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Quantitative proteomics workflow using Multiple Reaction Monitoring based detection of proteins from Human brain tissue

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TITLE:

Quantitative Proteomics Workflow using Multiple Reaction Monitoring Based Detection of Proteins from Human Brain Tissue

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KEYWORDS:

SRM, Transition, Skyline, Retention time, Quadrupole, Collision energy

SHORT ABSTRACT:

The protocol aims to introduce the use of a triple quadrupole mass spectrometer for Selected Reaction Monitoring (SRM) of proteins from clinical samples. We have provided a systematic workflow starting from sample preparation to data analysis for clinical samples with all the necessary precautions to be taken.

LONG ABSTRACT:

The proteomic analysis of the human brain tissue over the last decade has greatly enhanced our understanding of the brain. However, brain related disorders continue to be a major contributor of deaths around the world, necessitating the need for even greater understanding of their pathobiology. Traditional antibody-based techniques like western blotting or immunohistochemistry suffer from being low-throughput besides being labor-intensive and qualitative or semi-quantitative. Even conventional mass spectrometry-based shotgun approaches fail to provide conclusive evidence to support a certain hypothesis. Targeted proteomics approaches are largely hypothesis driven and differ from the conventional shotgun proteomics approaches that have been long in use. Multiple reaction monitoring is one such targeted approach that requires the use of a special mass spectrometer called the tandem quadrupole mass spectrometer or triple quadrupole mass spectrometer. In the current study, we have systematically highlighted the major steps involved in performing a successful tandem quadrupole mass spectrometry-based proteomics workflow using human brain tissue with an aim to introduce this workflow to a broader research community.

INTRODUCTION:

During the last decade, rapid developments in mass spectrometry (MS) coupled with increased understanding of chromatography techniques have greatly helped in advancement of MS-based proteomics. Molecular biology-based techniques such as western blotting and immunohistochemistry have long suffered from reproducibility issues, slow turnaround time, inter-observer variability and their inability to accurately quantify proteins, to name a few. To this end, the superior sensitivity of high-throughput proteomics approaches continues to offer molecular biologists an alternate and more reliable tool in their quest to better understand the roles of proteins in cells. However, shotgun proteomics approaches (Data dependent Acquisition or DDA) often fail to detect low abundant proteins in complex tissues besides being heavily reliant on the sensitivity and resolution of the instrument. Over the last couple of years, labs around the world have been developing techniques like Data Independent Acquisition (DIA) that require increased computing power and reliable software that can handle these highly complex datasets. However, these techniques are still a work in progress and not very user friendly. Targeted MS-based proteomics approaches provide a perfect balance between the high throughput nature of MS approaches and the sensitivity of molecular biology approaches like ELISA. A targeted mass spectrometry-based proteomics experiment focuses on detecting hypothesis driven proteins or peptides from discovery-based-shotgun proteomics experiments or through available literature[1,2]. Multiple Reaction Monitoring (MRM) is one such targeted MS approach that uses a tandem quadrupole mass spectrometer for accurate detection and quantification of proteins/peptides from complex samples. The technique offers higher sensitivity and specificity despite requiring the use of a low-resolution instrument.

A quadrupole is made of 4 parallel rods, with each rod connected to the diagonally opposite rod. A fluctuating field is created between the quadrupole rods by applying alternating RF and DC voltages. The trajectory of the ions inside the quadrupole is influenced by the presence of the same voltages across opposite rods. By applying the RF to DC voltage, the trajectory of the ions can be stabilized. It is this property of the quadrupole that allows it to be used as a mass filter

which can selectively let specific ions to pass through. Depending on the need, a quadrupole can be operated in either the static mode or the scanning mode. The static mode allows only ions with a specified m/z to pass through, making the mode highly selective and specific to the ion of interest. The scanning mode on the other hand allows ions across the entire m/z range to pass through. Thus, tandem quadrupole mass spectrometers can operate in 4 possible ways: i) the first quadrupole operating in the static mode while the second operating in scanning mode; ii) the first quadrupole operating in the scanning mode while the second operating in the static mode; iii) both quadrupoles operating in the scanning mode; and iv) both quadrupoles operating in static mode[3]. In a typical MRM experiment, both the quadrupoles operate in the static mode allowing specific precursors and their resulting products after fragmentation to be monitored. This makes the technique very sensitive and selective allowing accurate quantification.

For molecular biologists, the human brain tissue and its cells are a treasure trove. These remarkable units of an ever-interesting organ of the human body can provide molecular and cellular insights into its functioning. Proteomic investigations of the brain tissue can not only help us understand the systemic functioning of a healthy brain but also the cellular pathways that get dysregulated when inflicted by some disease[4]. However, the brain tissue with all its heterogeneity is a very complex organ to analyze and requires a concerted approach for a better understanding of the changes at the molecular level. The following work describes the entire workflow starting right from extracting proteins from brain tissue, creating and optimising the methods for MRM assay, to validation of the targets (**Figure 1**). Here, we have systematically highlighted the major steps involved in a successful MRM based experiment using human brain tissue with an aim to introduce the technique and its challenges to a broader research community.

PROTOCOL:

This study involves brain tissue samples from human participants, reviewed and approved by TMH and IITB IEC - (IITB-IEC/2018/019). The participants provided their informed and written consent to participate in this study.

1. Protein extraction from brain tissue

1.1 Weigh around 50 mg of brain tissue and wash the tissue with 300 µL of 1x phosphate buffer saline (PBS) using a micropipette.

NOTE: This step is performed to remove any blood on the external surface of the tissue and must be repeated if necessary. It is advisable to remove as much blood from tissue as possible as it interferes with downstream protein estimation and processing.

1.2 Following washes with PBS, add 300 µL of lysis buffer (Buffer A) to the tube containing the tissue. Buffer A contains 8 M urea, 50 mM Tris pH 8.0, 75 mM NaCl, 1 mM $MgCl_2$ and Protease inhibitor cocktail (as per manufacturer's instructions).

NOTE: Protein yield varies depending on several factors ranging from the conditions in which the samples are stored, the amount of starting material and the efficiency of handling the samples while processing. Reduce the volume of buffer A proportionately when working with amounts of tissue lower than 50 mg.

1.3 Lyse the tissue using a probe sonicator while keeping the tube on an ice bath. Use the following parameters for sonication: 40% amplitude, 5 seconds on and off cycle for 2:30 min.

1.4 Continue with tissue homogenization using a bead beater to completely lyse the tissue.

NOTE: This step should be performed at medium speed for 90 seconds followed by incubation on ice for 3-5 minutes.

1.5 Centrifuge the contents of the tube at 6,000 x g for 15 min at 4 °C. Transfer the supernatant into a fresh tube and mix homogeneously.

NOTE: Make aliquots of the sample and store at -80°C until further use.

2 Protein quantification and quality check

2.1 Quantify the samples prior to digestion using a standard graph made with known concentrations of BSA.

NOTE: Ensure that the assay being used for protein estimation is compatible with the buffer used for making the protein lysate. Check the quality of the protein lysate by running an SDS-PAGE gel.

3 Protein digestion

3.1 Take 50 µg of protein in a micro-centrifuge tube and reduce the proteins by adding TCEP such that the final concentration is 20 mM. Incubate the contents at 37 °C for 1 h.

3.2 Following incubation, alkylate the reduced proteins by adding iodoacetamide (IAA) to the tube such that its final concentration is 40 mM. Incubate the tube in dark for 30 minutes.

NOTE: Prepare IAA freshly right before its addition to the tube.

3.3 Add buffer B to the tube containing the reduced and alkylated proteins such that the final concentration of urea in the tube is less than 1 M.

NOTE: Buffer B is composed of 25 mM Tris (pH 8.0) and 1 mM $CaCl_2$ and is used for diluting the samples. Upon dilution, ensure that the contents of the tube have a pH of 8 for optimum protein digestion after addition of trypsin.

3.4 Add trypsin in a 1:30 enzyme to protein ratio and incubate the tubes overnight at 37 °C

with constant shaking.

3.5 Following digestion, concentrate the digested product in a vacuum concentrator. At this step, the peptides can either be reconstituted and desalted or stored at -80 °C for future use.

4 Desalting and peptide quantification

NOTE: Desalting or peptide clean-up is essential before loading the samples on any LC-MS/MS. Salts and other contaminants in the sample can clog the columns and cause damage to the instrument as well. The process can be performed using commercially available C18 stage-tips or columns.

4.1 Activate the stage tip with 20 µL of 50% acetonitrile (ACN) in 0.1% formic acid (FA). Centrifuge the contents at 1,000 x g for 1 minute and discard the flow through.

NOTE: Conditions for centrifugation are the same till the end of the procedure.

4.2 Add 20 µL of 100% ACN in 0.1% FA and centrifuge the contents as in step 4.1.

NOTE: Activation steps can be repeated a couple of times.

4.3 Following activation, pass 20 µL of reconstituted peptide sample through the stage-tip and centrifuge as performed in step 4.1.

NOTE: Do not discard the flow through in this step.

4.4 Repeat step 4.3 with the flow through at least 5 times to ensure maximal peptide binding to the stage-tip.

4.5 Pass 20 µL of 0.1% FA through the stage-tip and discard the flow through.

NOTE: Repeat this step two more times for better results.

4.6 Elute the bound peptides in a fresh microfuge tube by passing increasing concentrations of ACN, i.e., 40%, 60% and 80%, respectively.

4.7 Dry the peptides in a vacuum concentrator and proceed for peptide quantification.

4.8 Reconstitute the dried peptides in 0.1% FA and quantify using the Scopes method[5].

5 Transition list preparation of finalized targets

NOTE: A transition refers to the pair of precursors (Q1) to product (Q3) m/z values in an SRM experiment. A peptide can have one to many transitions, with the same Q1 value but different Q3 values. A triple quadrupole mass spectrometer requires information of the transitions for the peptides and their products to be detected. Hence, before starting a targeted experiment, a transition list needs to be prepared. This can be done using the online repository of SRMAtlas[6] (https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/GetTransitions) or an open source software called Skyline[7] (https://skyline.ms/project/home/software/Skyline/begin.view).

5.1 Download the recent human proteome FASTA file from UniProt (https://www.uniprot.org/) and create a background proteome file by inserting it in Skyline. In peptide settings, go to the **Background Proteome** dropdown and click on **Add**. Feed in the FASTA sequence file of the human proteome. Make sure this database is selected and the allowed missed cleavage value is set to 0 before proceeding to the next step.

5.2 Now, under the **Filter** tab in **Peptide Settings** delimit the length of accepted peptides from 8 to 25 amino acids.

5.3 In **Transition Settings**, under **Filter** tab set **Precursor Charges** as 2,3, set **Ion Charges** as 1 and set **Ion Types** to y. Product ions can be selected depending on the user's choice. Select **N-terminal to proline** for special ions and leave all other parameters as default.

NOTE: The peptide and transition settings can vary according to the experiment.

5.4 Insert the peptides or proteins of interest by clicking on **Edit** and moving to **Insert**. To insert proteins, copy their accession IDs and to insert specific peptides, copy the peptide sequences. The software maps the accession IDs to the background proteome and the transition list is created based on the peptide and transition settings.

5.5 Export the transition list. Ensure that in the dropdown for **Instrument Type**, the right instrument is selected. For the optimization experiments, one may choose to split the transition lists into smaller numbers by setting the desired number of transitions per file in Skyline. This will ensure that the instrument is not overwhelmed to screen too many transitions in a single run. The number of transitions needs to be further optimized to get a single method – this we mention henceforth under the method refinement section.

6 LC parameters

6.1 Use a binary solvent system with the aqueous solvent containing 0.1% FA (Solvent A) and the organic solvent containing 80% ACN (Solvent B).

6.2 Set the column temperature to 45 ˚C.

6.3 Set an LC gradient of 10 min with a 450 µL/min flowrate (as shown in **Table S1**).

7 MS parameters

NOTE: The explained assay has been developed and optimized for TSQ Altis Triple Quadrupole Mass Spectrometer.

7.1 Optimize runs parameters like Q1 and Q3 resolution, dwell time and cycle time – one parameter at a time. We find that 0.7 resolution for Q1 and Q3 works best. The cycle time or dwell time might need to be tweaked according to the number of transitions and average peak width of the peptides being monitored.

7.2 Use the MS parameters in **Table S2** and **Table S3**. The total method duration is 10 min.

NOTE: The parameters remain same for all the runs during and after method refinement, unless mentioned otherwise. For a fresh experiment, there may be a need to change certain parameters depending on the type of the sample and sample processing steps.

8 Run sequence and Instrument QC

8.1 Prepare a mixture of water: methanol: isopropanol: acetonitrile in 1:1:1:1 ratio. Use this mixture as a blank.

8.2 Prepare peptides for any standard sample that can be used to consistently monitor the performance of the instrument. This will be used as a QC standard. We detect peptides from BSA digests that have been optimized and give good and consistent response over several days (**Figure 2**).

8.3 To run samples, the sequence should start with a couple of blanks, followed by the QC standard and clinical samples. Always ensure there is one blank between two consecutive samples.

8.4 For the ease of comparison, ensure equal amounts and volumes of each sample are injected every time.

9 Method refinement

9.1 Analyze the preliminary data obtained from the pooled samples using Skyline. Look for the right peak, transitions, and parameters such as peak shape and intensity to select the best results. Save the file as a new Skyline project.

9.2 Use a library to find the best matching peak and consequently the best possible transition list is advised. A library is a set of MS/MS peaks available from literature or in-house experiments.

9.3 Export a fresh transition list from the newly saved Skyline project and use this transition list to make a fresh method. Acquire data from the new method created and repeat the process of refining the transitions using Skyline.

9.4 Once the list of peptides and transitions is finalized following data analysis using Skyline, fine tune the method by trying out different permutations of cycle time and dwell time.

NOTE: The use of heavy labelled synthetic peptides and indexed retention time (iRT) peptides makes method refinement easy[8, 9]. It is hence advisable that fine tuning of the method be performed using these peptides.

9.5 Using the retention time information from the refinement experiments, create a final method that is scheduled (having a defined acquisition window).

9.6 Prepare samples for individual subjects. Data will be acquired for these samples using the final scheduled method.

9.7 Once the data is acquired, further downstream analysis and group-wise comparison can be performed by importing the raw files to Skyline.

REPRESENTATIVE RESULTS:

We performed relative quantification of 3 proteins from 10 samples, 5 samples from each group of patients with abnormalities in the brain. These proteins included Apolipoprotein A-I (APOA-I), Vimentin (VIM) and Nicotinamide phosphoribosyltransferase (NAMPT) which are known to perform diverse roles in the brain cells. Post-run analysis of the data was performed using Skyline-daily (Ver 20.2.1.286). A total of 10 peptides corresponding to 3 proteins were monitored. These included 3 peptides for APOA-I, 4 peptides for VIM and 3 peptides for NAMPT. The total number of transitions from these 10 peptides amounted to 57. The samples were grouped into either of the two groups depending on the condition they belonged to. Using the group comparisons feature of skyline, the peak abundances of these peptides were compared, and relative quantification values were calculated (**Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: An overview of steps involved in a Selected Reaction Monitoring experiment.

A. Sample preparation for a typical proteomics experiment involves extraction of proteins (for illustration we have shown tissue sample) followed by digestion using trypsin. The digested peptides are ultimately desalted and made LC-MS ready. **B.** The steps involved in an MRM experiment include precursor and product ion selection based on their m/z values. Only the transitions showing good response are considered for analysis. **C.** The data analysis in an SRM experiment includes a detailed examination of peak shapes and peak areas. This is ultimately followed by statistical analysis of the results.

Figure 2: Consistency in response for BSA using an optimized SRM method.

A. Chromatogram for a representative peptide of BSA shows consistent peak shape and intensity

throughout the five days the experiment was performed. **B.** Retention time consistency observed for the peptide on all the five days of the experiment **C.** Peak areas for the peptide as seen over the course of five days in the week.

Figure 3: Differential regulation of three proteins in two groups of GBM tumor samples. **A.** Representative chromatograms for Apolipoprotein A-I and cumulative peak area as seen following inter-group comparison. **B.** Representative chromatograms for Vimentin and cumulative peak area as seen following inter-group comparison. **C.** Representative chromatograms for Nicotinamide phosphoribosyltransferase and cumulative peak area as seen following inter-group comparison.

Table S1: Details of 10-minute LC gradient to be used for all samples.

Table S2: Parameter settings for the ion source.

Table S3: Parameter settings for SRM method.

DISCUSSION:

Validation of protein targets for a long time was performed using antibody-based assays like immunohistochemistry, western blotting, etc, which were considered the gold-standard. These methods find use even today with minor modifications in the protocol and little dependence on technology making them very cumbersome and tedious techniques. Besides this, they also involve the use of expensive antibodies which do not always show the same specificity across batches and require a great deal of expertise. Additionally, only a small fraction of proteins identified using high throughput techniques like mass spectrometry, have compatible antibodies available, further complicating the whole procedure. Hence targeted proteomic assays are slowly being taken up as the new approach for validating targets[10]. With most of the target discovery happening on high-throughput omics platforms, panels of validated targets are also being considered for clinical screening applications[11–13].

The representative results in this article have validated the differential expression of proteins Apolipoprotein A-I (APOA-I), Vimentin (VIM) and Nicotinamide phosphoribosyltransferase (NAMPT) in two conditions (condition 1 and condition 2) of the brain tissue. Apolipoprotein A-I has been reported to play a pivotal role in maintenance of cerebrovascular integrity and reducing the risk of Alzhiemer's disease. Even though ApoA-I is not synthesized in the brain, its ability to cross the blood brain barrier (BBB) makes its presence in the brain vital[14]. The protein Vimentin has been studied in a number of roles inside the brain. However, one of the key functions of Vimentin is its involvement in microglia activation. Reduced expression of Vimentin was associated with impairment of microglial activation[15]. The protein NAMPT has been reported to play a key role in ageing related loss of neurons and cerebral vascular endothelial dysfunctions[16]. All the three proteins have been reported to play a multitude of roles in normal brain cells and brain related malignancies. Therefore, MRM based validation for these proteins and their peptides can find great use in clinical diagnosis related to various brain related disorders.

A fully optimized targeted assay can be easily used for high-throughput detection and quantification of a target panel. The rate limiting step is the initial method optimization which is tedious and varies based on the sample type, protein/peptide targets, instrument being used and the detection bias of certain peptides. It is crucial that the transition list is optimized for a robust assay. Any user interested in developing such an assay for human brain tissue samples, will find that the above explained protocol minimizes these variable factors. It gives an optimized protocol for peptide extraction from this unique and tedious sample and optimal parameters to be used in the instrument with special attention to crucial quality control steps at various points of the protocol. As with any new technology, the researchers have provided a set of guidelines, which authors need to furnish or what steps they need to follow during the experiment to ensure best overall output. To this front, in 2017, the MCP guidelines for reporting targeted proteomics assays and data were laid down[17]. These guidelines ensure that the reported work is reliable and reproducible, hence increasing the applicability of the method. By taking the right precautions and utilising the true potential of this assay, researchers would soon be able to come up with clinically relevant assays with immense potential in diagnosis and therapeutics.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors received support from Thermofisher for the publication fee.

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Figure 1

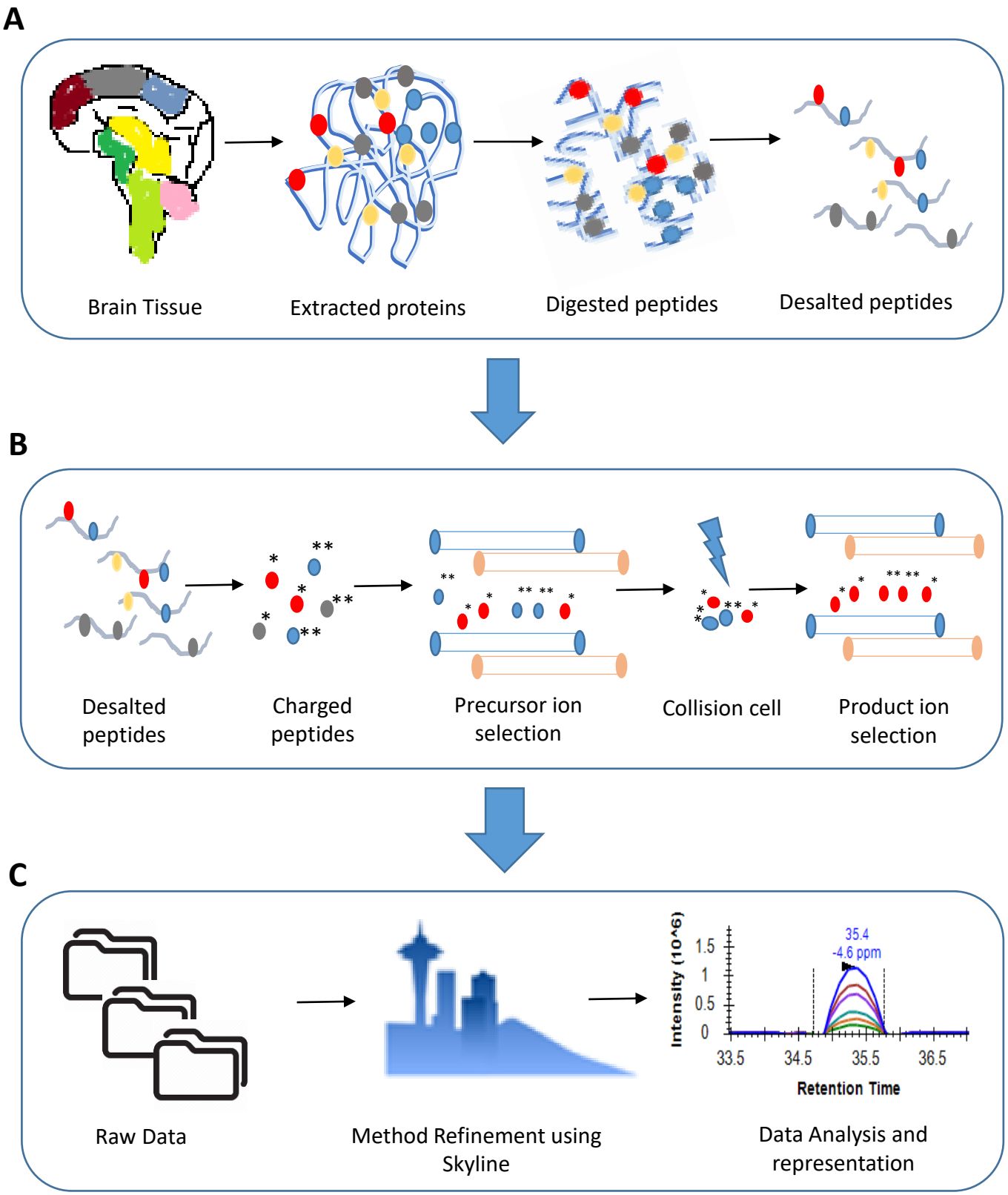


Figure 2

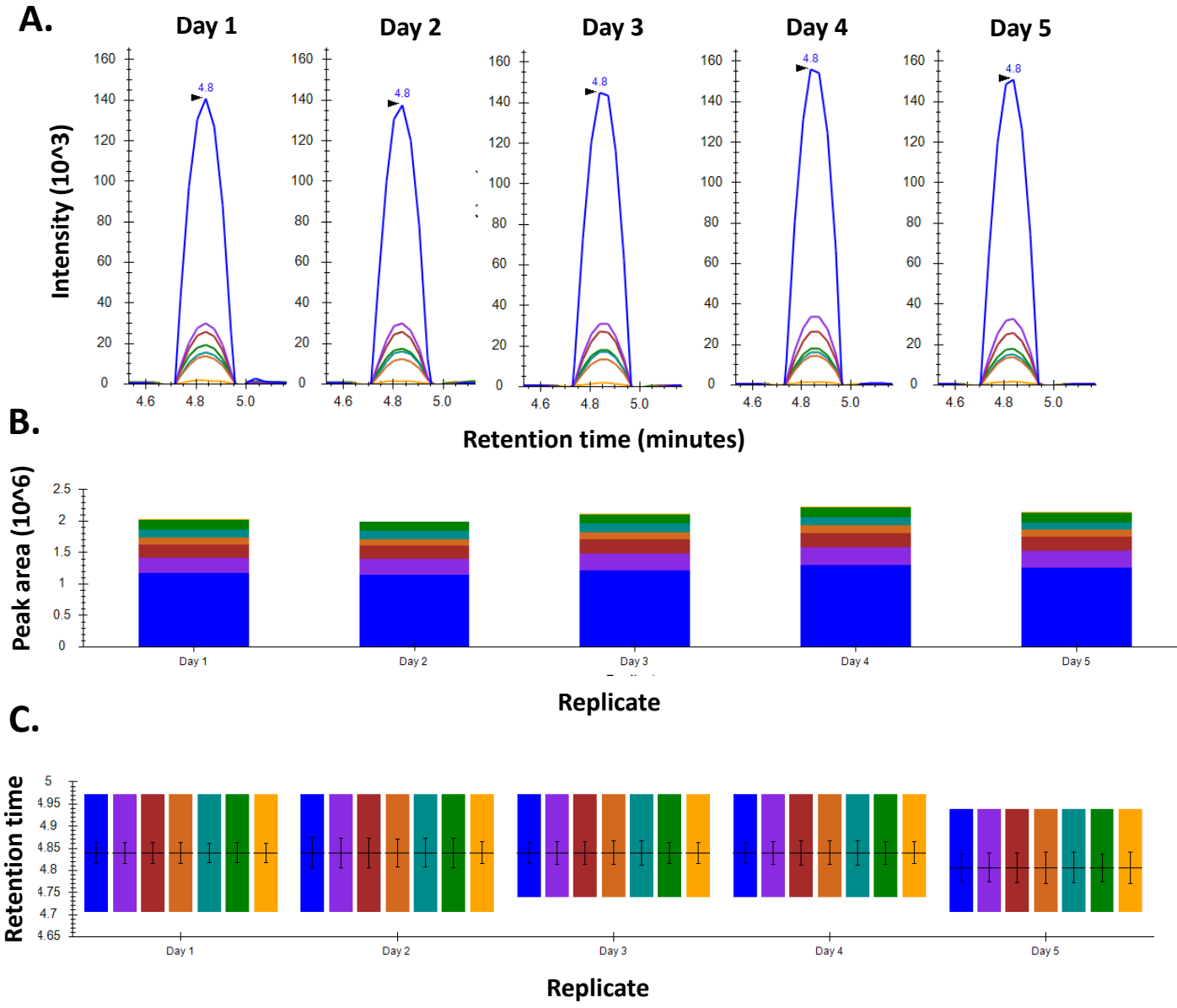
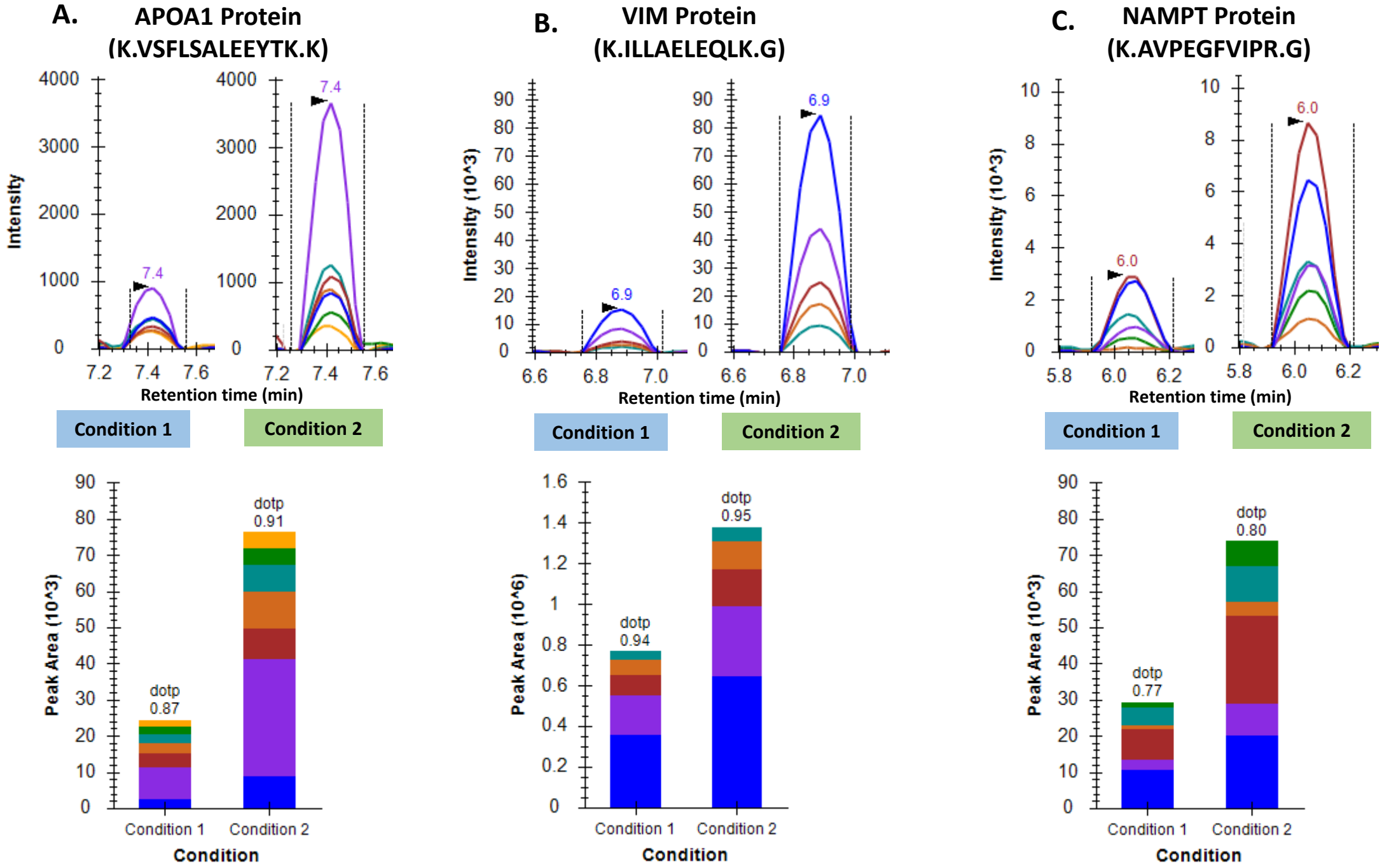


Figure 3



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JoVE61833 rebuttal letter

Dear Editors and Reviewers,

We thank you for your critical comments that have contributed in increasing the quality of our manuscript and video. We have addressed each of the comments sequentially.

Editorial and production comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We thank the editorial board for giving us this opportunity. We have gone through the whole manuscript and done the needful.

Revision made: A major part of the manuscript has been re-written. The current manuscript has been proof-read and all spellings and grammatical errors were rectified.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

Response: We have specifically checked for any such symbols throughout the manuscript and omitted them. We have also reduced commercial terminology from the text to a large extent.

Revision made: The manuscript was edited accordingly.

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "TSQ Altis" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

Response: We acknowledge your point in this respect and have reduced the number of instances the term "TSQ Altis" has been used in both the video and manuscript.

Revision made: The script for the introduction part of the video was changed and clip was reshot. The mention of the term "TSQ Altis" in the manuscript has now been limited to only wherever absolutely essential.

4. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Response: We have added an ethics statement supporting the use of human brain tissues used in the study

Following changes are incorporated in the text: Please refer to lines 101 to 104

"Ethics statement
This study involves brain tissue samples from human participants, reviewed and approved by TMH and IITB IEC - (IITB-IEC/2018/019). The participants provided their informed and written consent to participate in this study."

5. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.
Response: We have made a table of essential supplies according to the guidelines without missing any reagents or supplies required to reproduce the experiment.
Revision made: "Table of Materials" has been attached along with other files for submission.

6. Changes to be made by the Author(s) regarding the video:
i) Please increase the homogeneity between the video and the written manuscript. Ideally, the narration is a word for word reading of the written protocol.
For example: 3:51-4:30: describing step ("activation") in terms of its function, not in terms of how to execute it. Please ensure that all details to replicate the protocol are included.
Response: We have edited the script and manuscript accordingly. We have recorded the voice-over and edited into the video so that the homogeneity is maintained.
Edits made: Video voice-over re-recorded and edited.

ii) Video & Audio Editing
• 00:05 - 00:41, 11:04-end The interview of Saicharan Ghantasala is out of sync (audio is slightly delayed). Please check the audio-video synchronization
Response: We agree with your comment. We have reshot this segment and added audio so that there are no technical issues.
Edits made: Introduction and conclusion of the video were reshot and audio was synced.

• 2:51 please change this jump cut to at least a quick dissolve/fade
Response: We agree this jump cut seemed jarring to the eye. We have made the required edits.
Edits made: The suggested edit was made in the video.

•3:13-3:20, 4:30-4:33: There is a strange background noise (distorted birdsong?) at beginning and end of step. Consider re-recording this step
Response: We have recorded major parts of the narration again, with care to avoid disturbing background noise.
Edits made: The narration was recorded and suggested edits were made in the video.

•8:08 " please refer text" should be "please refer to the text
Response: We have edited this text at all the places in the video.
Edits made: The suggested edit was made in the video.

•8:38, 10:21 the screen capture software pop-up is briefly visible. Please cut or cover this.
Response: We checked the video and noticed the pop-ups pointed out here. We have made the required changes.

Edits made: The suggested edit was made in the video

•9:17-10:24 this screen cap appears to be a much lower resolution than the others.
Response: Due to some problem during file conversion this clip was changed to its low-resolution version. We have removed it and added the full resolution clip.
Edits made: The low-resolution clip was replaced with a clip of resolution matching all other screen captures.

• 11:05 this harsh cut from black to footage is jarring. please consider a fade here.
Response: We have added the fade here.
Edits made: The suggested edit was made in the video.

iii) Chapter Title Cards + Text
• 00:42 Consider adopting a single capitalization style for titles. For example, at this chapter card, ""from"" would be lowercase to follow Title Case style.
Response: We realised this problem with many of our title cards and have added the properly edited title cards to the video now.
Edits made: The suggested edit was made in the video.

• 1:16, 3:07, 8:07 please change "refer text..." to "refer to text" and consider keeping text overlays on screen for a couple more seconds to make them easier to read.
Response: We checked the video and noticed the pop-ups pointed out here. We have made the required changes.
Edits made: The suggested edits were made in the video.

• 2:16, 5:17, 7:42, 8:13, 8:18, 8:35, 8:37, 9:14, 9:16, 11:04 consider fading to a solid color like white before fading to the title to make the transition clearer and cleaner. For instance, at 2:19, the title fades to black before the footage fades up.
Response: Thank you for bringing this to our notice. We have added fade to white before each title card comes up.
Edits made: The suggested edits were made in the video.

• 8:36: consider holding this title card on screen for a few more seconds to give viewers a chance to read it.
Response: We have increased the time for which this title card appears.
Edits made: The suggested edits were made in the video.

Please upload a revised high-resolution video here:
https://www.dropbox.com/request/P8HqXpeDnRIitpdV9qr9?oref=e
Response: We have uploaded our high-resolution video in this link. We have also attached a low-resolution version with the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript by Ghantasala et al. describes standard peptide workflow for triple quadrupole LCMS instruments using Skyline SW. This is a common protocol and I think that it could be interesting for the audience to see it in JoVE. However, to make this valuable, some significant mistakes need to be corrected.

Major Concerns:

The manuscript contains parts that are clearly wrong, for instance description of the quadrupole modes (ln 55 and further). 1/The DC and RF is not connected in the way the authors describe. 2/RF mode in quadrupole means that the quadrupole is not selecting any ions, but allowing every ion as lack of DC leads to zero resolution. 3/ there are 4 modes of operation of a triplequad. The authors are only using SRM. If they want to describe triplequad theory, it needs to be done correctly.

Response: We thank the reviewer for pointing out the glaring mistakes in the introduction section of the previous version of the manuscript. We have made substantial changes and corrections to the introduction section in the current version of the manuscript. We believe that the manuscript in its current form correctly describes the theory of a triple quadrupole mass spectrometer in a simple language.

Edits made: The entire paragraph has been re-written and there is no overlap whatsoever between the older version and the new version of the manuscript.

Following changes are incorporated in the text: Line No 69 to 84

"A quadrupole is made of 4 parallel rods, with each rod connected to the diagonally opposite rod. A fluctuating field is created inside the quadrupole rods by applying alternating RF and DC voltages. The trajectory of the ions inside the quadrupole is influenced by the presence of the same voltages across opposite rods. By applying the RF to DC voltage, the trajectory of the ions can be stabilized. It is this this property of the quadrupole that allows it to be used as a mass filter which can selectively let specific ions to pass through. Depending on the need, a quadrupole can be operated in either the static mode or the scanning mode. The static mode allows only ions with m/z to pass through, making the mode highly selective and specific to the ion of interest. The scanning mode on the other hand allows ions across the entire m/z range to pass through. Thus, tandem quadrupole mass spectrometers can operate in 4 possible ways, i.e., i) the first quadrupole operating in the static mode while the second operating in scanning mode, ii) the first quadrupole operating in the scanning mode while the second operating in the static mode, iii) both quadrupoles operating in the scanning mode, and iv) both quadrupoles operating in static mode. In a typical multiple reaction monitoring experiment, both the quadrupoles operate in the static mode allowing specific precursors and their resulting products after fragmentation to be monitored. This makes the technique very sensitive and selective allowing accurate quantification."

For this type of manuscript, the description of the Skyline workflow and the peptide optimization is the most important part from didactical perspective. This is not sufficiently done. You need to clearly describe how the parameters are optimized, how is the CE optimized and if internal or optimize RF voltage on the electrodynamic funnel is used. The user/reader needs to be able to use the JoVE contribution for their full benefit. Better explanation of using direct dwell time vs cycle time is also needed, although on modern systems only the cycle time should be used for large assays.

Response: We thank the reviewer for the suggestions provided and agree that the additional information would benefit the readers. However, the main aim of our manuscript is to introduce the technique and its potential to a broader research community that is still reliant of molecular biology-based techniques such as western blotting, ELISA, Immunohistochemistry or FISH for validation of multiple target proteins. It is for this reason that we have used a simplified workflow that is not too overwhelming for a first-time reader.

Reviewer #2:

This manuscript is basically materials and methods only! Since this M&M is not a novel approach/technique /protocol, I can't see why it needs to be published !!? Many labs use similar protocols and mass spec techniques for protein extraction from brain tissues and other, but no one publishes the protocol only. Hence, I don't find this type or article will benefit others, and its not suitable for publishing ....

PS/ The English language needs improvement.

Response: We appreciate the criticism from the reviewer and thank him for providing us a perspective. We have re-written the manuscript and re-shot the video to provide the readers valuable information that may help them in setting up MRM experiments in the near future.

Reviewer #3:

1. In the introduction, the authors should mention that DDA or DIA performed on Qtof/orbitrap type of MS are extensively used for quantitative proteomics analysis of tissue/cell. MRM has the advantage of high specificity and sensitivity; and can be performed even with a low resolution, slow triple Quad MS.

Response: We have incorporated the suggestions in the revised version of the manuscript.
Following changes are incorporated in the text: Please refer to line 54 to 67

"However, shotgun proteomics approaches (Data dependent Acquisition or DDA) often fail to detect low abundant proteins in complex tissues besides being heavily reliant on the sensitivity and resolution of the instrument. Over the last couple of years, labs around the world have been developing techniques like Data Independent Acquisition (DIA) which require increased computing power and reliable software that can handle these highly complex datasets. However, these techniques are still a work in progress and not very user friendly. Targeted MS-based proteomics approaches provide a perfect balance between the high throughput nature of MS approaches and the sensitivity of molecular biology approaches like ELISA. A targeted mass spectrometry-based proteomics experiment focuses on detecting hypothesis driven proteins or peptides from past proteomics experiments or through

available literature[1,2]. Multiple reaction monitoring is one such targeted MS approach that uses a tandem quadrupole mass spectrometer for accurate detection and quantification of proteins/ peptides from complex samples. The technique offers higher sensitivity and specificity despite requiring the use of a low-resolution instrument.'

2. Line 123, "3.1 Take 100 µg of protein in a micro-centrifuge tube and add freshly prepared Digestion Buffer B* (500 mM ammonium bicarbonate, pH 8.0) to make up the volume to 20 µL.". It may be useful to mention that urea concentration should be diluted from 8M to preferably <1M. In this respect, the final volume of 20 µL seems small?

Response: We have edited this section of the manuscript and the error has been rectified

Edits made: Suggested changes made to the manuscript.

Following changes are incorporated in the text: Please refer to line 135 to 144

3.1 "Take 50 µg of protein in a micro-centrifuge tube and reduce the proteins by adding TCEP such that the final concentration is 20mM. Incubate the contents at 37 °C for 1 hour.

3.2 Following incubation, alkylate the reduced proteins by adding Iodoacetamide (IAA) to the tube such that its final concentration is 40mM. Incubate the tube in dark for 30 minutes. NOTE: Prepare IAA freshly right before its addition to the tube.

3.3 Add buffer B to the tube containing the reduced and alkylated proteins such that the final concentration of Urea in the tube is less than 1M.

NOTE: Buffer B is composed of 25mM Tris (pH- 8.0) and 1mM $CaCl_2$ and is used for diluting the samples. Upon dilution, ensure that the contents of the tube have a pH of 8 for optimum protein digestion after addition of trypsin."

3. Line 142, ´ "4.1 Prepare C18 zip-tips by taking a plug of C18 material from Empore C18 extraction disks in a 20 µL tip. Ensure that each tip has approximately the same amount of C18 to avoid 143 variations in the result." It is not zipTip; it is StageTip. I strongly advice the authors to show how they prepare the StageTip.

Response: We agree with comment and have incorporated the corrections pointed out to us. Additionally, considering the variations accompanying making of stage-tips *in-house* in comparison to the more reliable commercially available stage-tips or columns (C18 spin columns or Sep pak columns), we have made a conscious effort to refrain from introducing reader to the process of making an in-house stage-tip.

Edits made: Edits were made in the manuscript to replace the word zipTip with Stage tips.

Following changes are incorporated in the text: Please refer to line 151 to 171.

"Desalting and Peptide Quantification

NOTE: Desalting or peptide clean-up is essential before loading the samples on any LC-MS/MS. Salts and other contaminants in the sample can clog the columns and cause damage to the instrument as well. The process can be performed using commercially available C18 stage-tips or columns.

4.1 Activate the stage tip with 20uL 50% Acetonitrile (ACN) in 0.1% Formic acid (FA). Centrifuge the contents at 1000g for 1 minute and discard the flow through.

NOTE: Conditions for centrifugation are the same till the end of the procedure.

4.2 Add 20uL of 100% ACN in 0.1% FA and centrifuge the contents as in step 4.1.

NOTE: Activation steps can be repeated a couple of times.

4.3 Following activation, pass 20uL of reconstituted peptide sample through the stage-tip and centrifuge as performed in step 4.1.

NOTE: Do not discard the flow through in this step.

4.4 Repeat step 4.3 with the flow through at least 5 times to ensure maximal peptide binding to the stage-tip.

4.5 Pass 20uL of 0.1% FA through the stage-tip and discard the flow through.

NOTE: Repeat this step two more times for better results.

4.6 Elute the bound peptides in a fresh microfuge tube by passing increasing concentrations of ACN, i.e., 40%, 60% and 80%, respectively.

4.7 Dry the peptides in a vacuum concentrator and proceed for peptide quantification.

4.8 Reconstitute the dried peptides in 0.1% FA and quantify using the Scopes method[4]. "

4. "4.2 Activate 5 zip-tips for desalting 100 µg of digested peptide. Add 20 µL of 50% ACN in 0.1% FA to each of the 5 zip-tips and centrifuge briefly to let the solution pass through. Now add 20 µL of 100% ACN in 0.1% FA and pass through again". The authors should provide the speed and duration of centrifugation, which could be adjusted according to the nature of the sample and the input volume.

Response: We have added the details for centrifugation in the manuscript.
Edits made: The suggested edits were made.
Following changes are incorporated in the text: Please refer to line 151 to 171.

5. Line 169, " an open source software called Skyline", the website of Skyline should be included.

Response: We have now added the link to the websites in the manuscript. Also, we have already cited the original paper for the same. We found it fit to include the website link for SRMAtlas too.
Edits made: Link to the website added in the manuscript.
Following changes are incorporated in the text: Please refer to line 178 to 180.

"This can be done using the online repository of SRMAtlas[5,6] (https://db.systemsbiology.net/sbeams/cgi/PeptideAtlas/GetTransitions) or an open source software called Skyline[7] (https://skyline.ms/project/home/software/Skyline/begin.view)."

6. Line 170, "Download the recent human proteome FASTA file from UniProt", provide UniProt website.

Response: We have added the link to Uniprot home page.
Edits made: The suggested edits were made.
Following changes are incorporated in the text: Please refer to line 181 to 182

"Download the recent human proteome FASTA file from UniProt (https://www.uniprot.org/) and create a background proteome file by inserting it in Skyline."

7. Line 182, what is "The software b".

Response: Thank you for pointing this out. We realised this sentence was mistakenly left incomplete. We have now added the full sentence in the manuscript.
Edits made: The incomplete sentence was completed
Following changes are incorporated in the text: Please refer to line 194 to 195

"The software maps the accession IDs to the background proteome and the transition list is created based on the peptide and transition settings."

Supplemental Table 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Table S1.xlsx

Time [mm:ss]	Flow [µl/min]	%B	Curve
0:00	450	2	5
6:00	450	45	5
6:50	450	95	5
7:00	450	95	5
7:50	450	2	5
10:00	450	2	5

Supplemental Table 2

Ion Source Type	**NSI**
Spray Voltage	**Static**
Positive Ion (V)	**2100**
Negative Ion (V)	**600**
Sweep Gas (Arb)	**0**
Ion Transfer Tube Temp (°C)	**300**

Supplemental Table 3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Table S3.xlsx

Parameter	Comments
Use Cycle Time	**TRUE**
Cycle Time (sec)	**2**
Use Calibrated RF Lens	**TRUE**
Q1 Resolution (FWHM)	**0.7**
Q3 Resolution (FWHM)	**0.7**
CID Gas (mTorr)	**2.5**
Source Fragmentation (V)	**0**
Chromatographic Peak Width (sec)	**20**
Use Chromatographic Filter	**FALSE**
Use Retention Time Reference	**FALSE**
Display Retention Time	**FALSE**
Use Quan Ion	**FALSE**
Show Visualization	**FALSE**