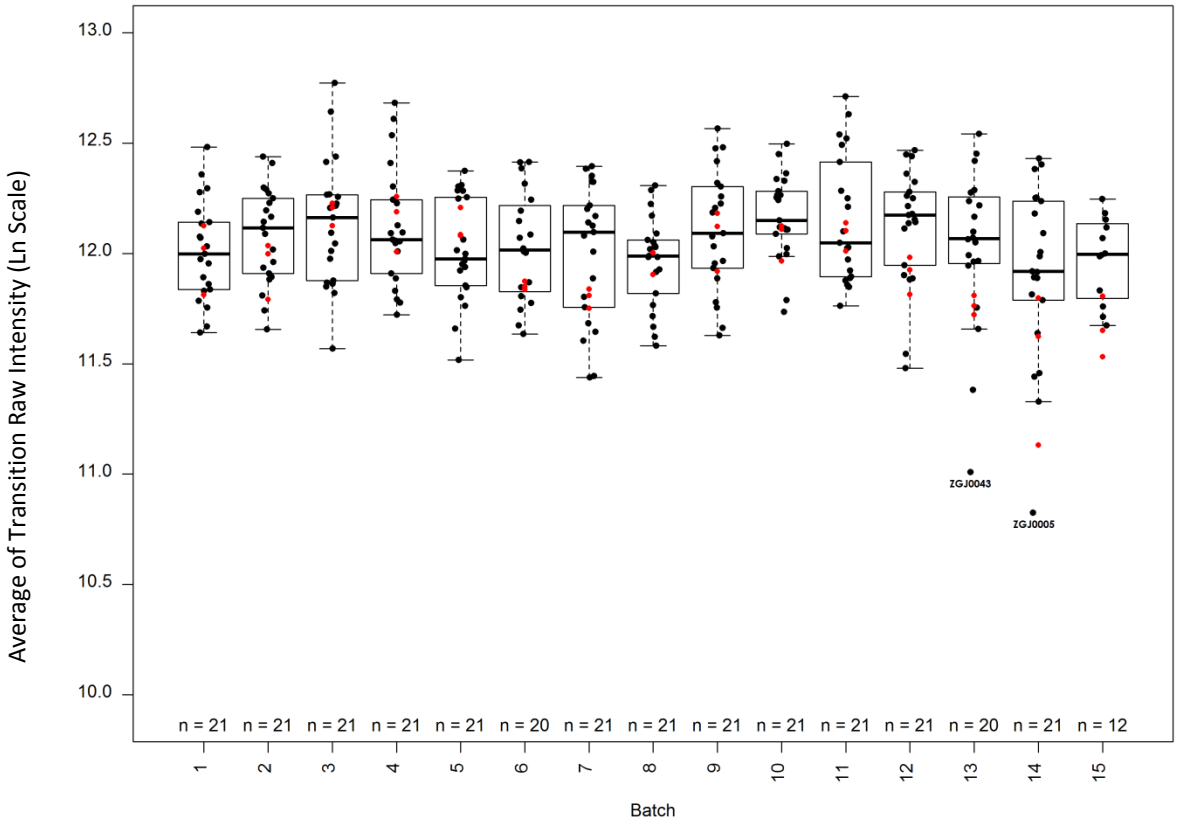

Figure 6. Sample average raw intensity of peak area. Samples are grouped by depletion batch. Black: Study samples, Red: HGS-CSF QC samples. Two samples with average intensity greater than 3 standard deviations from their group mean were flagged as outliers (ZGJ0043 (Batch 13) and ZGJ0005 (Batch 14). Boxes are delimited at the top by the third quartile (Q3) and at the bottom by the first quartile (Q1). The thick black line within a box represents the median (Q2). Whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range (Q3-Q1) from the box.

A standard Principal Component Analysis (PCA) was applied to the peak area data in order to identify patterns unrelated to biological variability. The PCA was performed as follows. If L is the matrix of peak areas with samples as rows and columns as peak areas, then we defined L* as the matrix of peak areas doubly centered, i.e. each cell of the matrix has both its column and row mean subtracted from it, to which we also add back the grand mean (the mean of all peak area values). This was performed to remove principal component dependencies to systematic shifts in intensity levels (i.e. transitions that are over all samples less or more intense than other transitions, or samples that are, over all transitions, more or less intense than other samples). The SVD $L^* = UDV^T$ was then performed, with U containing the components for samples. **Figure 7** displays the sample distribution based on

the first two principal components of the PCA performed on raw intensity data, using all transitions. No samples were flagged as outliers based on this analysis.

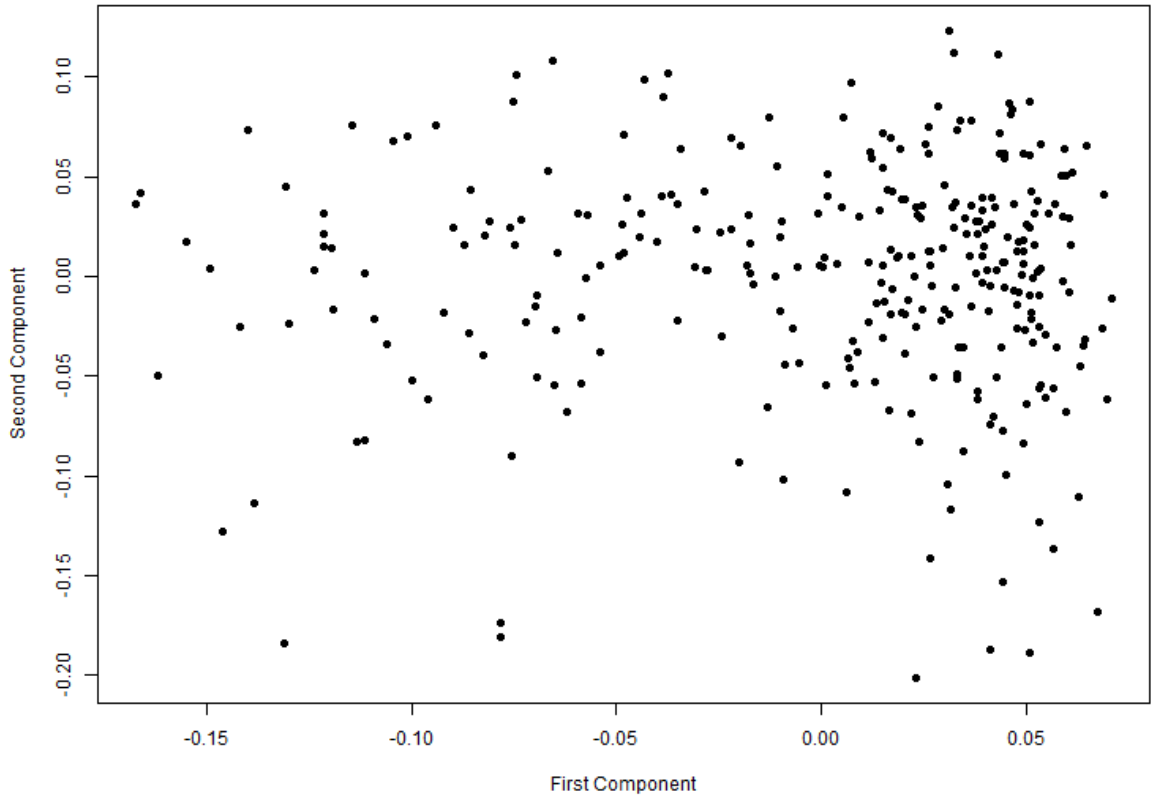

Figure 7. Sample distribution based on the first two principal components of a PCA performed on raw intensity data, using all transitions.

8. Normalization

A bimodal trend was observed when the sample average intensity was plotted against LC/MRM-MS injection order (**Figure 8**). The valley between the two modes corresponds to a change in depletion column.

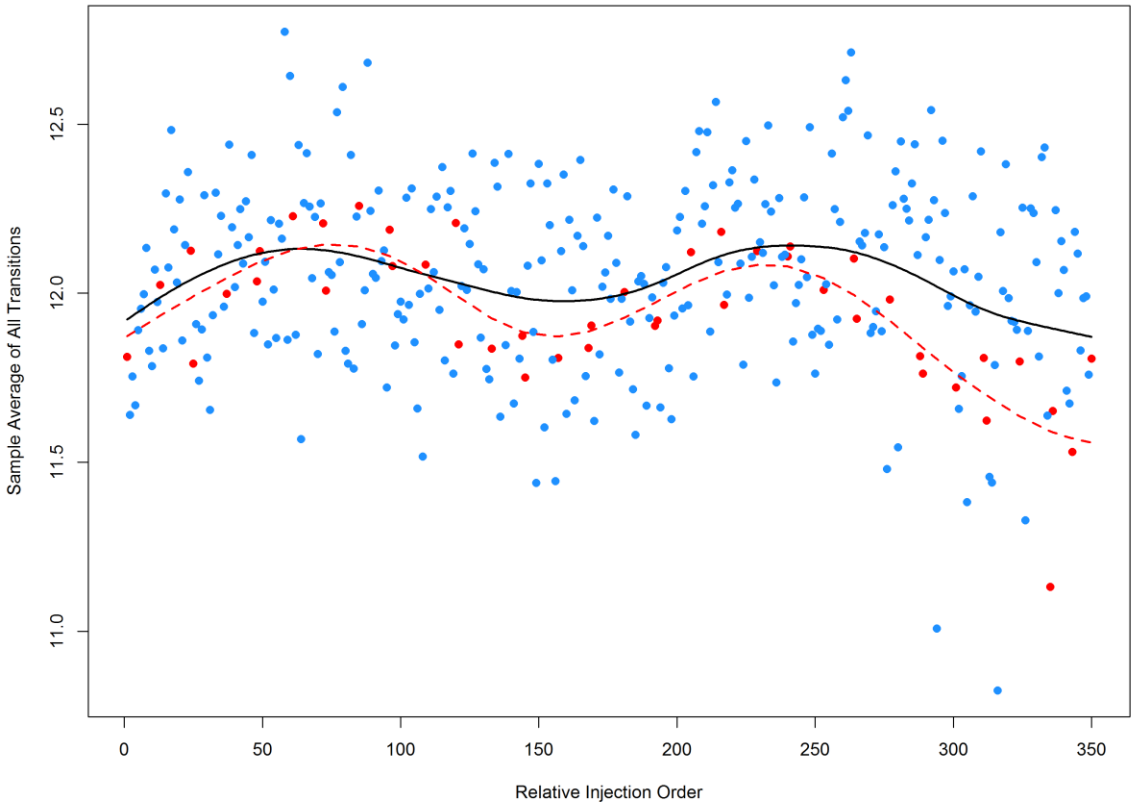

Figure 8. Sample average intensity plotted against LC/MRM-MS injection order. Study samples appear in blue, CSF-HGS in red. The black curve is a smoothing spline on the study samples and the red dashed curve is a similar spline on the CSF-HGS.

Additionally, the depletion method is known to introduce variability between samples processed on different days and this factor needs to be normalized. There are a number of different approaches to data normalization. The uploaded datasets reflect Caprion's normalization methodology as described below.

A two-step normalization procedure was applied to the raw peak area data. In the first stage, a correction was applied based on the trend observed for CSF-HGS samples only, to assure correction for process-related bias.

In order to do so, we first fitted a smoothing spline to the CSF sample averages (**Figure 8**). The smoothing was performed using the R function *smooth.spline* with the CSF sample injection order as the *x* parameter, the CSF sample log-intensity average (across all transitions) as the *y* parameter, and the default options[1] for the other parameters. Using this curve, we compute the predicted average log-intensity using the study samples injection order (note that the predicted average log-intensity for the CSF samples is simply the curve fit). For each sample and for a given transition, we subtracted the predicted value from the sample average log-intensity. This was done independently for each transition. To remain on the same original scale, the pre-normalized transition average was added back to each transition.

The second-step normalization was based on two regression models predicting intensity level:

$M1\colon C_{i1} = \mu_1 + \alpha D_i + \varepsilon_i, \hat{C}_{i1} = \mu_1 + \alpha D_i$

$M2\colon C_{i2} = \mu_2 + \varepsilon_i, \hat{C}_{i2} = \mu_2$

In each model, *C* is the log-intensity value of the CSF samples after step 1 normalization and *D* is the depletion day of the sample. The parameter □ is simply a baseline log-intensity level and □ is the coefficient of *D*, to estimate.

Once the parameters have been estimated, the normalization of the study samples is then applied to each transition.

$$I_{it} = I_i - \left(\hat{C}_{i1} - \hat{C}_{i2}\right)$$

In the equation, I_i and I_{it} are respectively the study samples log-intensity after step 1 normalization and after step 2 normalization.

The net effect of the second step of normalization is to move the daily sample average accounted for in M1 to the global sample average accounted for in M2.

Figure 9 shows the distribution of the sample average intensity after the two-step normalization. **Figure 10** shows the sample average intensity vs. injection order following normalization.

[1] For R 2.15.0, these are w = NULL, df, spar = NULL, cv = FALSE, all.knots = FALSE, nknots = NULL, keep.data = TRUE, df.offset = 0, penalty = 1, control.spar = list(), tol = 1e-6 * IQR(x).

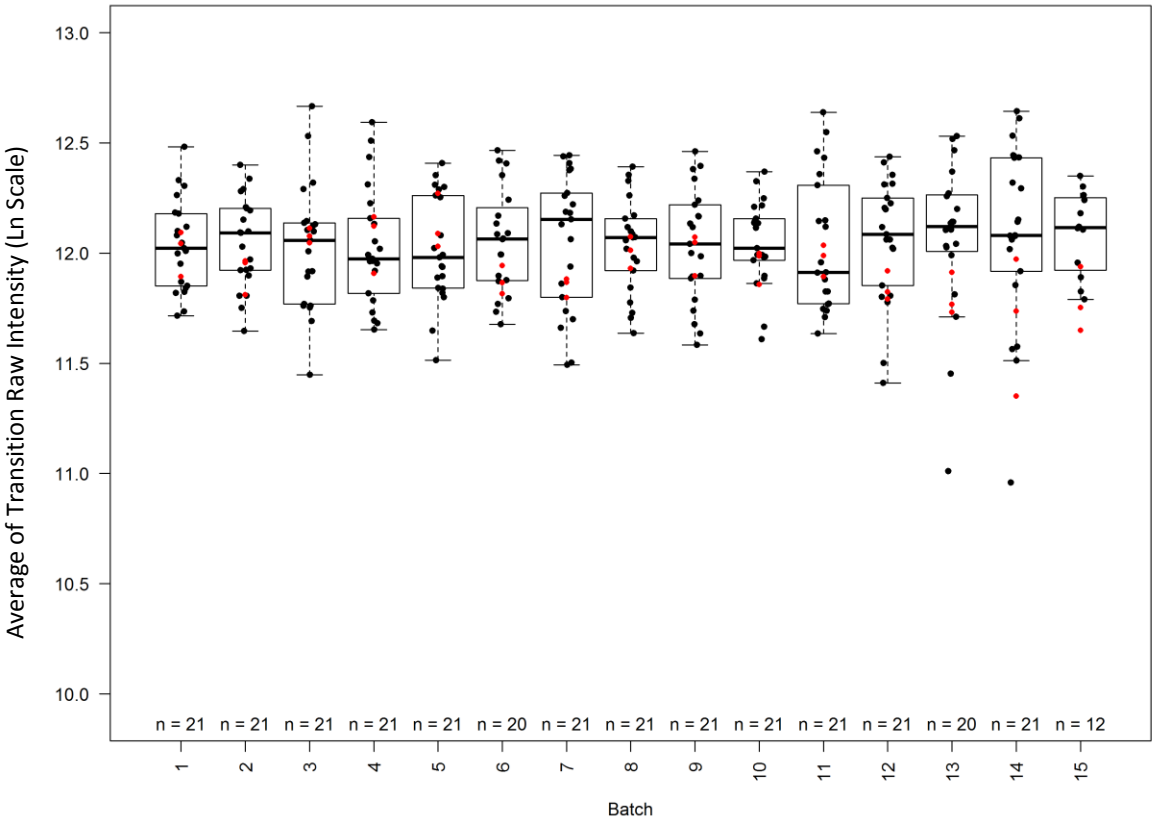

Figure 9. Sample average normalized intensity. Samples are grouped by depletion batch. Black: Study samples, Red: HGS-CSF QC samples. Boxes are delimited at the top by the third quartile (Q3) and at the bottom by the first quartile (Q1). The thick black line within a box represents the median (Q2). Whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range (Q3-Q1) from the box.

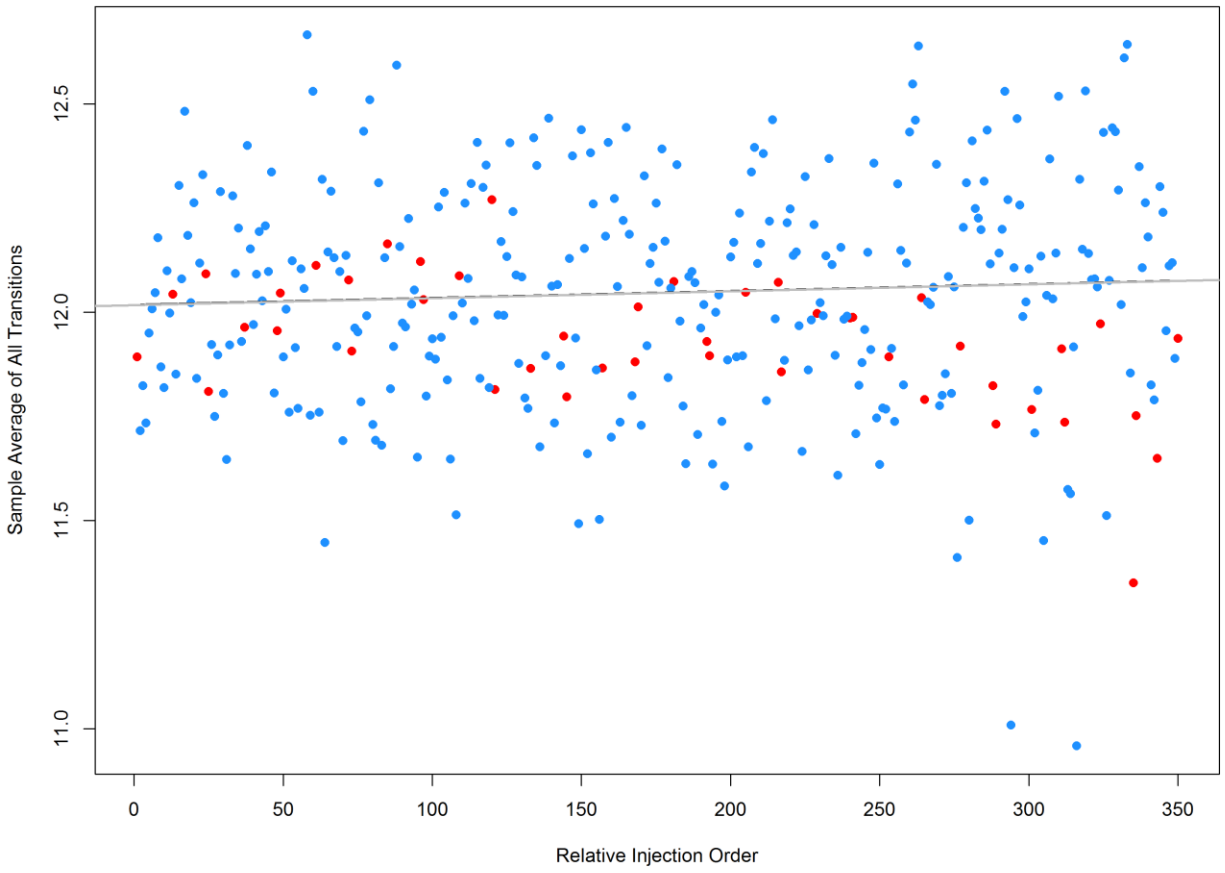

Figure 10. Sample average of normalized intensities plotted against LC/MRM-MS injection order. Study samples appear in blue, CSF-HGS in red. Almost indistinguishable, the thick black curve, which is a smoothing spline on the study samples, and the thick grey curve, which is a linear regression on the study samples as well. The equation for the linear regression is 12.01742 + 1.652038×10^{-4} x InjectionOrder. The *p*-value (*t*-test) to assess if the slope is 0 is 0.28, not rejecting the null hypothesis of slope 0.

Details of the R *smooth.spline* Algorithm

The *x* (injection order) values were first checked for duplicates, which in R 2.15.0 *smooth.spline*, is defined as values of *x* corresponding to duplicates of *xx* = *round*((*x* - *mean*(*x*))/*tol*). Note that as is, the *round* function does not consider digits. For the *tol* value, see footnote 1. As there are no injection order duplicates, this step has no impact.

The *x* vector was then scaled to a [0,1] range by the transformation *xbar* = (*x*-*min*(*x*))/(*max*(*x*)-*min*(*x*)). The actual data used by the function was a sorted version of *x*, but since the injection orders were already in increasing sequence, this has no impact.

With the default options, number of knots was defined as follow:
INPUT: n (number of unique *x*), $a_1 = \log_2(50)$, $a_2 = \log_2(100)$, $a_3 = \log_2(140)$, $a_4 = \log_2(200)$
If $n < 50$ Then $nknots = n$
Else
{

If $n < 200$ Then $a = 2^{a_1 + \frac{(a_2 - a_1)(n-50)}{150}}$
Else If $n < 800$ Then $a = 2^{a_2 + \frac{(a_3 - a_2)(n-200)}{600}}$
Else If $n < 3200$ Then $a = 2^{a_3 + \frac{(a_4 - 3)(n-800)}{2400}}$
Else Then $a = 200 + (n - 3200)^{0.2}$
$nknots = \text{trunc}(a)$

}

Knots placement is *xbar*[1], *xbar*[1], *xbar*[1], *xbar*[*seq.int*(1, *n*, *length.out=nknots*)], *xbar*[n], *xbar*[n], *xbar*[*n*]. The *seq.int*(1, *n*, *length.out=nknots*) function creates a vector of equally spaced values of length *nknots.* When used as a sub setting integer vector, values are then truncated to integers. Therefore, *xbar*[*seq.int*(1, *n*, *length.out=nknots*)] represents *nknots xbar* values, roughly equally spaced on their relative *xbar* position scale.

9. Peptide and Protein Quantitation

Prior to quantitation, we examined the transition detection rate, defined as the proportion of samples with a pre-normalized intensity value of at least 10,000 (See column "%raw transition sample values >10000" in the spreadsheet "**Normalized Intensity**". The transitions with detection in fewer than 50% of samples are listed in **Table 5**. These transitions include peptides that were intentionally included in the panel as markers of blood contamination, and so might be expected to be present in a small number of samples. Thus no filter was applied based on the number of CSF samples in which a transition was detected because peptides that are present in only a small number of samples may still be informative and should be available for subsequent analysis.

Table 5. Transitions detected in fewer than 50% of samples.

Transition_ID	TransitionFullName	PeptideSequence	Protein
3	AVLTIDEK_444.76_605.3	AVLTIDEK	A1AT_HUMAN
4	AVLTIDEK_444.76_718.4	AVLTIDEK	A1AT_HUMAN
7	LSITGTYDLK_555.81_696.4	LSITGTYDLK	A1AT_HUMAN
8	LSITGTYDLK_555.81_797.4	LSITGTYDLK	A1AT_HUMAN
97	TGISPLALIK_506.82_654.5	TGISPLALIK	APOB_HUMAN
103	IAELSATAQEIIK_693.90_314.2	IAELSATAQEIIK	APOB_HUMAN
104	IAELSATAQEIIK_693.90_960.5	IAELSATAQEIIK	APOB_HUMAN
117	CLAVYQAGAR_526.27_665.3	CLAVYQAGAR	APOE_HUMAN
118	CLAVYQAGAR_526.27_835.4	CLAVYQAGAR	APOE_HUMAN
123	LGADMEDVR_503.24_649.3	LGADMEDVR	APOE_HUMAN
124	LGADMEDVR_503.24_892.4	LGADMEDVR	APOE_HUMAN
143	ETPAATEAPSSTPK_693.84_616.3	ETPAATEAPSSTPK	BASP1_HUMAN
144	ETPAATEAPSSTPK_693.84_917.5	ETPAATEAPSSTPK	BASP1_HUMAN
193	LFAYPDTHR_373.86_413.2	LFAYPDTHR	CATA_HUMAN
194	LFAYPDTHR_373.86_625.3	LFAYPDTHR	CATA_HUMAN
281	VDNAPDQQNSHPDLAQEEIR_759.36_329.1	VDNAPDQQNSHPDLAQEEIR	CNTP2_HUMAN
282	VDNAPDQQNSHPDLAQEEIR_759.36_400.2	VDNAPDQQNSHPDLAQEEIR	CNTP2_HUMAN
289	TGLQEVEVK_501.78_603.3	TGLQEVEVK	CO3_HUMAN
290	TGLQEVEVK_501.78_731.4	TGLQEVEVK	CO3_HUMAN
293	TELRPGETLNVNFLLR_624.68_662.4	TELRPGETLNVNFLLR	CO3_HUMAN
294	TELRPGETLNVNFLLR_624.68_875.5	TELRPGETLNVNFLLR	CO3_HUMAN
295	LSINTHPSQKPLSITVR_631.03_575.4	LSINTHPSQKPLSITVR	CO3_HUMAN
296	LSINTHPSQKPLSITVR_631.03_785.5	LSINTHPSQKPLSITVR	CO3_HUMAN
297	VPVAVQGEDTVQSLTQGDGVAK_733.38_775.4	VPVAVQGEDTVQSLTQGDGVAK	CO3_HUMAN
298	VPVAVQGEDTVQSLTQGDGVAK_733.38_975.5	VPVAVQGEDTVQSLTQGDGVAK	CO3_HUMAN
361	LGAEVYHTLK_565.81_661.4	LGAEVYHTLK	ENOG_HUMAN
362	LGAEVYHTLK_565.81_760.4	LGAEVYHTLK	ENOG_HUMAN
430	ALAAELNQLR_549.82_914.5	ALAAELNQLR	GFAP_HUMAN
443	DQLVIPDGQEEEQEAAGEGR_724.00_357.2	DQLVIPDGQEEEQEAAGEGR	GOLM1_HUMAN
444	DQLVIPDGQEEEQEAAGEGR_724.00_456.2	DQLVIPDGQEEEQEAAGEGR	GOLM1_HUMAN
447	EYPGSETPPK_552.76_341.2	EYPGSETPPK	GRIA4_HUMAN
448	EYPGSETPPK_552.76_812.4	EYPGSETPPK	GRIA4_HUMAN
463	EFTPPVQAAYQK_689.85_580.3	EFTPPVQAAYQK	HBB_HUMAN
621	SGETVINTANYHDTSPYR_675.65_738.3	SGETVINTANYHDTSPYR	LPHN1_HUMAN
622	SGETVINTANYHDTSPYR_675.65_875.4	SGETVINTANYHDTSPYR	LPHN1_HUMAN
737	SAPAAAIAAR_449.76_572.4	SAPAAAIAAR	NGF_HUMAN
738	SAPAAAIAAR_449.76_643.4	SAPAAAIAAR	NGF_HUMAN
757	LESLEHQLR_562.81_682.4	LESLEHQLR	NPTX2_HUMAN
842	LYGPSEPHSR_381.52_712.3	LYGPSEPHSR	PLDX1_HUMAN
878	HLSVNDLPVGR_603.83_956.5	HLSVNDLPVGR	PRDX3_HUMAN
890	LSILYPATTGR_596.34_878.5	LSILYPATTGR	PRDX6_HUMAN
891	LIALSIDSVEDHLAWSK_633.01_491.3	LIALSIDSVEDHLAWSK	PRDX6_HUMAN
892	LIALSIDSVEDHLAWSK_633.01_985.5	LIALSIDSVEDHLAWSK	PRDX6_HUMAN
959	SLPVDVFAGVSLSK_709.90_590.4	SLPVDVFAGVSLSK	SLIK1_HUMAN

Combining Transitions into a Peptide/Protein Result

Input

$I = \begin{bmatrix} I_{11} & \dots & I_{1T} \\ \vdots & \ddots & \vdots \\ I_{n1} & \dots & I_{nT} \end{bmatrix}$: Rows are the study samples from 1 to *n* and columns are transitions from 1 to *T*

Algorithm

1. For each peptide (or protein), take

$I_p = \begin{bmatrix} I_{1i_1} & \dots & I_{1i_P} \\ \vdots & \ddots & \vdots \\ I_{ni_1} & \dots & I_{ni_P} \end{bmatrix}$

from *I*. The selected columns are the transitions that belong to the peptide (or protein) of interest and pass the detection rate filtering criteria. Then loop through steps 2-5 for all peptides (or proteins).

2. Compute the sample covariance of I_p *:*

a. For each transition: *i* = 1, …, *T:* $\overline{I_\iota} = \frac{1}{n}\sum_{j=1}^{n} I_{ji}$

b. The element *i,k* of the sample covariance matrix, denoted Σ is calculated as

$$\Sigma_{ik} = \frac{1}{n-1}\sum_{j=1}^{n}\left(I_{ji} - \overline{I_\iota}\right)\left(I_{jk} - \overline{I_k}\right)$$

3. Perform the eigendecomposition of Σ to obtain matrices Q and Λ such that $\Sigma = Q\Lambda Q^T$. The entries of the diagonal matrix Λ are the eigenvalues of the covariance matrix, Σ, and the columns of Q are the eigenvectors.

4. Define λ_m as the maximum value of the diagonal of Λ and Q_m the corresponding column of Q. Qm is therefore the eigenvector that captures most of the inter-sample variability reported by the individual transitions, a.k.a. the 1st principal component.

5. The peptide (or protein) vector intensity (each entry represents a sample) is given by $I = I_p Q_m$. Since the orientation of eigenvectors is arbitrary, it is convenient to make sure that the sign of the elements in Q are positive. i.e. If all the elements are negative, set them to positive.

6. A metric to report for each peptide (or protein) is the % variance explained by the largest component. This is generated by dividing λ_m by the trace of Λ. If all the transitions are completely collinear without noise, the value would be 1. Lower values would indicate less confidence in the rolled-up intensity which could be related to low signal/noise, isoform

differences between different peptides of a protein, analytical interferences, etc. This metric is reported in the column "VariancePC1" in the **"log(Peptide Intensity)"** and **"log(Protein Intensity)"** spreadsheets. The average % variance explained by the first component for the peptide roll-up was 0.97, indicating strong correlation between the transitions for a given peptide over all peptides.

Note: The following APOE peptides on the MRM panel were designed to detect specific alleles of APOE. These peptides were not aggregated to the protein level.

APOE_HUMAN	**CLAVYQAGAR**	**E2**
APOE_HUMAN	**LAVYQAGAR**	**E3, E4**
APOE_HUMAN	**LGADMEDVR**	**E4**

10. Estimating Concentrations Using External Standard Peptides

A mix of synthetic standard peptides in buffer, each at 200 pmol/mL, was analyzed before (n=5) and after (n=5) the study samples. The non-normalized transition intensity values for these 10 replicates are included in the spreadsheet **"External Standards"**. At the transition level, an estimate of protein concentration in the study samples can be calculated as follows:

$$\text{Protein concentration in ng/mL (Transition level)} = \frac{\text{Transition intensity in study sample x Inj. Volume (5 µL) x Synthetic peptide conc. (200 pmol/mL) x MW protein}}{\text{Average transition intensity in peptide mix x 1000 x CSF volume injected (36 µL)}}$$

These estimates are included in the spreadsheet **"Estimated Concentrations."** Because the transition intensities from the standard mixes were not included in the roll-up into peptide and protein intensities, no concentration estimates at the peptide or protein level were calculated. The reported protein concentrations at the transition level are considered as an illustrative estimate and have not been demonstrated to be accurate or precise. Subsequent studies using heavy-isotope labeled internal standard peptides are required to generate robust concentration measurements.

11. Data quality control based on the 16 ADNI test/re-test samples

Data quality was assessed using the ADNI CSF test/re-test samples using the peptide-level intensity data for all 320 peptides contained in spreadsheet "**log(Peptide Intensity)**".

Reproducibility between each of the 16 replicate pairs was assessed graphically and by computing Spearman correlation and concordance coefficients between each pair (See **Appendix I**). Overall very high reproducibility was observed, with correlation between technical replicates above 0.959 for all the 16 pairs. Most of the peptides with larger variability had relatively lower expression values.

Based on the exploratory analyses of test-retest data, we developed a statistic to flag less reliable peptides. In Step 1, the most variable peptides were empirically identified in the test-retest dataset, based on differences in expression level between technical duplicates. **Figure 11** shows the distribution of the maximum difference in any of the 16 pairs for each peptide. A bi-modal distribution of differences could be observed. An arbitrary cut-off of 5 was then applied, identifying 24 peptides. Column "Step 1" in spreadsheet "**Test-Retest Flagged Peptides**" lists these 24 peptides. These peptides showed a difference of greater than 5 on the log scale within at least one of the 16 technical replicate pairs. A column was added to the "**log(Peptide Intensity)**" spreadsheet to indicate these 24 test/re-test flagged peptides. However, we noted that several of these peptides were included in the panel as potential markers of blood contamination (HBA, HBB, APOB). If blood contamination was minimal in the 16 CSF samples represented in the test/re-test set, these peptides would be expected to have low and noisy signal but they might still have utility in detecting rare blood contaminated samples in the full sample set. Most of the other flagged peptides had low signal in the test/re-test samples. Given the small number of flagged peptides and the potential for these peptides to still be informative in a small number of samples in full sample set, we decided *not* to exclude the 24 flagged peptides in the final dataset, included as spreadsheet "**CSV Export**".

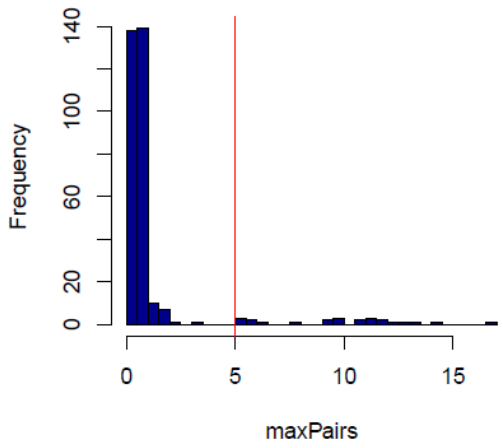

Figure 11. Distribution of peptide maximum intensity differences within the 16 technical pairs. Based on the bimodal distribution observed, an empirical cut-off of maximum difference >5 was selected to flag peptides that were outliers in the test/re-test analysis.

To further understand the relationship of test/re-test performance to expression levels, we explored various metrics on the test-retest dataset to identify outliers at the expression level. The metrics and corresponding rules are summarized in **Figure 12**. These included the standard deviation, the maximum, the median absolute deviation and the minimum for each peptide computed across the 32 samples in the test-retest dataset.

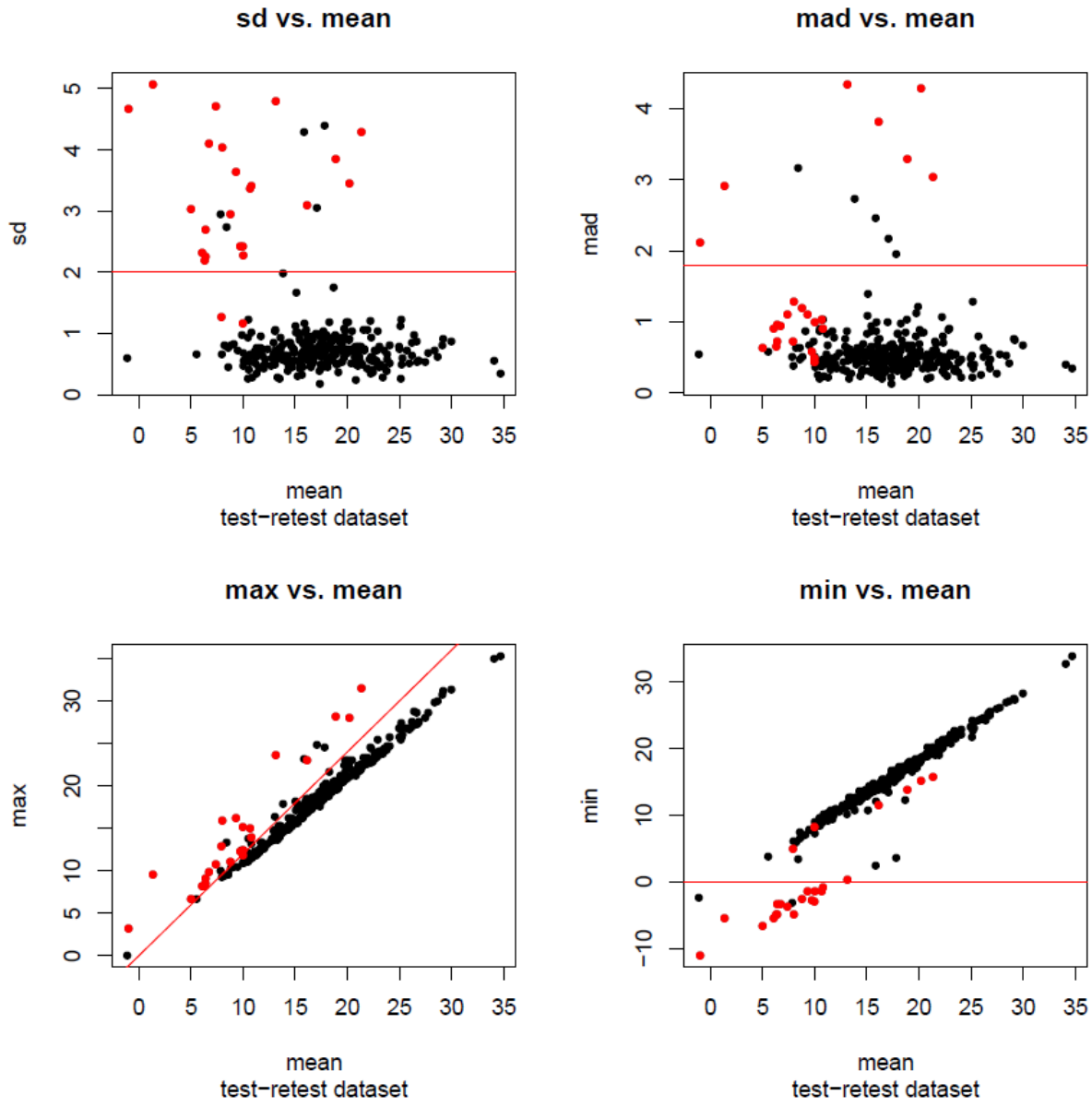

Figure 12. Evaluating different metrics to predict outlier peptides as defined in Step 1. The x-axes represent the average expression value over the 32 samples of the test-retest dataset, while y-axes represent standard deviation (sd), median absolute deviation (mad), max and min expression values in the 32 samples. Each dot represents a peptide. Red dots represent technical outlier peptides as defined in **Figure 11**.

These statistics are shown plotted against the mean in **Figure 12**. Of the four statistics, the minimum appeared to be the most consistent in identifying the same peptides identified in **Figure 11**. We use a threshold of 0 for the minimum log(roll-up) value in at least one of the 32 samples to flag peptides. 19 peptides were identified, and of these, 17 were among the 24 peptides flagged as technical outliers in Step 1 and two additional peptides seen as black dots below the red line in **Figure 12**. Column "Step 2" shows the peptides identified.

In a final step to explore these relationships, we applied this rule of identifying all negative log(roll-up) values to the entire dataset of 304 samples. **Figure 13** shows the minimum *vs.* mean plot for the complete dataset. This rule applied to the entire dataset identified 27 peptides, including 19 of the 24 technical outliers as defined in Step 1. All peptides identified in this step are reported in Column "Step 3".

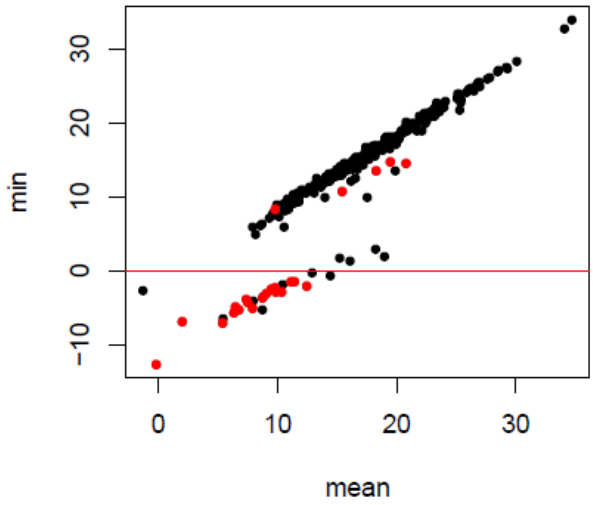

Figure 13. Applying the outlier detection rule on peptides log(roll-up) expression values on the whole ADNI CSF dataset. The x-axis represents the average expression value over the 304 samples while y-axis represents minimum expression values in the 304 samples. Each dot represents a peptide. Red dots represent technical outlier peptides as defined in Figure 11.

In summary, the test-retest dataset allows us to estimate the technical reproducibility of the MRM technology on the ADNI CSF dataset. All 16 pairs (technical replicates) showed a comparable and very high correlation and concordance. The most variable peptides were associated with low expression in both the test-retest dataset (32 samples) and the entire dataset (304 samples). Defining a threshold of a minimum log(roll-up) value in any of the samples below zero allows us to capture 79% of the technically variable peptides flagged in the test/re-test evaluation. However, given that a number of the peptides that were flagged using these metrics were in fact specifically included in the panel as blood contamination markers and might have utility in a small subset of samples, all 320 peptides in Spreadsheet "**log(Peptide**

Intensity)" were accepted as "QC Pass" in the Test/Re-test analysis and subsequently included in the CSV export. Flagging information is included for informational purposes.

12. What is posted on the ADNI Website and cautionary notes to data analysis:

What is posted:

- The final QC'd dataset of peptide quantitations is uploaded to LONI as **CSFMRM.csv**
- Details on the samples, run-order, transitions, results from standards, the raw intensity values, and all steps in processing the data, including the test/re-test metrics are included in the Excel document "**CSFMRM Consolidated Data.xlsx**". For convenience, the final QC'd dataset is replicated in this document as Spreadsheet "**CSV Export**".
- This Primer Document and Appendices, including the proposed Statistical Analysis Plan, are also included in the posting.

Cautionary notes:

- For the 16 CSF samples with replicates, data from both aliquots are included in the datasets.
- Given the potential for differences between the levels of peptides from a single a protein and the unknown biological relevance of any such differences, we do not recommend combining peptide quantitation into protein quantitation without further analysis of the data to address these issues.
- The Peptide Intensity values are in arbitrary units on a natural log scale. No heavy isotope labeled internal standards were used in this phase of the project. These relative peptide intensities are intended for use in comparing across samples - no comparisons of intensities *between* peptides should be made.