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](http://www.editorialmanager.com/jove" \t "_blank) and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.  
  
  
  
Sincerely,  
  
  
  
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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.

* 1. *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

* 1. *Remove the peptides that are below the limit of detection from the assay.*
  2. *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

* 1. *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

* 1. *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

* 1. *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.

* 1. *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