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A High Throughput, Multiplexed and Targeted Proteomic CSF Assay to quantitate Neurodegenerative Biomarkers and Apolioprotein E Isoform Status

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Abstract:	Currently there are no effective treatments for many neurodegenerative diseases. Reliable biomarkers for identifying and stratifying these diseases will be important in the development of future novel therapies. Often potential new biomarkers are not further investigated or developed and translated into clinical practice due to limitations and costs in their development into robust clinical assays. However targeted proteomics using multiple reaction monitoring liquid chromatography- tandem mass spectrometry (MRM LC-MS/MS) specifically using triple quadrupole mass spectrometers, is one method that can be used to rapidly evaluate biomarker validation for clinical translation to diagnostic laboratories. Traditionally this platform has been used extensively for measurement of small molecule analysis in clinical laboratories, but it is the potential to analyse proteins that makes it an attractive alternative to

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Jaydev Uppondi
Science Editor of JoVE

RE: JoVE54541**, "A High Throughput, Multiplexed and Targeted Proteomic CSF Assay to quantitate Neurodegenerative Biomarkers and Apolioprotein E Isoform Status"**

Dear Dr. Uppondi,

Thank you very much for the opportunity to resubmit our manuscript. The manuscript has been modified with all the reviewers' suggestions as requested. Please find included in the re-submission our point by point responses to the reviewer's comments. We now feel that we have addressed all concerns and made all the changes suggested.

Yours sincerely,

Dr Kevin Mills

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A High Throughput, Multiplexed and Targeted Proteomic CSF Assay to Quantify Neurodegenerative Biomarkers and Apolioprotein E Isoform Status

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KEYWORDS:
CSF, targeted proteomics, Multiple Reaction Monitoring, Apolipoprotein E isoform, dementia, biomarker, tandem mass spectrometry, multiplex

SHORT ABSTRACT:
We describe a high-throughput, multiplex and targeted proteomic cerebrospinal fluid (CSF) assay developed with potential for clinical translation. The test can quantitate potential markers and risk factors for neurodegeneration as well as phenotype patients for

apolipoprotein E variants (E3, E2 and E4) and measure the quantitative allelic expression.

LONG ABSTRACT:
Currently there are no effective treatments for many neurodegenerative diseases. Reliable biomarkers for identifying and stratifying these diseases will be important in the development of future novel therapies. Often potential new biomarkers are not further investigated or developed and translated into clinical practice due to limitations and costs in their development into robust clinical assays. However targeted proteomics using multiple reaction monitoring liquid chromatography-tandem mass spectrometry (MRM LC-MS/MS) specifically using triple quadrupole mass spectrometers, is one method that can be used to rapidly evaluate biomarker validation for clinical translation to diagnostic laboratories. Traditionally this platform has been used extensively for measurement of small molecule analysis in clinical laboratories, but it is the potential to analyze proteins that makes it an attractive alternative to ELISA-based methods. We describe here how targeted proteomics can be used to measure multiplexed markers of dementia including the detection and quantitation of known risk factor apolipoprotein E isoform E4 (ApoE).

In order to make the assay suitable for translation, it is designed to be rapid, simple in sample preparation, highly specific and cost effective. To achieve this, every step in the development of the assay must be optimized for the individual proteins and tissues they are analyzed in. This method describes a typical workflow including various tips and tricks of developing a targeted proteomics MRM LC-MS/MS for translation.

Method development is preferred using custom synthesized versions of tryptic quantotypic peptides. These peptides are needed to optimize the MS for detection and then spiked into CSF to determine correct identification of the endogenous peptide in the chromatographic separation prior to analysis in the mass spectrometer. To achieve absolute quantitation stable isotope labelled internal standard versions of the peptides with short amino acid sequence tags and containing a trypsin cleavage site are included in the assay.

INTRODUCTION:
The growing impact of neurodegenerative diseases such as Alzheimer's, Lewy Body Dementia and Parkinson's disease is becoming a socioeconomic issue for many countries[1]. There is a need for further biomarkers that can be used to identify and stratify patients earlier and to monitor any potential new treatments. The overall goal of this method is to create a generic pipeline for a streamlined, economic and faster way of validating potential CSF markers of neurodegeneration. The rationale is to use targeted proteomics or peptide MRM LC-MS/MS as an easily amendable method to assess multiple potential protein biomarkers from biomarker discovery experiments. These can be multiplexed over a rapid chromatographic separation (<10 mins) and assessed. Within this multiplex screen for neurodegenerative markers we have included the known dementia risk factor apolipoprotein isoform E4 (ApoE) so we can determine simultaneously the isoform status and level of expression eliminating the need for separate genotyping tests[2]. LC MS/MS is routinely used as the method of choice for accurately quantitating small molecules over other methods such as ELISA or radioimmunoassay (RIA). This

shift in the use of MS technology to analyzing proteins has been driven mainly by issues with immuno-based technologies. These include cross-specificity, batch to batch variation, limited shelf life and cost. Therefore targeted proteomics is rapidly becoming a growing alternative to antibody based methods such as Western blotting, RIA and ELISA. However the ability to multiplex many markers into one assay is the major advantage over immuno-based technologies[3]. The technique is applicable to many tissues and has been used as a validation strategy for many proteomics studies including plasma[4] and urine[5,6].

The technique can be applied to any laboratory that has access and expertise in using triple quadrupole mass spectrometers. Peptide design is relatively simple with use of open source databases. There is a competitive market in synthesis of custom peptides which makes purchasing these affordable. Heavy peptides however are expensive and the markers should ideally be assessed on small cohorts for use before investing in these. There is growing potential for the technique to be used in the clinical diagnostic setting with most large hospitals having triple quadrupole based platforms used for small molecule diagnostics and which can easily be adapted to run targeted proteomic assays. One such application of the method making it into the routine diagnostic setting is its recent application to newborn blood spot screening for sickle cell disease[7].

PROTOCOL:

Note: A schematic of the overall protocol described here is given in figure 1. All samples used of the development of this method are surplus clinical diagnostic samples and have ethical approval from the London Bloomsbury Ethics committee.

[Place figure 1 here]

1. Peptide selection and design.

Note: Criteria for a marker peptide is that it must be unique (**proteotypic**) and represent the quantitative abundance of the protein (**quantotypic**). To determine if a peptide is unique the 'blast' search tool on the Uniprot website (http://www.uniprot.org/blast/) can be used.

1.1. Define a list of target proteins (markers), *i.e.* the ApoE (see table 2).

Note: If the marker has been identified from previous proteomic profiling experiments[8] then select the peptide that gives the best response from that data set.

1.2. If this information is unavailable then use the open source websites such www.thegpm.org to select appropriate peptides. Alternatively, perform *in silico* trypsin digestion of target proteins using a software tool such as MS-Digest.

1.3. Choose the peptide which is tryptic and not susceptible to post translational modifications.

NOTE: This information can be checked on the Uniprot website www.uniprot.org. Avoid peptides prone to chemical modification during LC-MS sample preparation.

1.4. Order the custom synthesis of chosen peptides.

Note: Marker peptides can be custom synthesized by various commercial companies. For ApoE the quantotypic peptide used to measure total ApoE levels was determined to be AATVGSLAGQPLQER. The proteotypic peptide sequences used to determine the E2, E3 and E4 variants are the tryptic peptides for position 112 LGADMEDVCGR and LGADMEDVR (E4) and position 158 RLAVYQAGAR and CLAVYQAGAR (E2) are selected.

2. Preparation of standard peptides.

Note: The best quantitative transitions need to be selected which can only be determined when optimizing the detection in the matrix (CSF). The most efficient way of optimizing multiplexed peptides is to create pools of the peptides at known concentrations. These pools can then be used for method development and standard curves.

2.1. Resuspend synthetic peptides (peptide details are given in Table 2) to 1 mg/ml stock concentration according to manufactures instructions. By default if instructions are not available, resuspend peptides in 50:50 (v:v) acetonitrile (ACN)/ H_2O.

2.2. Prepare the 1:10 dilutions of the peptide from the stock concentration and pool 1000 pmoles of each peptide into a low binding microcentrifuge tube. Dry down in a speed-vac concentrator the final pool and store at -20 °C. Prepare several pools for future use.

2.3. Aliquot 100 µl of CSF into low binding tubes. Freeze-dry the CSF.

2.4. Resuspend an aliquot of pooled 1000 pmol peptides in digestion buffer (100 mM Tris HCl, pH 7.8, 6 M Urea, 2 M Thiourea, 2% ASB14) to obtain concentrations of 10 and 1 pmol/µl.

2.5. Spike the pooled peptides into the freeze-dried 100 µl aliquots of CSF at 0, 1, 2, 5, 10 and 15 pmol concentrations. Add 20 ng of intact unrelated protein such as yeast enolase to act as an internal standard and control for digestion efficiency of trypsin.

2.6. Top up the CSF aliquots with digest buffer to a final amount of 20 µl. Vortex.

2.7. Add 1.5 µl of dithiothreitol (30 mg in 1 ml of 100 mM Tris pH 7.8) and shake at room temperature for 1 hour.

2.8. Add 3 µl of iodoacetamide (35 mg in 1 ml of 100 mM Tris pH 7.8) and shake at room temperature for 45 min in the dark.

2.9. Add 165 µl of ddH_2O.

2.10. Add 10 µl of 0.1 µg/µl sequencing grade modified trypsin solution re-suspended in 50 mM ammonium bicarbonate buffer pH 7.8. Incubate in a water bath at 37 ^oC overnight and stop the digestion by freezing samples. Store digests at -20 ^oC until ready to analyze.

3. Optimization of MS peptide detection.

3.1. Copy and paste the sequence of the peptide to be optimized in to appropriate software *i.e.* Skyline[9]. Click on the peptide sequence to obtain the information of expected precursor ion mass and product ions.

3.2. Dilute the peptides from stock concentration (see section 2.1) further to 1 ng/ml concentration for MS method development.

3.3. Directly infuse the peptides in the mass spectrometer at optimized flow rate (usually 0.1-0.8 ml/min) and with 0-5% collision energy.

3.4. Acquire MS spectrum in order to identify the experimental multiply charged precursor ions[8]. Choose the precursor ion (m/z) that gives the most intensity (precursor ions doubly or triply charged are advised).

3.5. Re-infuse the peptide and apply collision energy to fragment the peptide by collision-induced-dissociation (CID). Optimize the cone and collision energies to obtain the best fragmentation pattern (fragment ions singly or doubly charged and mass of fragment ion preferably bigger than parent ion are advised).

3.6. Verify that the experimentally obtained transitions match transitions generated *in silico* (as described in step 3.1). Save the transition list in the MRM method file using at least 2 most intense transitions per precursor ion. Include 2 transitions: 1 for quantitation and 1 for confirmation in the final assay.
Note: Some MS manufacturers have a function (*i.e.* Waters Intellistart) for automated MRM or SRM analysis optimization. If possible infuse peptides with combined mobile phases at 50-70% ACN with 0.1% FA.

4. LC-MRM method development.

Note: Analyze the mixture of synthetic peptides by a UPLC system coupled to triple quadrupole mass spectrometer. Ensure the source is clean. Solvent A is ddH_20 with 0.1% FA; Solvent B is ACN with 0.1% FA.

4.1. Use the LC-MS system equipped with a UPLC column packed with C-18 phase (1.6 µm diameter, 90 Å pores, 2.1 mm x 50 mm length) and attached to a pre-column of the same phase.

4.2. Defrost CSF digests on ice, centrifuge at 16,000 g for 10 min and transfer 60 µl into 300 µl glass insert vials and store the rest back at -20^{o}C.

4.3. Inject the highest concentration from standard curve point using 10 min 1-40 % ACN linear gradient (see table 1 for gradient settings)

4.4. Open the resulting chromatogram and note retention time and top two most intense

(quantitative) transitions per peptide with all transitions created in step 3.

4.5. Based on this information update a 10 min MRM method (created in step 3.6) with timed channels to measure peptides (see figure 2 for an example). To maintain sensitivity keep each channel with points per peak greater than 8 and dwell time greater than 0.01 sec for at least one transition for each peptide.

4.6. Include 'solvent delays' in the MRM method: one at the beginning until 10 sec before peak first elution and another at the end of the method 20 sec after the last peak elution. Do this by selecting "solvent delays" in method events in MS method file.

4.7. Run the standard curve through the timed MRM method and ensure there are no interfering nonspecific peaks with transitions (generated in step 3.6) by checking for linearity.

4.8. Determine if the peptides are detectable by running non-spiked pooled control and disease CSF through the method.

4.9. Remove the peptides that are below the limit of detection from the assay.

[place figure 2 here]

5. Addition of internal standards

Note: As described previously[10], stable isotope labelled internal standards can be included in the assay. Due to the expense of these standards it is advised to first assess the peptides in the matrix.

5.1. Determine the ideal peptides optimized for detection in CSF: choose the peptides which are the most recurrent, with the highest intensity and without interference peaks.

5.2. Design corresponding peptides to include heavy ^{13}C ^{15}N amino acid labels that increase the mass of the peptide by a least >6 Da relative to endogenous peptide. Also add a tag of 4-6 additional amino acids to either the N- or C-terminus of the heavy peptide to control for tryptic digestion.

5.3. Dilute stable isotope labelled internal standards in digestion buffer (see step 2.4). Determine the ideal amount of stable isotope labelled internal standard which will be spiked in CSF by spiking in various levels depending on the abundancies previously observed during development. Aim to achieve approximately 1:1 ratio of stable isotope labelled standard to endogenous peptide.

Note: Stable isotope labelled internal standards can be synthesized by various commercial companies.

6. LC-MRM Assay of CSF Patient Samples

6.1. Spike optimized amount of stable isotope labelled standards into 100 µl of CSF and

freeze-dry the mixture.

6.2. Resuspend the CSF in 20 µl of digest buffer and perform the digestion as described in points 2.7-2.10).

6.3. Analyze the samples using LC-MRM method developed in step 4.

6.4. For quantitative analysis, run the standard peptides in concentrations 0-15 pmols in a standard curve. See step 2.5 for the preparation of standard curve.

7. Data Analysis

Note: Quantitative data is based on the intensity ratio of baseline peak internal standard (heavy labeled peptides). Detailed information regarding SRM/MRM data analysis was previously described[10]. Ratio data can then be used in a standard curve to determine absolute levels or calculated from the added concentration of heavy labelled peptide.

7.1. Analyze LC-MRM data using the mass spectrometers manufacturers' standard software[10]. Alternatively use Skyline software to analyze quantitative MRM data[10].

7.2. Check the sensitivity of the run by checking the response of an internal standard such as the spiked yeast enolase or a stable isotope labelled standard in each run. Ensure that the coefficient of variation (CV) is not >25%.

7.3. Manually review the data annotation to ensure accuracy. Analyze each peptide and ratio to an appropriate stable isotope labelled internal standard. *I.e.* use the stable isotope labelled version of the peptide if available.

7.4. To obtain absolute values of pmol per 100 µl of CSF run the ratio data through appropriate standard curves that were run at the same time.

7.5. Calculate the coefficient of variation (CV). CV for each peptide should be <25% and <15% for high abundant peptides. NOTE: Absolute value pmol per 100 µl CSF values can used in subsequent downstream statistical analysis.

8. Apolipoprotein E isoform status

Note: To determine ApoE isoform status the presence of the corresponding peptides can be performed by determining the presence of each isoform.

8.1. Consider the ApoE in 100 µL of CSF threshold of > 1000 signal to noise as positive for that peptide. See figure 5 for the peptides required/absent to determine a patient's isoform status. Determine the allelic expression by % of each isoform to total ApoE expression.

REPRESENTATIVE RESULTS:

Using the method described above, a high throughput 10 min multiplex assay consisting of 74

peptides from 54 proteins was developed as an assay for markers of the neurodegenerative disorders Alzheimer's disease and Lewy Body Dementia (LBD)[8]. Figure 3 shows a multiplex chromatogram published previously [8] ofthe significant peptide markers from the assay. The peptides included in the assay and their quantitative transitions are given in table 2. The data generated by this method gives standardized ratios to the relevant stable isotope labelled internal standard. These values can then be put through a standard curve to determine absolute pmol/100 µl CSF concentrations. These values can then be statistically analyzed for changes in clinical samples.

As described previously[8], quantitation of the 74 peptides included in this CSF assay revealed that 25 of these markers were altered significantly in the CSF of dementia patients. To illustrate the effectiveness of this assay results from previously described dementia markers pro-orexin and YKL chitnase 3 like protein (YKL-40)[11,12] are given in figure 4. The integrated ApoE assay identifies the ApoE isoform/allele status of the patient as well. Apo E4 is a known risk factor for Alzheimer's disease therefore integrating this into the assay also will provide valuable information. The detection of the ApoE isoforms is explained in figure 5 and is based on the detection of the corresponding peptides for the amino acid changes for isoforms E2 (R158C) and E4 (C112R). Figure 5A shows the peak pattern expected for each isoform combination and figure 5B shows the result of a CSF tested with the assay on patient samples.

FIGURE LEGENDS:

Figure 1. Schematic illustrating the overall process of creating a targeted CSF MRM LC-MS/MS assay. Candidate marker peptides for evaluation are selected from protein targets. Through the use of custom synthesized peptides a targeted LC-MS/MS multiplex method is created. After evaluation the assay can be used to assess the efficacy of potential markers of neurodegeneration.

Figure 2. Example of a dynamic MRM MS method. Timed channels of peptide transitions can be grouped according to established retention times. By enabling MRMs to be incorporated into a timed fashion as the selected marker peptides elute from the chromatography column minimizes the number of transitions over a time period and increases the sensitivity of the assay.

Figure 3. Representative overlaid MRM chromatograms. Reprint from Heywood *et al.*[8] Biomarkers significant for the neurodegenerative disorders Lewy Body Dementia and Alzheimer's disease[8] are shown over a 10 min LC gradient.

Figure 4. Example data. Reprint from Heywood *et al.*[8] Graphs show of how the described MRM LC-MS/MS method can reliably quantitate and discriminate from controls the known neurodegenerative markers such as YKL chitinase 3 like protein (YKL-40)[11,12] and the AD marker orexin [13]. AD = Alzheimer's disease, LBD = lewy body dementia and PD = Parkinson disease. Data previously published.

Figure 5. Illustration of how the Apo E isoform status of a patient can be determined. A.

Indicates the peptides covering the 112 amino acid sequence LGADMEDVCGR for neutral (E3a) or LGADMEDVR for presence of E4 and for position 158 to detect RLAVYQAGAR the neutral (E3b) or CLAVYQAGAR for the E2 isoform. B. Peptides from the ApoE sequence are shown in the left hand panels. The different combinations of the peptides detected in CSF can indicate the ApoE isoform status.

Table 1. UPLC gradient settings for a 10 min method. A = ddH_20 0.1% FA, B= ACN, 0.1% FA

Table 2. Peptides Included in the CSF MRM LC MS/MS assay.
Table 2 describes all included peptides used in the method described and published [8]. Indication of whether the marker was reliably detected in 100 µl of CSF is indicated. Transitions labelled in bold are the ones used for quantitative data.

DISCUSSION:
As with all MS based assays the critical steps in the method are the determination of the appropriate and accurate amounts of internal standard. If absolute quantitation is being used, then the correct amounts of spiked peptides in the standard curve are also critical.

Our assay does not require the precipitation of the CSF or the use of any type of clean up or desalting steps prior to MS analysis., it is entirely a one pot reaction method. Due to the small volume of CSF and its limited complexity (compared to plasma) it has been found that these steps can be removed from the final protocol; thereby simplifying the assay making it more suitable for translation to a diagnostic setting. The inclusion of a pre-column to the main analytical column and inclusion of solvent delays in the MS method appears to be sufficient to maintain sensitivity during the MS run for more than 500 sample runs. As the assay is over a short UPLC run time it is important to unequivocally identify the correct peak in the CSF digest matrix.

This can only be achieved with the use of spiked synthesized peptides and a standard curve. If there is an uncertainty of a peptide matched to the correct chromatographic peak then it may be useful to check to run the sample through multiple transitions and check the transition intensity pattern with the synthetic standards. All our assays developed contain at least 2 peptides transitions to confirm the identity of peptide.

The assay has been developed for markers detected in up to 100 µl of CSF. For some other markers of dementia a larger volume of CSF maybe needed. Another limitation of the assay is the issue of dynamic range of protein expression. Some abundant markers need to be injected on the mass spectrometer in a smaller amount whilst some low abundant markers require a larger injection volume, therefore the sample may have to be injected twice.

The significance of this technique is the ability to multiplex and the increased specificity of the 3 levels of identification over antibodies (retention time, precursor and product m/z). From the perspective of clinical translation the biggest advantage is the potential cost saving on antibodies though an initial outlay for mass spectrometry equipment and trained personnel is

necessary. Another asset is the speed in which the method can be translated onto the triple quadrupole based systems. This speeds up the ability to translate and validate an assay significantly compared to all immuno-based technologies. Finally as this platform and expertise has been routinely used to measure small molecules clinically, many large hospital centers already have this infrastructure in place. In the field of neurodegeneration there is a lot of focus and need for new biomarkers in CSF and serum. This method provides a platform for a high-throughput assay where future markers can be added (and removed) to be assessed for efficacy *etc*. This technique has further application to other tissues for ApoE isoform identification such as plasma which has already been described[2] and even bloodspots.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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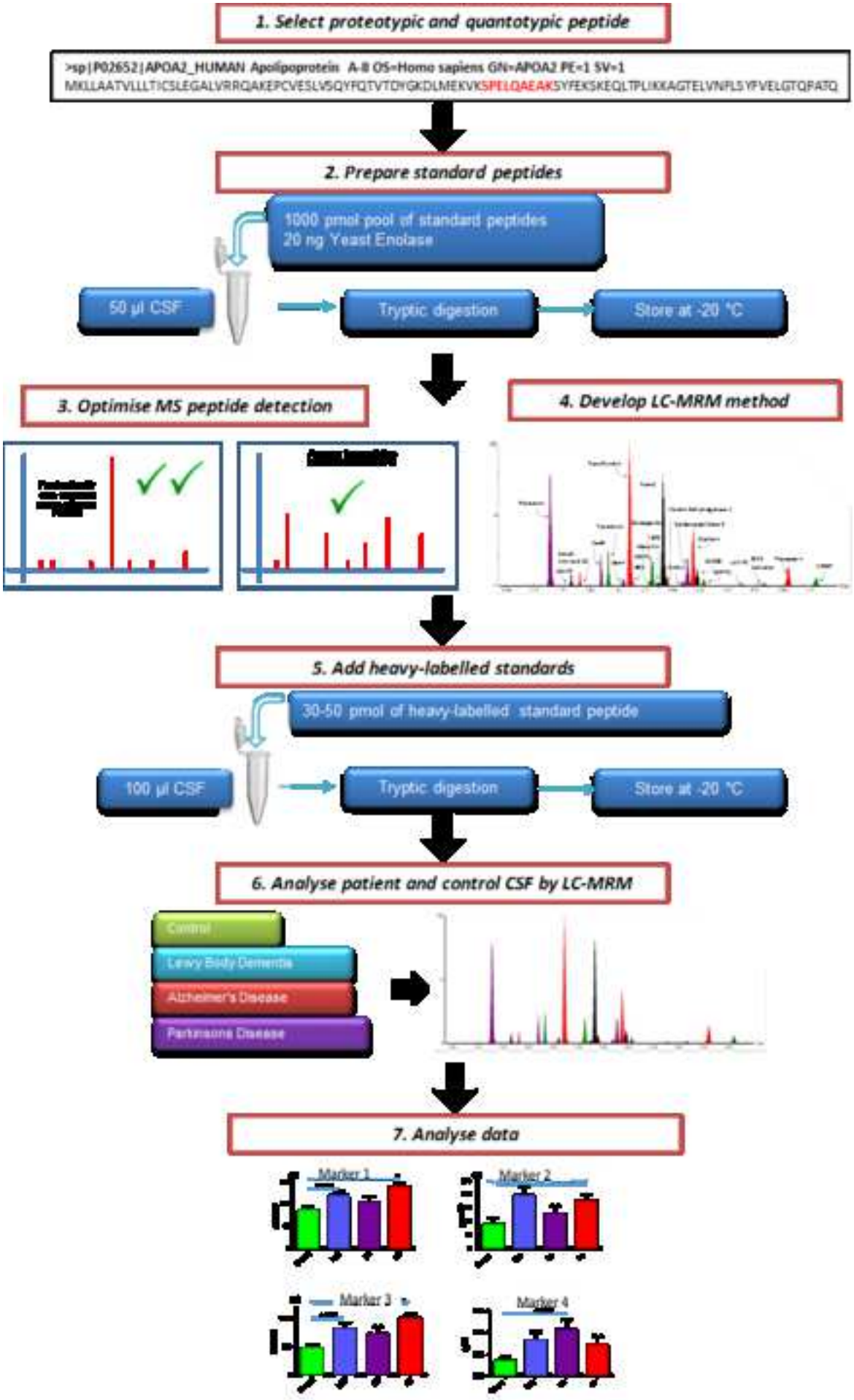

Figure 2

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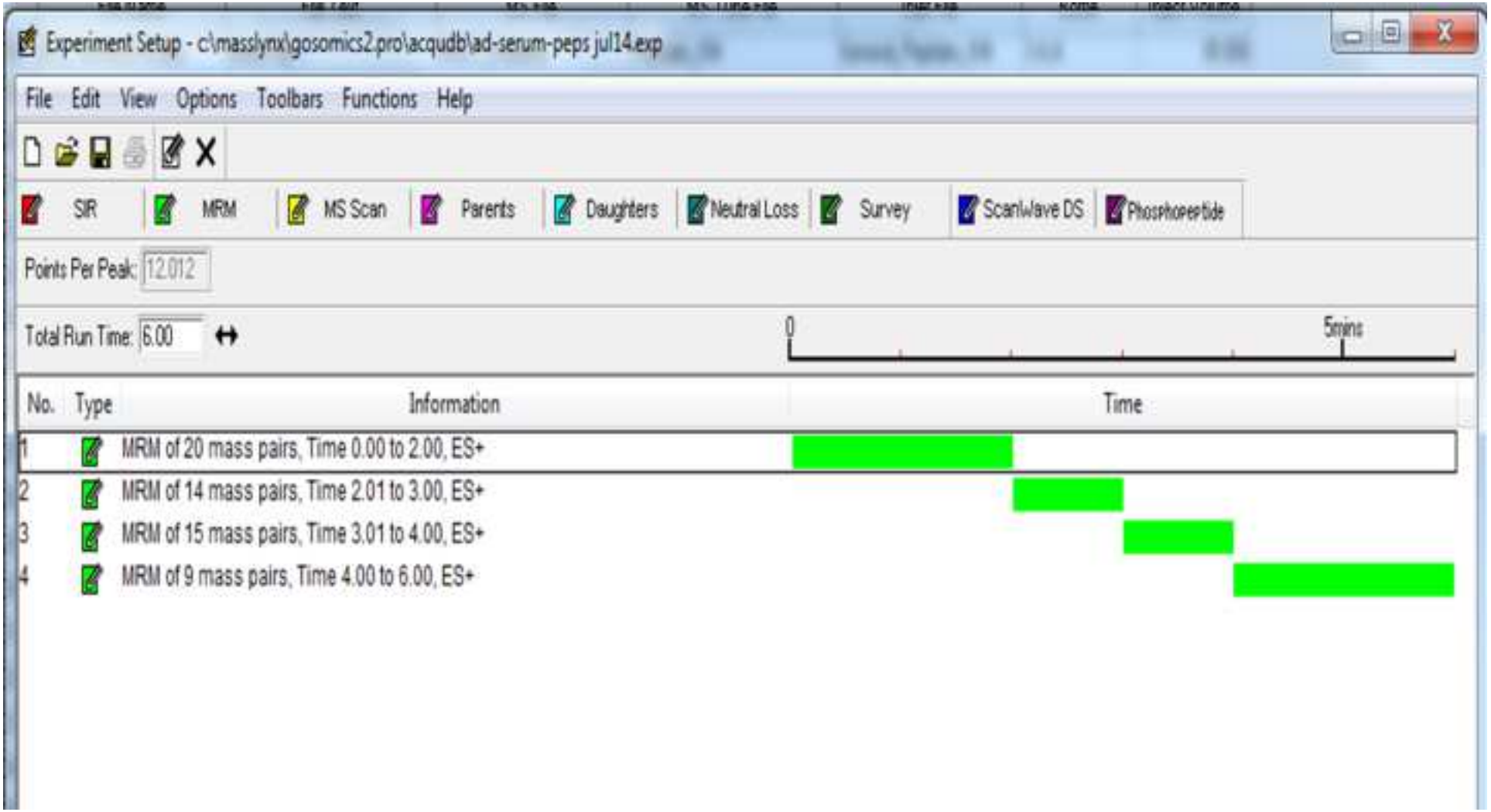

Figure 3

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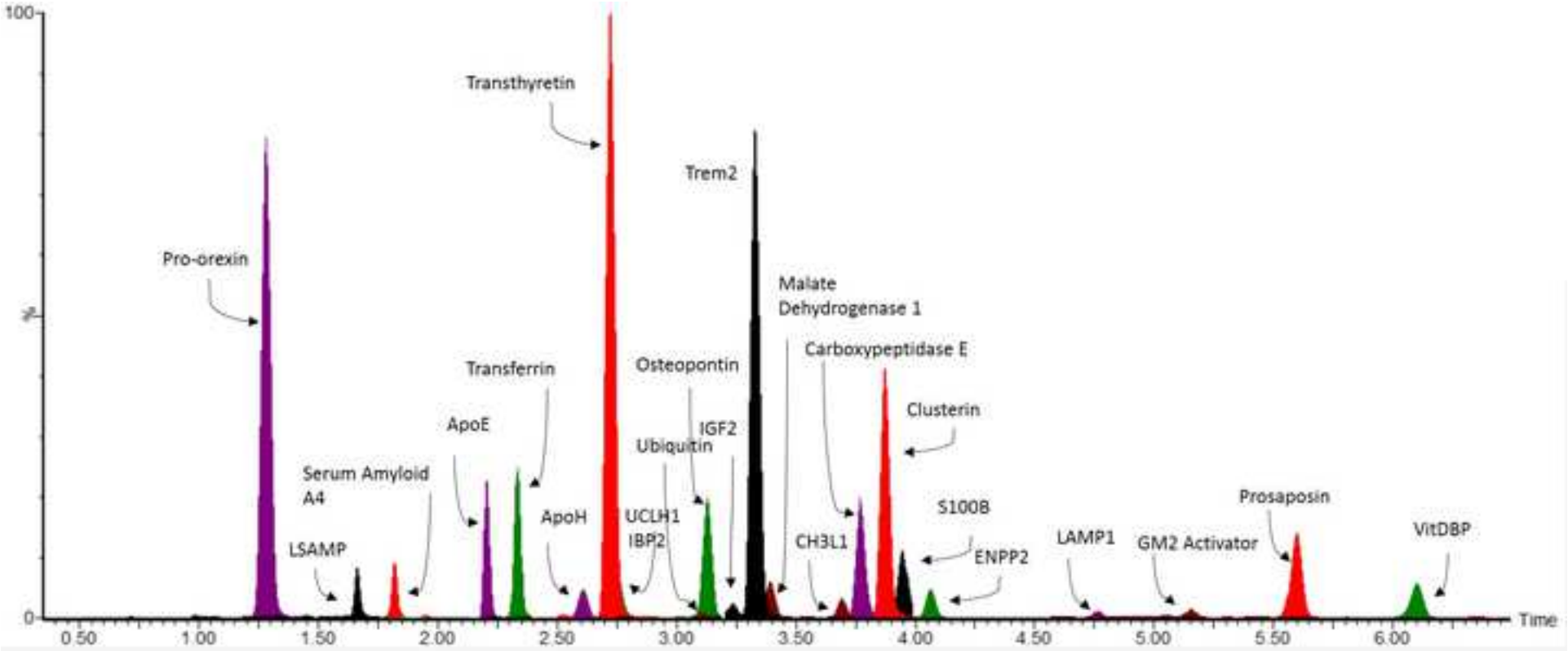

Figure 4

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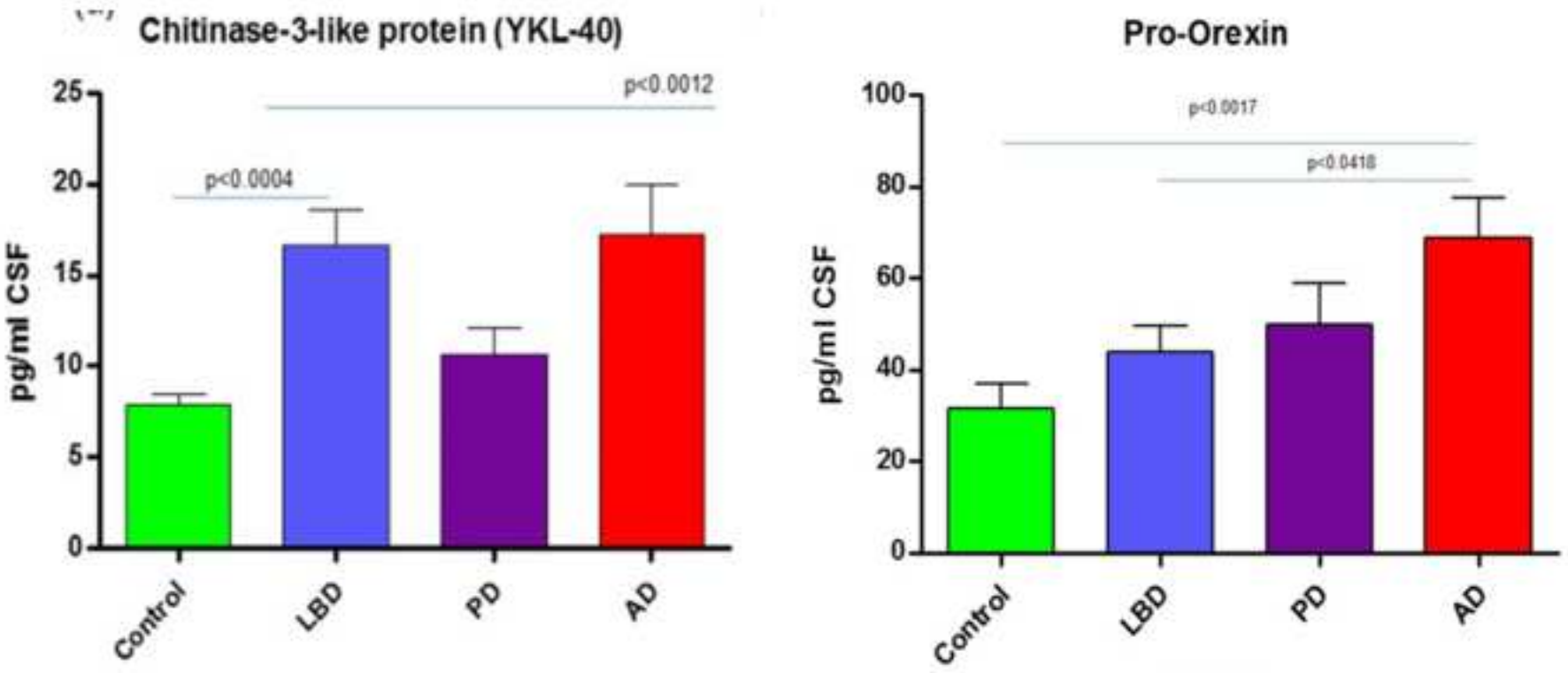

Figure 5

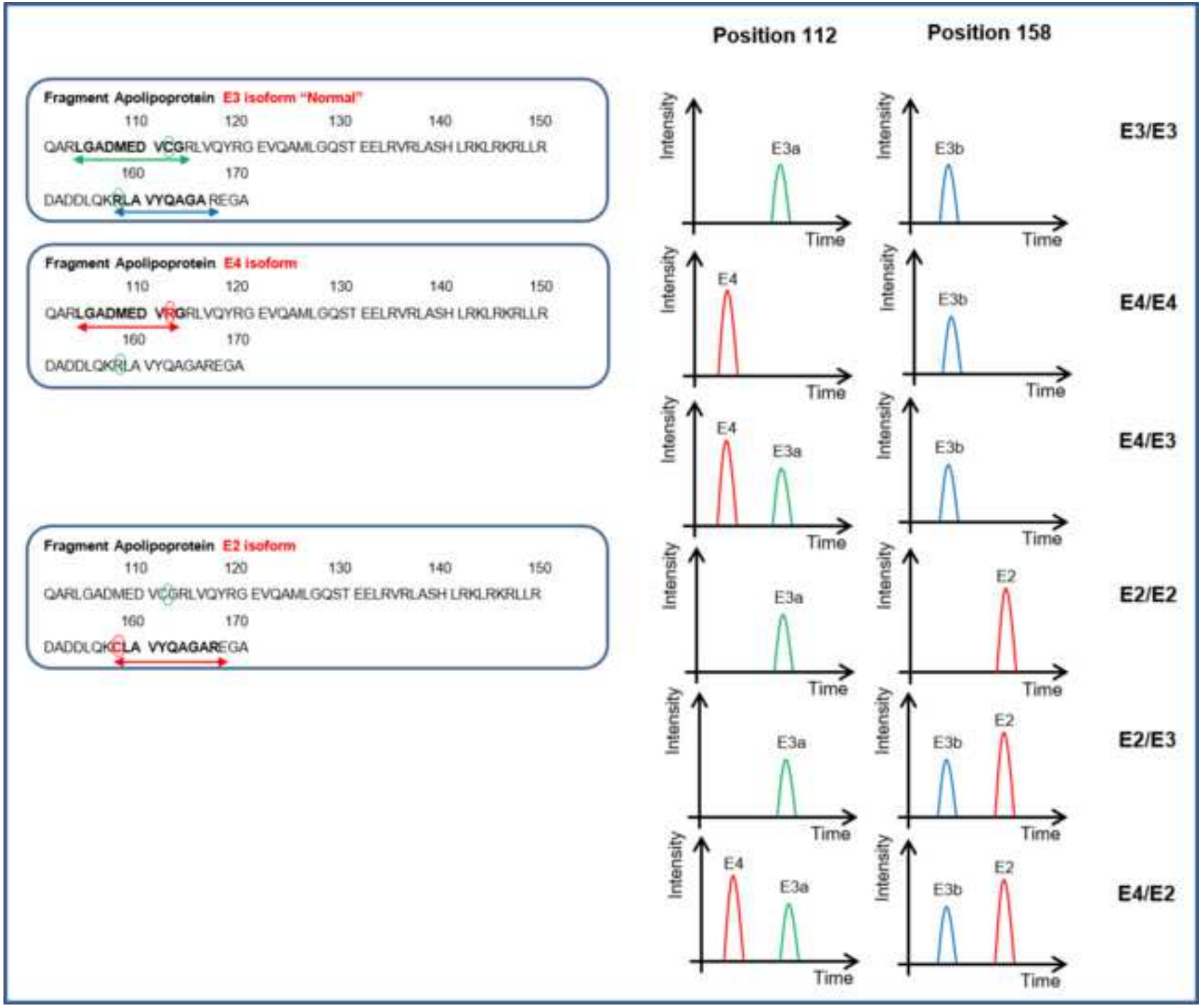

Table 1

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Time	Flow (mL/min)	% A	% B	Curve
Initial	0.8	97	3	initial
0.2	0.8	97	3	6
7	0.8	60	40	6
7.01	0.8	0.1	99.9	6
8	0.8	0.1	99.9	6
8.01	0.8	97	3	1
10	0.8	97	3	1

Table 2 revised

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Uniprot accesssion no.	Name	Precursor m/z	Product m/z
P05062	Aldolase B	715.17	709.14
P05062	Aldolase B	715.17	858.77
P37840	alpha synuclein	643.73	**339.35**
P37840	alpha synuclein	643.73	346.30
P02652	Apolipoprotein AII	487.05	659.46
P02652	Apolipoprotein AII	487.05	**788.56**
P02649	Apolipoprotein E (total)	749.79	642.43
P02649	Apolipoprotein E (total)	749.79	**827.63**
P02649	Apolipoprotein E 112 E3	611.76	**491.24**
P02649	Apolipoprotein E 112 E3	611.76	606.27
P02649	Apolipoprotein E 158 E3	475.09	502.34
P02649	Apolipoprotein E 158 E3	475.09	**665.44**
Q8TCZ8	Apolipoprotein E E2 isoform	554.79	**345.14**
Q8TCZ8	Apolipoprotein E E2 isoform	554.79	835.55
Q8TCZ8	Apolipoprotein E E4 isoform	503.52	835.49
Q8TCZ8	Apolipoprotein E E4 isoform	503.52	892.50
Q8TCZ8	Apolipoprotein E E4 isoform Aqua peptide	506.52	841.51
Q8TCZ8	Apolipoprotein E E4 isoform Aqua peptide	506.52	898.52
	Apolipoprotein E Heavy peptide	753.16	649.27
	Apolipoprotein E Heavy peptide	753.16	834.34
P02749	Apolipoprotein H (beta2microglobulin)	796.08	**503.33**
P02749	Apolipoprotein H (beta2microglobulin)	796.08	531.80
P05067	b-amyloid 38	444.46	**363.16**
P05067	b-amyloid 38	444.46	525.30
P05067	b-amyloid 40	543.57	412.31
P05067	b-amyloid 40	543.57	**561.32**
Q96KN2	Beta-Ala-His dipeptidase	633.63	533.41
Q96KN2	Beta-Ala-His dipeptidase	633.63	**1065.67**
Q96KN2	Beta-Ala-His dipeptidase	506.09	284.14
Q96KN2	Beta-Ala-His dipeptidase	506.09	**497.34**
P16870	Carboxypeptidase E	817.22	356.26
P16870	Carboxypeptidase E	817.22	**820.17**
Q13740	CD166	838.29	**397.04**
Q13740	CD166	838.29	829.14
P36222	Chitinase 3 Like protein 1 (YKL40)	546.09	439.80
P36222	Chitinase 3 Like protein 1 (YKL40)	546.09	**577.36**
P36222	Chitinase 3 Like protein 1 (YKL40)	761.76	654.46
P36222	Chitinase 3 Like protein 1 (YKL40)	761.76	**1008.60**
Q13231	chitotriosidase 1	501.65	**311.20**
Q13231	chitotriosidase 1	501.65	646.20
P10909	Clusterin	625.66	**585.94**
P10909	Clusterin	625.66	686.50
P01034	Cystatin C	614.13	300.20
P01034	Cystatin C	614.13	**610.38**

Q99LX0	DJ1	554.22	674.15
Q99LX0	DJ1	554.22	**723.72**
Q13822	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	772.46	**929.45**
Q13822	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	772.46	1171.49
Q9UNN8	Endothelial protein C receptor	516.06	**433.26**
Q9UNN8	Endothelial protein C receptor	516.06	816.61
P02671	Fibrinogen A	532.19	621.25
P02675	Fibrinogen beta chain	846.65	333.18
P17900	Ganglioside GM2 activator	775.62	769.64
P17900	Ganglioside GM2 activator	775.62	**213.18**
P14136	Glial fibrillary acidic protein	589.59	**616.50**
P14136	Glial fibrillary acidic protein	589.59	779.60
P04062	glucosylceramidase 1	731.16	**1100.60**
P04062	glucosylceramidase 1	731.16	1199.67
P78417	Glutathione S transferase omega	661.18	**553.39**
P78417	Glutathione S transferase omega	661.18	658.37
P01308	Insulin B	651.40	521.37
P01308	Insulin B	651.40	797.32
P01344	Insulin like Growth Factor 2	906.96	**315.21**
P01344	Insulin like Growth Factor 2	906.96	363.05
P05019	Insulin like Growth Factor 2	770.10	**347.16**
P05019	Insulin like Growth Factor 2	770.10	606.30
P18065	Insulin-like growth factor-binding protein 2	808.70	644.39
P18065	Insulin-like growth factor-binding protein 2	808.70	**758.34**
Q13449	Limbicsystem associated membrane protein	529.90	**521.10**
Q13449	Limbicsystem associated membrane protein	529.90	831.62
P11279	Lysosome-associated membrane glycoprotein 1	923.92	458.27
P11279	Lysosome-associated membrane glycoprotein 1	923.92	**571.33**
P11279	Lysosome-associated membrane glycoprotein 1	897.35	364.20
P11279	Lysosome-associated membrane glycoprotein 1	897.35	1071.73
P113473	Lysosome-associated membrane glycoprotein 2	656.91	829.45
P113473	Lysosome-associated membrane glycoprotein 2	656.91	**359.21**
P40925	Malate dehydrogenase	697.73	546.36
P40925	Malate dehydrogenase	697.73	**848.57**
P01033	Metalloproteinase inhibitor 1	617.55	404.12
P01033	Metalloproteinase inhibitor 1	617.55	**717.39**
P02686	Myelin basic protein	487.89	285.12
P02686	Myelin basic protein	487.89	616.50
P13591	Neural Cell Adhesion Molecule 1	662.68	532.91
P13591	Neural Cell Adhesion Molecule 1	662.68	**597.00**

P13591	Neural Cell Adhesion Molecule 1	759.28	1093.69
P13591	Neural Cell Adhesion Molecule 1	759.28	**1240.87**
P07196	Neurofilament light protein	578.20	**387.29**
P07196	Neurofilament light protein	578.20	942.72
Q9HCG7	Non-lysosomal glucosylceramidase	663.53	514.40
Q9HCG7	Non-lysosomal glucosylceramidase	663.53	888.35
P10451	Osteopontin	927.90	511.36
P10451	Osteopontin	927.90	**835.81**
Q13219	Pappalysin-1	488.30	365.41
Q13219	Pappalysin-1	488.30	495.57
Q13219	Pappalysin-1	802.00	**786.59**
Q13219	Pappalysin-1	802.00	1095.86
P28799	progranulin	492.22	237.14
P28799	progranulin	492.22	413.23
O43612	Pro-orexin	451.48	**512.51**
O43612	Pro-orexin	451.48	641.14
P07602	Prosaposin	865.46	215.18
P07602	Prosaposin	865.46	**910.53**
P04271	protein S100B	569.99	**703.76**
P04271	protein S100B	569.99	753.28
Q15771	Ras-related protein Rab-30	585.28	442.29
Q15771	Ras-related protein Rab-30	585.28	**487.27**
	Serotransferrin	426.50	387.21
P35542	Serum Amyloid A4	567.06	363.22
P35542	Serum Amyloid A4	567.06	**535.31**
P02743	Serum Amyloid P	578.97	508.39
P02743	Serum Amyloid P	578.97	**708.53**
P17600	Synapsin 1	727.70	**443.32**
P17600	Synapsin 1	727.70	541.51
P10636	Tau protein (MAPT)	655.71	**472.32**
P10636	Tau protein (MAPT)	655.71	896.60
	Tau protein (MAPT) heavy peptide	659.22	**479.32**
	Tau protein (MAPT) heavy peptide	659.22	903.60
P02766	Transthyretin	698.13	**606.36**
P02766	Transthyretin	787.82	**1002.30**
Q03403	Trefoil 2	791.72	377.13
Q03403	Trefoil 2	791.72	782.75
Q03403	Trefoil 2	848.84	332.11
Q03403	Trefoil 2	848.84	1202.73
Q07654	Trefoil 3	726.86	715.20
Q07654	Trefoil 3	726.86	721.12
Q07654	Trefoil 3	424.96	529.74
Q07654	Trefoil 3	424.96	587.30
Q9NZC2	Triggering receptor expressed on myeloid cells 2	492.64	**632.07**
Q9NZC2	Triggering receptor expressed on myeloid cells 2	492.64	688.62

Q9NZC2	Triggering receptor expressed on myeloid cells 2	562.01	**937.18**
Q9NZC2	Triggering receptor expressed on myeloid cells 2	562.01	1050.27
P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	532.73	**747.30**
P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	532.73	894.31
P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	615.11	**734.51**
P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	615.11	856.57
P62987, P62979,P0CG47,P0CG48	Ubitqutin species derived peptide	894.75	298.19
P62987, P62979,P0CG47,P0CG48	Ubitqutin species derived peptide	894.75	**1002.55**
P02774	VITAMIN D BINDING PROTEIN	789.54	**657.24**
P02774	VITAMIN D BINDING PROTEIN	789.54	1053.93
P04004	Vitromectin	712.18	435.31
P04004	Vitronectin	712.18	**647.44**
P04004	Vitronectin	835.43	310.23
P04004	Vitronectin	835.43	**423.30**
Q8TAG5	Vset transmembrane domain 2	1008.03	627.26
Q8TAG5	Vset transmembrane domain 2	1008.03	**1198.26**
P00924	Yeast enolase internal standard	790.16	661.49
P00924	Yeast enolase internal standard	790.16	805.57
P00924	Yeast enolase internal standard	790.16	918.67
P00925	Yeast enolase internal standard	614.58	306.28
P00926	Yeast enolase internal standard	614.58	514.88
P00927	Yeast enolase internal standard	614.58	547.88
P00928	Yeast enolase internal standard	614.58	821.35
P00929	Yeast enolase internal standard	709.06	377.29
P00930	Yeast enolase internal standard	709.06	451.47
P00931	Yeast enolase internal standard	709.06	948.68

Peptide sequence	Reliably quantitated in 100ul CSF?
IADQCPSSLAIQENANALAR	No
EQVTNVGGAVVTGVTAVAQK	Yes
SPELQAEAK	Yes
AATVGSLAGQPLQER	Yes
LGADMEDVCGR	Yes
LAVYQAGAR	Yes
CLAVYQAGAR	Yes
LGADMEDVR	Yes
AAQARLGADMED[V(13C5; 15N)]R	Yes
AATVGSLAGQP(L13C5; 15N)QERAQAW	
ATFGCHDGYSLDGPEEIECTK	Yes
GAIIGLMVGG	yes
GAIIGLMVGGVV	Yes
TVFGTEPDMIR	Yes
WNYIEGTK	Yes
ELLVIELSDNPGVHEPGEPEFK	Yes
QIGDALPVSCTISASR	Yes
	Yes
LVMGIPTFGR	
TLLSVGGWNFGSQR	Yes
ADGLYPNPRER	no
LFDSDPITVTVPVEVSR	Yes
	Yes
ALDFAVGEYNK	

GLIAAICAGPTALLAHEIGFGSK	Yes
WWGGQPLWITATK	Yes
	yes
TLAFPLTIR	
HPDEAAFFDTASTGK	yes
MGPTELLIEMEDWK	Yes
	Yes
SEFVVPDLELPSWLTTGNYR	
LADVYQAELR	yes
NFVDSPIIVDITK	Yes
GSAPPGPVPEGSIR	yes
FVNQHLCGSHLVEALYLVCGER	no
SCDLALLETYCATPAK	Yes
GPETLCGAELVDALQFVCGDR	Yes
TPCQQELDQVLER	Yes
INSANGLEIK	Yes
FFLQGIQLNTILPDAR	Yes
NMTFDLPSDATVVLNR	yes
	Yes
GILTVDELLAIR	
FVEGLPINDFSR	yes
GFQALGDAADIR	Yes
TQDENPVVHFFK	No
FIVLSNNYLQI	Yes

YIFSDDSSQLTIK	Yes
VLEAELLVLR	not very well due to interfering peak
	no
AIPVAQDLNAPSDWDSR	Yes
LDGSTHLNIFFAK	not quantifiable due to peak interference
VSFSSPLVAISGVALR	Yes
VHCCPHGAFCDLVHTR	no
AGAEPAPRPCLGR	yes
EIVDSYLPVILDIIK	Yes
AMVALIDVFHQYSGR	yes
QNTLVNNVSSPLPGEGK	Yes
EALQGVGDMGR	Yes
VGEYSLYIGR	Yes
EMLSSTTYPVVVK	no
LQTAPVPMPDLK	Yes
SRLQTAPVPMPD[L(13C6; 15N)]K	
AADDTWEPFASGK	Yes
YTIAALLSPYSYSTTAVVTNPK	Yes
QESDQCVMEVSDR	no
NCGYPGISPEECASR	no
IPGVPWCFKPLQEAECTF	No
VDCGYPHVTPKECNNR	no
VLVEVLADPLDHR	

VVSTHNLWLLSFLR	Yes
LGFEDGSVLK	Yes
MPFPVNHGASSEDTLLK	Yes
TITLEVEPSDTIENVK	Yes
VPTADLEDVLPLAEDITNILSK	Yes
FEDGVLDPDYPR	Yes
SIAQYWLGCPAPGHL	Yes
GPEDLDPGAEGAGAQVELLPDR	
	Yes
AVDDFLISLDGTANK	
R.GNPTVEVELTTEK.G [15, 27]	

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acetonitrile (ACN), LC-MS grade	Fisher	A955-1	
Formic acid, LC-MS grade,	Fisher	A117-50	
Dithiothreitol (DTT)	Sigma	D5545-5G	
Hydrochloric acid, 37% w/w	VWR	BDH3028-2.5LG	
Iodoacetamide	Sigma	I1149-5G	
Sodium hydroxide (NaOH)	Fisher	S318-500	
Trypsin, sequencing grade, modified	Promega	V5113	
Trifluoroacetic acid (TFA), LC-MS grade	Fisher	A116-50	
Urea	Sigma	U0631-500g	
Water, LC-MS ULTRA Chromasolv	Fluka	14263	
Custom synthesised peptides desalted 1-4mg	Genscript	custom	
heavy labelled amino acid [C13 N15] custom peptides	Genscript	custom	
ASB 14	Merck Millipore	182750-25gm	
Thiourea	Sigma	T7875-500G	
Tris base	Sigma	T6066	
VanGuard precolumn	Waters	186007125	
Cortecs UPLC C18+ 1.6um 2.1 x50mm column	Waters	186007114	
Yeast Enolase	Sigma	E6126	
300ul clear screw top glass vials	Fisher scientific	03-FISV	
Y slit screw caps	Fisher scientific	9SCK-(B)-ST1X	
Freeze dryer	Edwards Mudulyo	Mudulyo system	
Concentrator/Speed vaccum	Eppendof	concentrator plus 5301	
Xevo -TQ-S mass spectrometer	Waters		
Acquity UPLC system	Waters		

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Author(s): Heywood Wendy E, Baud Anna, Bliss Emily, Sirka Ernestas, Schott Jonathan M, Zetterberg Henrik, Galimberti Daniela, Sebire Neil J and Mills Kevin

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AUTHOR:

Name: Dr Kevin Mills

Department: Institute of Child health

Institution: University College London

Article Title: A High Throughput, Multiplexed and Targeted Proteomic CSF Assay to quantitate neurodegenerative biomarkers and apolioprotein E isoform status

Signature:  Date: 29JAN2016

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MS # (internal use):

JoVE54541R1- Comments to reviewers. Comments have been addressed line by line in red font.

Dear Dr. Mills,

Your manuscript JoVE54541R1 "A High Throughput, Multiplexed and Targeted Proteomic CSF Assay to quantitate Neurodegenerative Biomarkers and Apolioprotein E Isoform Status" has been peer-reviewed and the following comments need to be addressed. Please keep JoVE's formatting requirements and the editorial comments from previous revisions in mind as you revise the manuscript to address peer review comments. Please maintain these overall manuscript changes, e.g., if formatting or other changes were made, commercial language was removed, etc.

Please track the changes in your word processor (e.g., Microsoft Word) or change the text color to identify all of the manuscript edits. When you have revised your submission, please also upload a separate document listing all of changes that address each of the editorial and peer review comments individually with the revised manuscript. Please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.

Your revision is due by **Mar 28, 2016.** Please note that due to the high volume of JoVE submissions, failure to meet this deadline will result in publication delays. To submit a revision, go to the JoVE Submission Site and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.

Sincerely,

Jaydev Upponi, Ph.D.

Science Editor

JoVE

1 Alewife Center, Suite 200, Cambridge, MA 02140

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Editorial comments:

- ***NOTE: Please download this version of the Microsoft word document (File name: 54541_R1_021716) for any subsequent changes.***

- Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

Formatting:
Please include commas between first and last names if author names are listed Last, First.

This has been done.
Please remove underlining from text in the protocol.

This has been done
Please include spaces between all numbers and units.

This has been done
Please refer to “steps” and “sections” of the protocol rather than “points”. For example, in 6.3, “section 4” should be cited.

This has been done
Please use the Greek symbol Mu to indicate “micro” rather than a lowercase “u”. (See 4.2, 7.5 as an example.)
This has been done

Grammar:
3.4 – Please correct “spectrum the in order”

This has been done

Acquire MS spectrum in order to identify the experimental multiply charged precursor ions

4.6, line 368, line 393 – Please use complete sentences.

4.6 Include 'solvent delays' in the MRM method: one at the beginning until 10 sec before peak first elution and another at the end of the method 20 sec after the last peak elution. This can be done by selecting "solvent delays" in method events in MS method file.

This has been done

4.9, 8.1 – Please use imperative tense.

This has been done

4.9 Remove the peptides that are below the limit of detection from the assay.

8.1 Consider the ApoE in 100 µL of CSF threshold of > 1000 signal to noise as positive for that peptide. See figure 5 for the peptides required/absent to determine a patient's isoform status. Determine the allelic expression by % of each isoform to total ApoE expression.

Additional detail is required:

4.3 – Please clarify "highest standard curve point." Do you mean highest concentration?

Yes- this has been clarified in the text

4.3 Inject the highest concentration from standard curve point using 10 min 1-40 % ACN linear gradient (see table 1 for gradient settings)

4.6 – How does one include solvent delays? What is clicked on in the software?

Further details have been included in 4.6 regarding solvent delays

4.6 Include 'solvent delays' in the MRM method: one at the beginning until 10 sec before peak first elution and another at the end of the method 20 sec after the last peak elution. This can be done by selecting "solvent delays" in method events in MS method file.

4.7 – Please be more specific about what is done to "re-assess transitions."

Further detail regarding assessing of transitions is given in 4.7

4.7 Run the matrix standard curve through the timed MRM method and ensure there are no interfering non-specific peaks in your transitions (generated in step 3.6) by checking for

linearity.

7.2 – What is "CV?" It should be defined here.

This has been done.

7.2 Check the sensitivity of the run by checking the response of an internal standard such as the spiked yeast enolase or a stable isotope labelled standard in each run. Ensure that the coefficient of variation (CV) is not >25%.

Results: Please discuss the data in figure 3, 4, and 5 in more detail. What to the results mean? Please correct the file name of figure 3 so that it isn't "figure 2". Please define the error bars in figure 4 (SD, SEM, etc.).

- If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.

Figure 3 and 4 are previously published we have included this statement in the results paragraph and further explanation has been added for figure 5. Re-print permissions have already been provided in the submission.

- JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

  This has been done

- NOTE: Please copyedit the entire manuscript for any grammatical errors you may find. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol. Please thoroughly review the language and grammar of your article text prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

- NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript describes the experimental steps involved in the development of a targeted proteomic assay for protein biomarkers of neurodegenerative diseases. The methodology is based around multiple reaction monitoring liquid chromatography-tandem mass spectrometry (MRM LC-MS/MS) to quantify peptides that are both proteotypic and quantotypic for proteins of interest in cerebrospinal fluid (CSF). Appropriate stable isotope labelled synthetic peptides are added to samples as internals standards. This is an interesting article that provides considerable detail of the outlined experimental approach. The manuscript is well written although the authors make wish to consider the following minor amendments:
1. Page 1. Title. Change the word 'Quantitate' to 'Quantify'.

This has been done.

A High Throughput, Multiplexed and Targeted Proteomic CSF Assay to Quantify Neurodegenerative Biomarkers and Apolioprotein E Isoform Status

2. Page 3. Line 97. There is an extra space between 'chromatography-' and 'tandem'.

This has been done.

However targeted proteomics using multiple reaction monitoring liquid chromatography-tandem mass spectrometry

3. Page 4, Lines 149-150. No need to capitalise 'Newborn Blood Spot Screening……'.

This has been done.

newborn blood spot screening

4. Table 2. For the target peptides precursor and product mass to charge ratio (m/z) listed. These values are given with varying numbers of decimal places. The notation should be consistent. It could be argued that as the analysis is performed on a triple quadrupole mass spectrometer nominal m/z values should be listed.

The values of the precursor and product m/z have been changed to give just 2 decimal places.

5. Apolipoprotein E is sometimes abbreviated to ApoE whilst on other occasions the full term is used. Be consistent. It is suggested that the abbreviation is defined the first time that it is written in the text and used thereafter.

The manuscript has been checked through and abbreviations changed where required.

6. Throughout the manuscript the term heavy labelled internal standard is used. This seems a mixture of terms. It is suggested that either 'heavy' internal standard/peptide or **stable isotope labelled internal standard/peptide is used.**

This has been changed throughout the manuscript

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The author has described the pipeline for the development of a multiplexed targeted mass spectrometry method that can be used for biomarker validation and ultimately for clinical translation to diagnostic laboratories. The author has also shown how this method has been used effectively to measure multiple markers of dementia and determine the isoform status of the known dementia risk factor apolipoprotein isoform E4.
The article was very well written and easy to understand. The method has been described in a detailed way that could easily be repeated by other scientists wanting to use this technique and apply to their disease of interest.
A great advantage of this method is that multiple proteins can be assayed in a single analytical run (less than 10 minutes), allowing for a rapid and high-throughput method that would be highly advantageous in a clinical setting. The fact that this protocol has been developed without the need for any type of clean up step prior to MS analysis also improves greatly the throughput of this method.
To select the correct peptide for quantitation, the matrix (CSF) was spiked with standard peptides to confirm the correct retention time of the endogenous peptide in CSF. Multiple transitions were also used for each peptide. These steps strengthen the fact that this method was developed in a thorough, concise and reliable way to ensure the correct peptides were selected.
The author also used their method to measure dementia markers that had been described

previously, which confirmed the validity and effectiveness of this method further.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
I had some minor suggestions regarding extra information that could be added when describing the method:
Page 4, line 160: How do you determine if the marker peptide is unique within the species being studied? Do you do a Blast search?

Yes we've npow mentioned this in the method

1. Peptide selection and design.
*Note: Criteria for a marker peptide is that it must be unique (**proteotypic**) and represent the quantitative abundance of the protein (**quantotypic**). To determine if a peptide is unique the 'blast' search tool on the Uniprot website (http://www.uniprot.org/blast/) can be used.*

Page 7, line 268: What solvents should heavy peptides be in for spiking and optimization?

We've included details to this section

Dilute stable isotope labelled internal standards in digestion buffer (see step 2.4). Determine the ideal amount of stable isotope labelled internal standard which will be spiked in CSF by spiking in various levels depending on the abundancies previously observed during development

Page 7, line 278: More details could be included regarding how the standard curve is prepared and/or refer to Step 2.5.

Sufficient detail on creating the standard curve is in section 2.5

Page 10,line 401: Add reference if this was described in a previous study.

A reference has been added for the method developed in plasma.

Minor typos:
Page 5, line 201: Insert 'the' to the sentence '45 min in the dark'.

This has been done
Page 6, line 215: Move 'the' to before 'MS spectrum' and not after.

This has been done
Page 6, line 243: Should this refer to 3.6 not 3.4?

This has been changed
Page 8, line 313: Insert µl instead if ul.

This has been done
Page 9, line 334: Make 'transition' plural.

This has been changed to 'transitions'
Figure 1: I think it should be 100 µl CSF instead of 50 µl under 'Prepare standard peptides' to match what it says in the text.

This has been changed

Figure 5: I could not find separate labels for Figure 5A and 5B in the figure or legend, yet it is mentioned in the text.

The legend for figure 5 has been amended.

Figure 5. Illustration of how the Apo E isoform status of a patient can be determined. *A. Indicates the peptides covering the 112 amino acid sequence LGADMEDVCGR for neutral (E3a) or LGADMEDVR for presence of E4 and for position 158 to detect RLAVYQAGAR the neutral (E3b) or CLAVYQAGAR for the E2 isoform. B. Peptides from the ApoE sequence are shown in the left hand panels. The different combinations of the peptides detected in CSF can indicate the ApoE isoform status.*

Reviewer #3:
Manuscript Summary:
A very well written and informative manuscript covering the essentials of quantitative mass spectrometry.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

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