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Comprehensive Workflow of Mass Spectrometry-based Shotgun Proteomics of Tissue Samples

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TITLE:

Comprehensive Workflow of Mass Spectrometry-based Shotgun Proteomics of Tissue Samples

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KEYWORDS:

label-free proteomics, label-based proteomics, tissue proteomics, mass spectrometer, in-solution digestion, data analysis

SUMMARY:

The described protocol provides an optimized quantitative proteomics analysis of tissue samples using two approaches: label-based and label free quantitation. Label-based approaches have the advantage of more accurate quantitation of proteins, while a label-free approach is more cost-effective and used to analyze hundreds of samples of a cohort.

ABSTRACT:

Recent advances in mass spectrometry have resulted in deep proteome analysis and robust, reproducible datasets. However, despite the considerable technical advancements, sample preparation from biospecimens such as patient blood, CSF, and tissue specimen still poses considerable challenges. For identifying biomarkers, tissue proteomics often provides an attractive sample source to translate the research findings from the bench to the clinic. It can reveal potential candidate biomarkers for early diagnosis of cancer and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, etc. Tissue proteomics also yields a wealth of systemic information based on the abundance of proteins and helps to address interesting biological questions.

Quantitative proteomics analysis can be grouped into two broad categories: a label-based approach and a label-free approach. In the label-based approach, proteins or peptides are

labeled using stable isotopes such as SILAC (stable isotope labeling with amino acids in cell culture) or by chemical tags such as ICAT (isotope-coded affinity tags), TMT (tandem mass tag) or iTRAQ (isobaric tag for relative and absolute quantitation). Label-based approaches have the advantage of more accurate quantitation of proteins and using isobaric labels, multiple samples can be analyzed in a single experiment. The label-free approach provides a cost-effective alternative to label-based approaches. Hundreds of patient samples belonging to a particular cohort can be analyzed and compared with other cohorts based on clinical features. Here, we have described an optimized quantitative proteomics workflow for tissue samples using label-free and label-based proteome profiling methods, which is crucial for applications in life sciences, especially biomarker discovery-based projects.

INTRODUCTION:

Proteomics technologies have the potential to enable the identification and quantification of potential candidate markers that can aid in the detection and prognostication of the disease[1]. Recent advancement in the field of mass spectrometry has accelerated clinical research at the protein level. Researchers are trying to address the challenge of complicated pathobiology of several diseases using mass spectrometry-based proteomics, which now offers increased sensitivity for protein identification and quantification[2]. Accurate quantitative measurement of proteins is crucial to comprehend the dynamic and spatial cooperation among proteins in healthy and diseased individuals[3]; however, such analysis at the proteome-wide scale is not easy.

One major limitation of proteomic profiling of clinical specimens is the complexity of biological samples. Many different types of samples have been investigated to study the disease proteome, such as cell lines, plasma, and tissues[4,5]. Cell lines are widely used as models in *in vitro* experiments to mimic different stages of disease progression. However, one major limitation with cell lines is that they easily acquire genotypic and phenotypic changes during the process of cell culture[6]. Body fluids such as plasma could be an attractive source for biomarker discovery; however, due to the highly abundant proteins and dynamic range of protein concentration, plasma proteomics is a bit more challenging[7]. Here, peptides originated from the most abundant proteins can suppress those derived from the low abundant proteins even if the mass/charge ratio is the same[6]. Although there have been advancements in the depletion and fractionation technologies in the last few years, getting good coverage still remains a major limitation of plasma proteomics[8,9]. The use of tissues for proteomic investigation of disease biology is preferred as tissue samples are most proximal to the diseases and offer high physiological and pathological information to provide better insights into the disease biology[10,11].

In this manuscript, we have provided a simplified protocol for the quantitative proteomics of tissue samples. We have used a buffer containing 8 M urea for the tissue lysate preparation as this buffer is compatible with mass spectrometry-based investigations. However, it is mandatory to clean the peptides to remove salts before injecting them into the mass spectrometer. One important point to remember is to reduce the urea concentration to less than 1 M before adding trypsin for protein digestion as trypsin exhibits low activity at 8 M urea concentration. We have explained two approaches of quantitative global proteomics: label-based quantification using iTRAQ (isobaric tags for relative and absolute quantification) and label-free quantification (LFQ).

The iTRAQ-based quantitative proteomics is mainly used for comparing multiple samples varying in their biological condition (e.g., normal versus disease or treated samples). The approach utilizes isobaric reagents to label the N-terminal primary amines of peptides[12]. The iTRAQ reagents contain one N-methyl piperazine reporter group, a balancer group, and one N-hydroxy succinimide ester group that reacts with N-terminal primary amines of peptides[13]. Digested peptides from each condition are labeled with a particular iTRAQ reagent. Following the labeling, the reaction is stopped and labeled peptides from different conditions are pooled into a single tube. This combined sample mixture is analyzed by mass spectrometer for identification and quantification. After the MS/MS analysis, reporter ion fragments with low molecular masses are generated and the ion intensities of these reporter ions are used for the quantification of the proteins.

Another approach, label-free quantification is used to determine the relative number of proteins in complex samples without labeling peptides with stable isotopes.

PROTOCOL:

This study was reviewed and approved by institutional review boards and the ethics committee of the Indian Institute of Technology Bombay (IITB-IEC/2016/026). The patients/participants provided their written consent to participate in this study.

1. Tissue lysate preparation

NOTE: Perform all the following steps on the ice to keep the proteases inactive. Make sure the scalpels and any tubes used are sterile to avoid any cross-contamination.

1.1. Take ~30 mg of tissue in a bead beating tube, add 200 µL of 1x phosphate buffer saline (PBS) and vortex it.

NOTE: In this study, fresh frozen human brain tumor tissues were taken for the lysate preparation. The protocol can be used for any fresh frozen tissue with some changes depending on the type of tissues (soft or hard tissues) and cellular complexity of the tissues.

1.2. After that, spin the tube to settle the tissue and carefully remove the PBS using a pipette. Perform another PBS wash if there are still traces of blood left in the tissue.

1.3. Add 300 µL of urea lysis buffer (8 M urea, 50 mM Tris pH 8.0, 75 mM NaCl, 1 mM $MgCl_2$) and protease inhibitor cocktail (PIC) as per the manufacturer's protocol.

NOTE: The volume of the lysis buffer should be enough to grind the tissue during the sonication process and to suspend what is extracted. Too little lysis buffer may result in inefficient tissue lysis, while too much lysis buffer will dilute the protein lysate.

1.4. Place the tube on ice and sonicate the tissue at an amplitude of 40% for 2.5 min with pulse

cycles of 5 s (ON/OFF, respectively).

1.5. Add zirconium beads to the tubes and homogenize the tissue using a bead beater for 90 s with 5 min incubation on ice. Repeat this step twice.

1.6. Once the tissue is adequately homogenized, incubate the tube on ice for 10 min.

1.7. After incubation, centrifuge the sample at 6,018 x *g* for 15 min at 4 °C to separate the cell debris from the supernatant.

1.8. Collect the supernatant in the fresh labeled tube and store at -80 °C as aliquots until further use.

2. Protein quantification and quality check of tissue lysates

2.1. Quantify the protein concentration in the tissue lysate using Bradford's reagent as described in the **Supplementary File 1**.

2.2. Following the protein quantification, run 10 µg of tissue lysate on a 12% SDS-PAGE gel to check the quality of the lysate.

NOTE: Further downstream processing must be carried out only for the lysates clearing the quality checks.

3. Enzymatic digestion of proteins

NOTE: The steps for enzymatic digestion are shown in **Figure 1a**.

3.1. For digestion, take 50 µg of proteins and add ddH_2O to make up the volume to 20 µL.

3.2. Now, prepare 20 mM Tris (2-carboxyethyl) phosphine (TCEP) from the stock (0.5 M TCEP) by adding 0.8 µL from stock to the protein lysate to reduce the disulfide bonds in the proteins and incubate the sample at 37 °C for 60 min.

3.3. Prepare 40 mM iodoacetamide (IAA) in ddH_2O and add 1.6 µL to alkylate the reduced cysteine residues. Incubate in the dark for 10 min at room temperature.

3.4. Add dilution buffer containing 25 mM Tris pH 8.0 and 1 mM $CaCl_2$ in a 1:8 ratio to dilute the urea concentration to less than 1 M in the sample. At this point, check the pH.

NOTE: If using trypsin as a digestion enzyme, make sure the concentration of urea is less than 1 M.

3.5. To perform digestion, add trypsin at an enzyme/substrate ratio of 1:50. Incubate the

tubes at 37 °C in a shaking dry bath for 16 h for overnight digestion.

NOTE: The trypsin enzyme is a highly reactive protease that is prone to self-digestion. Take extra care and perform the addition of trypsin swiftly over the ice.

3.6. After 16 h of incubation, dry the digested peptides in a vacuum concentrator.

4. Desalting of digested peptides

NOTE: To perform the desalting of peptides, use C18 stage tips.

4.1. Activate the C18 stage tip by adding 50 µL of methanol. Centrifuge the tip at 1,000 x *g* for 2 min at RT. Discard the filtrate collected at the bottom of the tube. Repeat twice.

4.2. Add 50 µL of acetonitrile in 0.1% formic acid to wash the stage tip. Centrifuge the tube at 1,000 x *g* for 2 min at RT. Discard the filtrate collected at the bottom of the tube. Repeat this step twice.

4.3. Add 50 µL of 0.1% (v/v) FA to equilibrate the column. Again, perform the centrifugation at 1,000 x *g* for 2 min at RT and discard the filtrate.

4.4. Reconstitute the dried digested peptides in 50 µL of 0.1% formic acid.

NOTE: Avoid air bubble formation inside the stage tips while passing the sample. The stage tips should not be completely dried during the centrifugation step, as drying can lead to peptide loss.

4.5. Add the reconstituted peptides into the activated stage tip and pass the sample through the stage tip by centrifugation at 1,000 x *g* for 2 min. Repeat this step at least four times. Store the flow-through at 4 °C.

4.6. To wash the sample, add 50 µL of 0.1% (v/v) formic acid. Repeat the centrifugation step and discard the filtrate.

4.7. For the elution of peptides, add 50 µL of 40% (v/v) ACN in 0.1% formic acid (v/v) and pass it through the stage tip by centrifugation. Collect the filtrate in a fresh tube. Repeat the step with 50% and 60% ACN in 0.1% formic acid and collect the filtrate in the same fresh tube.

4.8. Dry the desalted peptides collected in the fresh tube using a vacuum concentrator.

NOTE: The dried desalted peptides are ready to be injected, or it can be stored at -20 °C for 6 months. For long-term storage (>6 months), store the peptides at -80 °C.

5. Quantification of desalted peptides

5.1. Reconstitute the dried desalted peptides in 0.1% FA.

5.2. Wipe the photometric measurement plate with lint -free tissue using 70% ethanol.

5.3. Use 2 µL of 0.1% FA to set the blank.

5.4. Add 2 µL of reconstituted samples onto the plate in replicates.

5.5. Place the plate in the spectrophotometer and measure the absorbance at 205 nm and 280 nm.

5.6. Calculate Molar Absorptivity (ε) using the following formula:

$\varepsilon = 27 / [1 - 3.85 * A280 / A205]$

NOTE: Molar absorptivity (ε) is a measure of the probability of the electronic transition or how well a species absorbs the particular wavelength of radiation that is being shined on it. The value of ε should be in the range of 31 mL $mg^{-1}cm^{-1}$ to 33 mL $mg^{-1}cm^{-1}$. If the value does not fall in the range, this indicates that the samples are not properly digested.

5.7. Calculate the peptide concentration in µg/µL using the following formula:

Concentration of peptide = Net OD (205) / 0.051 * ε

6. Label-free quantitation (LFQ) of the digested peptides

NOTE: For label-free quantitation, use the LC and MS parameters mentioned in the **Supplementary File 2**. A high coverage data was obtained when three biological replicates of the same type of the sample were run in the mass spectrometer.

6.1. Liquid chromatography setup

6.1.1. After the quantification of desalted peptides, take 2 µg of peptides in a vial and make up the volume to 10 µL using 0.1% FA. The concentration of desalted peptides will be 200 ng/µL.

6.1.2. Open the auto-sampler of the liquid chromatography system (see **Table of Materials**) and place the vial inside the autosampler.

6.1.3. Use 0.1% (v/v) FA to equilibrate the pre-column and analytical column. Repeat this step five times.

6.1.4. Take 1 µg of desalted digested peptide from the vial and load it onto the column.

6.1.5. Set the LC gradient according to the sample complexity. In this experiment, LC gradient

was used for 120 min for label-free quantitation of the tissue samples.

6.2. MS setup: Before optimizing any proteomics assays, perform a quality control check of the instrument by monitoring some peptides of Bovine Serum Albumin (BSA) sample using any software for system suitability and analyzing coverage of BSA (**Figure 2A,B**). The acquisition parameters were set into the instrument using the MS data acquisition software (see **Table of Materials**).

6.2.1. Open the software, double click on **Instrument Set Up** and select the template from peptides-ID with default parameters.

6.2.2. Set the MS parameters using **Supplementary File 2** and **Save** it as a new method.

6.2.3. Now, open the software to fill the sample details; double click on the **Sequence Setup**, and fill in the details such as sample type, sample name, file save location, instrument method file, the volume of injection, and position of the sample.

6.2.4. Once all the information is filled, select the row and start the **Run**.

7. Label-based quantitation (iTRAQ) of digested peptides

NOTE: Label-based quantification can be performed using different isobaric labels such as iTRAQ or TMT reagents, etc. Here, iTRAQ 4-plex was used for the labeling of digested peptides from three tissue samples. The procedure of iTRAQ 4-plex labeling is mentioned below.

7.1. Labeling of digested peptides using iTRAQ reagents.

NOTE: In this experiment, peptides from three tissue samples are used. From each tissue sample, 80 µg of digested peptides are taken in four tubes for labeling with iTRAQ reagents (114, 115, 116, and 117) (see **Supplementary File 3** for the detailed experimental parameters).

7.1.1. Before using the iTRAQ reagent, bring each vial of the reagent to room temperature (approximately 5 min). Give a brief spin of approximately 30 s to bring the solution at the bottom of each vial.

NOTE: Make sure that in each vial, 10–15 µL solution should be present.

7.1.2. For iTRAQ labeling, reconstitute the dried peptides in 20 µL of dissolution buffer provided in the iTRAQ labeling kit.

7.1.3. Reconstitute the labels by adding 70 µL of ethanol from the vial provided in the kit and mix the solution for 30 s and spin it for 10 s.

NOTE: It is advisable that all the steps be carried out as per the manufacturer's instructions.

7.1.4. Add the homogeneously mixed iTRAQ labels (114, 115, 116, and 117) to their respective tubes containing peptide samples and allow for the labeling reaction to take place.

7.1.5. Mix the components of each tube by vortexing the tube for 30 s, and then spin the tube for 10 s to bring the mixture back to the bottom of the tube.

NOTE: Check the pH of the solution using pH paper. pH should be greater than 8; if not, add up to 10 µL of the dissolution buffer to adjust the pH.

7.1.6. Incubate each tube at room temperature for 90 min. At the end of the reaction, quench any excess unbound label in the tube by adding MS grade water.

7.1.7. Incubate the tubes at room temperature for 30 min to 1 h.

7.1.8. Once the incubation is over, transfer all the labeled contents into a single tube and dry the labeled peptides in a vacuum concentrator.

NOTE: A similar labeling procedure can be followed for TMT labeling.

7.2. Liquid chromatography setup

7.2.1. Reconstitute the samples in 0.1% formic acid, open the autosampler of nano LC, and place the samples inside the autosampler. Use the parameters mentioned in **Supplementary File 2** for LC setup.

7.2.2. Set the LC gradient according to the complexity of the sample. LC gradient of 180 min was used in this experiment for label-based quantitation (iTRAQ) of the tissue samples.

NOTE: For less complex samples, short gradient can efficiently separate most peptides. However, if the sample is very complex, use a longer gradient for better separation of peptides.

7.3. MS setup for iTRAQ technique

7.3.1. Set up all the MS parameters for label-based quantitation in the same way as used for the label-free quantitation except for the collision energy, which was set to 35% for MS/MS fragmentation in the label-based quantitation.

8. Data analysis

8.1. Analyze the raw (MS/MS spectrum) files obtained from LC-mass spectrometer using a commercially available analysis software (see **Table of Materials**).

NOTE: The Human Reference Proteome database from Uniprot (UP000005640) comprising

71,785 proteins sequences was used to obtain protein identities using Sequest HT and Mascot (v2.6.0) search engines. The parameters for label-free quantitation and label-based quantitation are described in **Supplementary File 4**.

REPRESENTATIVE RESULTS:

We have used two different approaches for discovery proteomics: label-free and label-based proteomics approaches. The protein profile of tissue samples on SDS-PAGE showed the intact proteins and could be considered for proteomic analysis (**Figure 2A**). The quality control check of the instrument was monitored *via* system suitability software and it showed the day-wise variation of the instrument performance (**Figure 2B**). We observed 91% sequence coverage of the BSA sample in 30 min of LC gradient (**Figure 2C**). The LC gradient was optimized using 500 ng of commercial HeLa cell digest and we observed 2425 proteins in a 2 h gradient as compared to 1488 proteins in a 1 h gradient (**Figure 2D**). We were able to identify, on an average, 2428 proteins across all three technical replicates of a pool tissue sample (**Figure 2E**).

The optimized LC and MS parameters were applied to three different biological tissue samples (**Figure 3** and **Supplementary File 2**). The chromatogram showed good reproducibility between three different biological tissue samples. We identified 2725, 2748, and 2718 quantifiable proteins from tissue samples 1, 2, and 3, respectively, using a label-free based approach. We observed that 151 proteins were common in the first and second LFQ experiments, 163 proteins were shared between the second and third LFQ experiments, and 187 proteins were shared between the first and third experiments, while 2190 proteins were common in all three tissue samples (**Figure 4A**).

We inspected the chromatogram and checked the iTRAQ labels and found it to be present in almost all MS/MS spectrums. The three sets have been run for the iTRAQ experiment. Proteins number obtained from each set were 2455, 2285, and 2307, respectively. 287 proteins were found to be common in sample 1 and sample 2, 183 proteins were common in sample 2 and sample 3, and 195 proteins were common in sample 1 and sample 3. The total number of proteins common in all three samples was 1557 (**Figure 4B**).

We compared total peptide spectral matches (PSMs), peptide groups, total proteins, protein groups, and the number of proteins obtained after 1% FDR from the LFQ and iTRAQ experiment (**Figure 4C**).

FIGURE LEGENDS:

Figure 1: Workflow for tissue proteomics. (**A**) The sample processing steps to prepare samples from tissue lysate for the MS analysis. (**B**) Steps for label-free quantitation. (**C**) Steps for label-based quantitation. (**D**) Steps for data analysis using a proteome discoverer.

Figure 2: Quality check control of tissue samples and reproducibility of the instrument. (**A**) Quality check of tissue lysates on 12% SDS-PAGE (**B**) Monitoring of some peptides of BSA using Panorama to check the instrument variability across the different days. (**C**) The sequence coverage of BSA in three technical replicates. (**D**) Optimization of LC parameters for tissue

samples. (**E**) The number of peptide spectral matches, peptides, and proteins in three different biological samples.

Figure 3: LC and MS parameters for proteomics analysis of tissue sample. (**A,B**) The liquid chromatography gradient used to separate the peptides for label-free quantitation (**A**) and label-based quantitation (**B**) of the tissue sample. (**C**) The MS parameters for label-free quantitation and label-based quantitation.

Figure 4: Label-free and label-based quantitation of tissue sample. (**A**) Venn diagram represents the common and exclusive proteins in tissue samples 1, 2, and 3 of the label-free experiment. (**B**) Venn diagram represents the common and exclusive proteins in tissue samples 1, 2, and 3 of the label-based experiment. (**C**) The comparative analysis of the number of peptide spectral matches (PSMs), peptide groups, total proteins, protein groups, and protein number after 1% FDR in label-free quantitation (LFQ) and label-based experiment (iTRAQ).

DISCUSSION:

Tissue proteomics of biological samples enables us to explore new potential biomarkers associated with different stages of disease progression. It also explains the mechanism of signaling and pathways associated with disease progression. The described protocol for tissue quantitative proteomics analysis provides reproducible good coverage data. Most of the steps have been adapted from the manufacturer's kit instructions. In order to obtain high-quality data, the following steps are most crucial. Hence, extra care should be given while performing these steps.

The incomplete digestion of proteins and contamination of keratin may provide less coverage of proteins ($n < 1000$), thereby affecting the overall experiment. The pH of samples (pH 8) and concentration of urea in the samples (less than 1 M) will ensure the efficient digestion of proteins. The use of fresh buffer and handling of samples with care will reduce the chances of keratin contamination. iTRAQ reagents are extremely costly and it requires a sophisticated MS platform to perform the MS/MS and software to analyze the data. The proteomics experiments are sensitive to contamination from salts, peptide quantification, and labeling efficiency of iTRAQ/TMT reagents. Before the MS/MS analysis, ensure digested peptides are properly desalted to reduce the background noise in the data. In the case of the iTRAQ technique, fragmentation of the attached tag generates a low molecular mass reporter ion that can be used to relatively quantify the peptides and the proteins from which they have originated, whereas for label-free approach, the area under the curve is considered for quantitation. To increase the confidence in the quantitation of proteins, independent validation experiments especially, MRM/PRM should be performed.

The analysis of tissue samples using two quantitation methods (label-free and label-based proteomics) has been described to obtain a good coverage of proteins. The label-free quantitative proteomics approach offers several advantages for its use in clinical studies. The samples are run independently, and this is particularly important for studies that are undertaken for a patient cohort as there are a large number of samples to be analyzed *via* mass spectrometer.

Using a technical replicate such as a pool of optimized peptides, one can ensure good reproducibility even if the samples are run at different time points. This approach has been used in large cohort studies such as the CPTAC, which is an effort of many international communities[14].

The potential targets emerging from the study could be considered for validation using targeted proteomics approaches. We conclude that the projects based on tissue samples analysis could be heavily benefitted from the detailed workflows of quantitative proteomics provided in this study. The mentioned steps will help to optimize the method and map the proteome of tissue samples. The selection of quantitative proteomic techniques may depend upon the number of samples, availability of MS platforms, and the biological question to be addressed.

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DISCLOSURES:

The authors have nothing to disclose.

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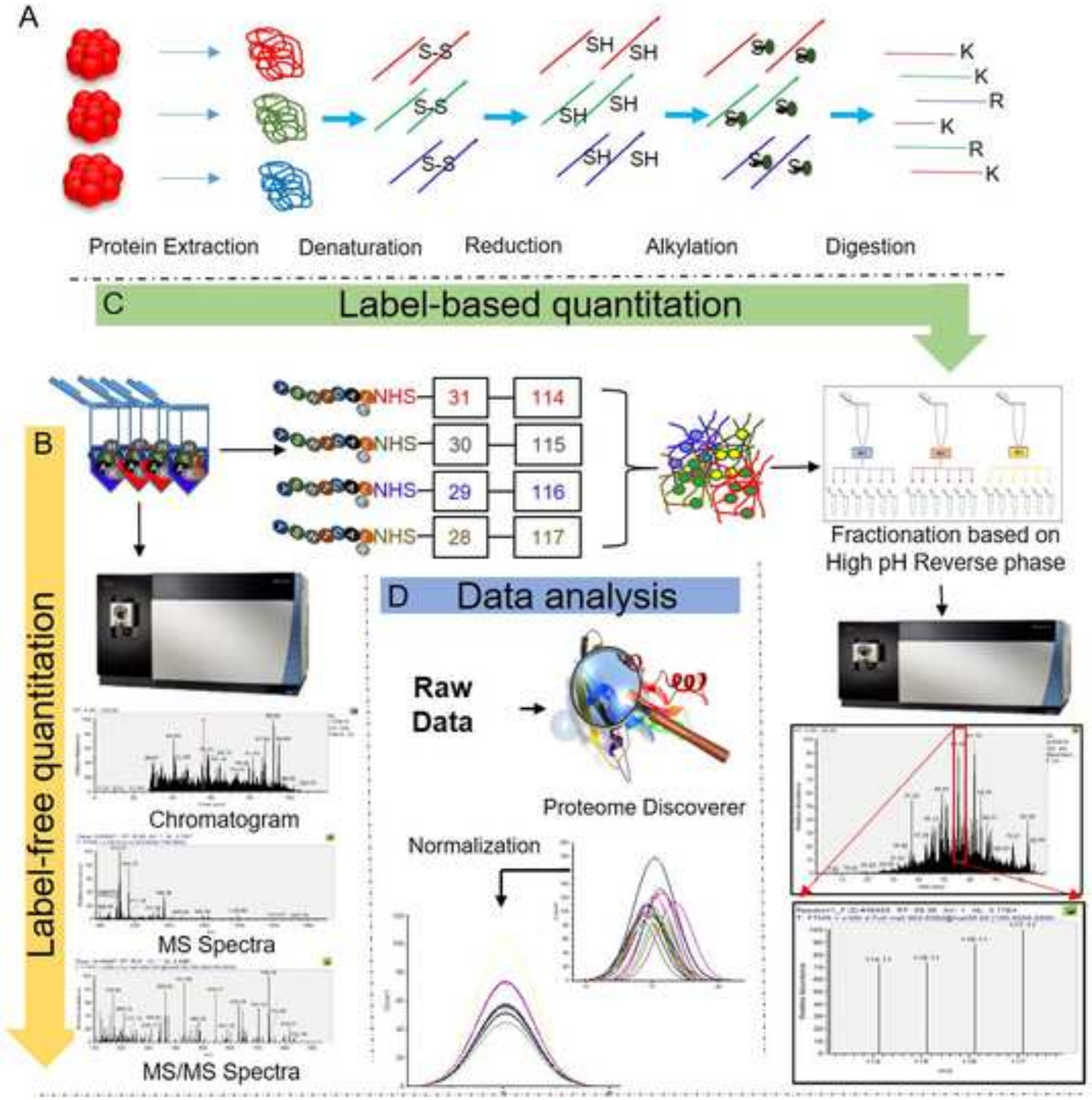

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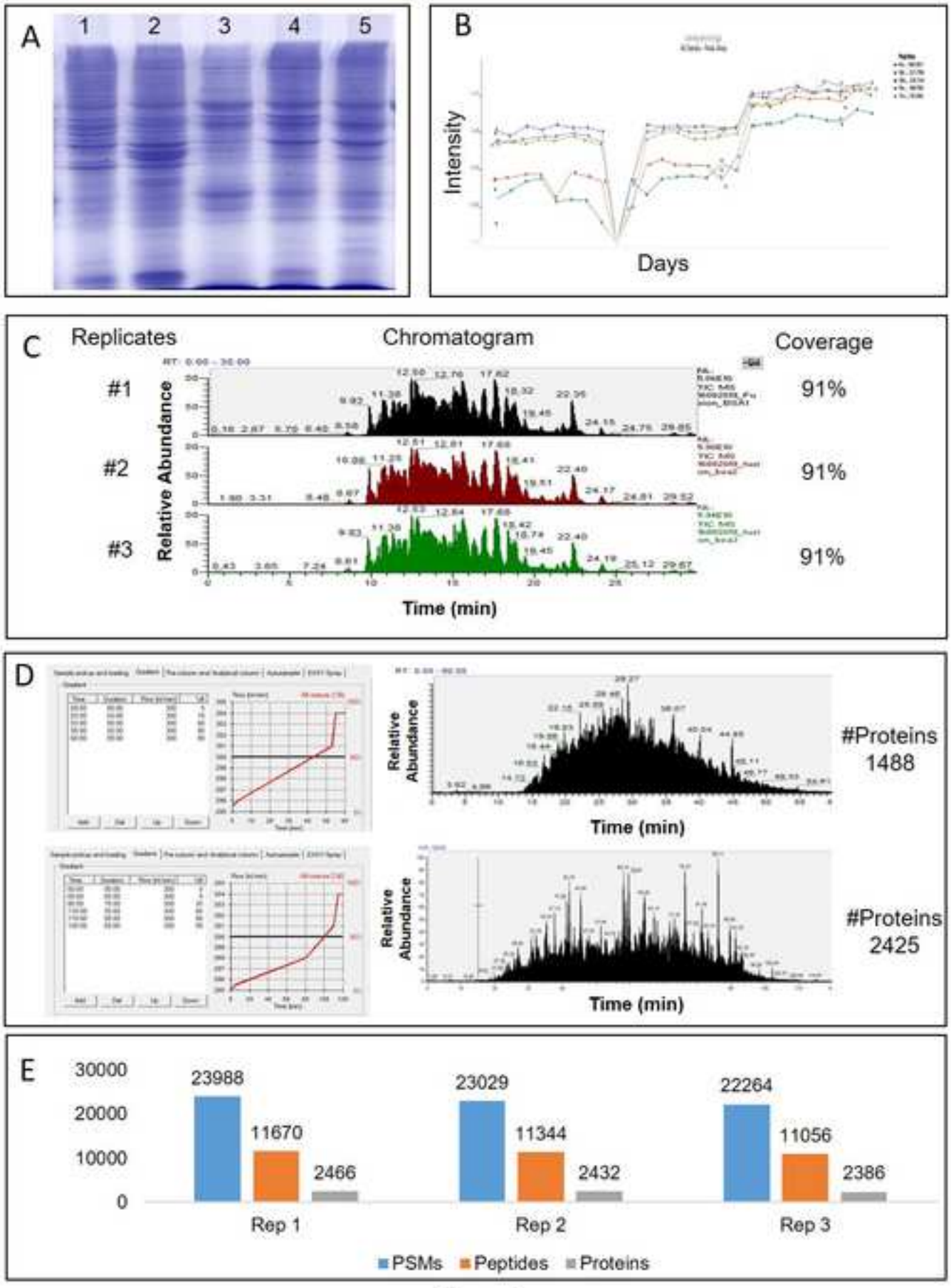

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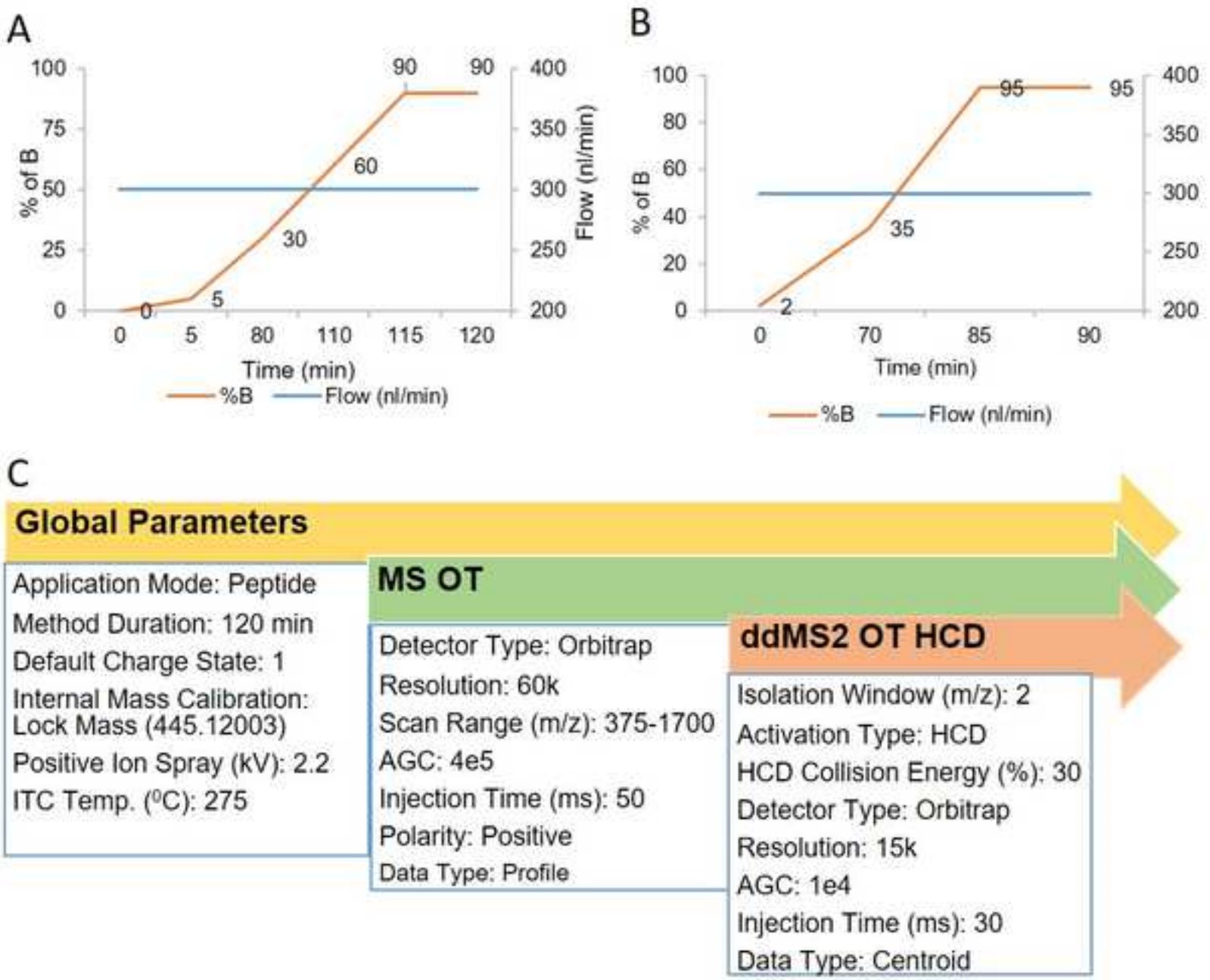

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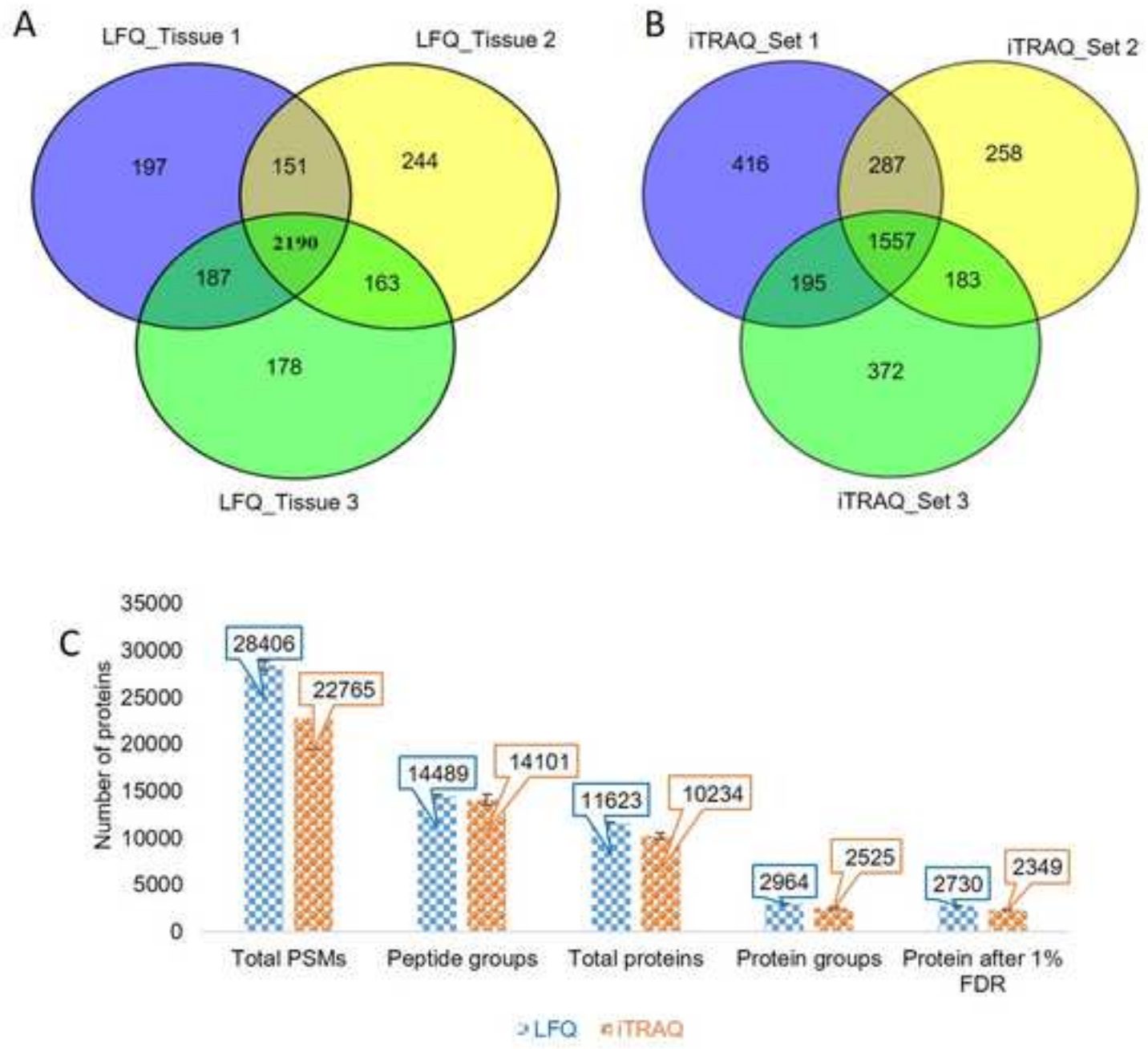

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2. Audio Editing and Pacing:
• Audio Levels are too high. Please decrease the audio levels, i.e.-6db or 50%, and ensure that the audio level peaks average around -9 dB.

Response: Audio levels are adjusted as per the suggestions in the revised video.

Once done please ensure that the video is no more than 15 min in length and please upload a revised high-resolution file here:
https://www.dropbox.com/request/i8AKOGeRBocGxubwG3Tl?oref=e

Response: Revised video was uploaded in the given dropbox folder.

Supplemental File (Figures, Permissions, etc.) Click here to access/download;Supplemental File (Figures, Permissions, etc.);Revised Supplementary file_30.07.2021.docx

Supplementary file 1

Quick Start™ Bradford Protein Assay

Reagent used: 1x Bradford Reagent

Introduction: The Bradford assay is a method to estimate the protein concentration in a sample. It involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford 1976). Under acidic conditions, the dye exists in a doubly protonated red cationic form (Amax = 470 nm). When it binds to the protein, it is converted to a stable unprotonated blue form (Amax = 595 nm). This blue form (protein-dye complex) can be detected at 595 nm using a spectrophotometer.

Standard preparation: Prepare the stock solution of BSA (Bovine serum albumin) with 2 mg/ml concentration by weighing 2 mg of BSA and dissolving it in 1 ml Mili Q water. Prepare the standards of varying concentration by serially diluting the stock solution of BSA.

Protocol:

1. Add 20 µl of each standard and unknown sample into clean tubes.
2. Add 1 ml of 1x Bradford reagent to each tube and vortex it.
3. Incubate the tubes at room temperature for 5 min. and measure the absorbance at 595 nm after the blank correction.
4. Prepare a standard curve by plotting the absorbance of standards versus their concentration in µg/µl.
5. Use the standard curve to determine the protein concentration in each sample.

Supplementary file 2

LC-MS parameters for Label-free quantitation (LFQ) and iTRAQ experiments

A. Sample pickup and loading: Sample pickup and loading parameters for the experiments are mentioned below. It summarizes the sample volume, flow and pressure of the column.

Sample pickup:	
Volume (µl)	2.00 µl for LFQ, 3.00 µl for iTRAQ
Flow (µl/min)	5.00
Sample loading:	
Volume (µl)	10.00 µl for LFQ, 12.00 µl for iTRAQ
Flow (µl/min)	Unspecified
Max. pressure (Bar)	800.00 µl for LFQ, 750.00 µl for iTRAQ

B. Chromatography gradients: Below is the liquid chromatography gradient used for the experiments. Tables contain the time duration for solvent B at different intervals of time with constant flow rate of 300 nl/min.

(i) For label-free quantitation (LFQ):

Time [mm:ss]	**Duration [mm:ss]**	**Flow [nl/min]**	**Mixture [%B]**
00:00	00:00	300	0
05:00	05:00	300	5

80:00	75:00	300	30
110:00	30:00	300	60
115:00	05:00	300	90
120:00	05:00	300	90

(i) For iTRAQ experiment:

Time [mm:ss]	Duration [mm:ss]	Flow [nl/min]	Mixture [%B]
00:00	00:00	300	0
70:00	70:00	300	35
77:00	07:00	300	95
90:00	13:00	300	95

(ii) Column equilibration: 0.1% (v/v) FA was used to equilibrate the column with below mentioned parameters for LFQ and iTRAQ experiments.

Pre-column equilibration:		
1.	Volume (µl)	10.00 µl for LFQ, 20.00 µl for iTRAQ
2.	Flow (µl/min)	Unspecified
3.	Max. pressure (Bar)	750.00

Analytical column equilibration:		
1.	Volume (µl)	8.00 µl for LFQ, 3.00 µl for iTRAQ
2.	Flow (µl/min)	Unspecified for LFQ, 0.30 µl for iTRAQ
3.	Max. pressure (Bar)	750.00 for LFQ, unspecified for iTRAQ
Autosampler wash:		
1.	Flush volume (µl)	100

(iii) **OT-HCD-OT MS/MS Method:** MS parameters used for the experiments are mentioned below:

Global Settings:		
1.	Method Duration (min)	120 min for LFQ and 90 min for iTRAQ experiment
2.	Application Mode	Peptide
3.	Default Charge State	1
4.	Internal mass calibration	445.12003 m/z
5.	Experiment	1
6.	Experiment Name	MS
7.	Start Time (min)	0

8.	End Time (min)	120
9.	Cycle Time (sec)	3

Scan Master Scan:		
1.	MSn Level	1
2.	Use Wide Quad Isolation	True
3.	Detector Type	Orbitrap
4.	Orbitrap Resolution	60K
5.	Mass Range	Normal
6.	Scan Range (m/z)	375-1700
7.	Maximum Injection Time (ms)	50
8.	AGC Target	400000
9.	Microscans	1
10.	RF Lens (%)	60
11.	Use ETD Internal Calibration	False
12.	Data Type	Profile
13.	Polarity	Positive

14.	Source Fragmentation	False
15.	Scan Description	Filter MIPS
16.	MIPS Mode	Peptide

Filter Charge State:		
1.	Include charge state (s)	2-6
2.	Include undetermined charge states	False
3.	Include charge states 25 and higher	False
Filter Dynamic Exclusion:		
1.	Exclude after n times	1
2.	Exclusion duration (s)	40
3.	Mass Tolerance	ppm
4.	Mass tolerance low	10
5.	Mass tolerance high	10
6.	Exclude isotopes	True
7.	Perform dependent scan on single charge state per precursor only	False
Filter Intensity Threshold:		

1.	Maximum Intensity	1E+20
2.	Minimum Intensity	5000 for LFQ and 20000 for iTRAQ experiment
3.	Relative Intensity Threshold	0
4.	Intensity Filter Type	Intensity Threshold
Data Dependent Properties:		
1.	Data Dependent Mode	Cycle Time
2.	Scan Event	1

Scan ddMSn Scan:		
1.	MSn Level	2
2.	Isolation Mode	Quadrupole
3.	Isolation Offset	Off
4.	Isolation Window	2 for LFQ and 1.2 for iTRAQ experiment
5.	Reported Mass	Original Mass
6.	Multi-notch Isolation	False
7.	Scan Range Mode	Auto Normal
8.	First Mass	100

9.	Scan Priority	1
10.	Activation Type	HCD
11.	Collision Energy Mode	Fixed
12.	Collision Energy (%)	30 % for LFQ and 35% for iTRAQ experiment
13.	Detector Type	Orbitrap
14.	Orbitrap Resolution	15K
15.	Maximum Injection Time (ms)	30
16.	AGC Target	10000
17.	Inject ions for available parallelizable time	True
18.	Microscans	1
19.	Use ETD Internal Calibration	False
20.	Data Type	Centroid
21.	Polarity	Positive
22.	Source Fragmentation	False

Supplementary file 3

Experimental plan for iTRAQ experiment: Following plan was followed while performing iTRAQ experiment.

Figure 1. Tissue sample labeling plan for iTRAQ experiment

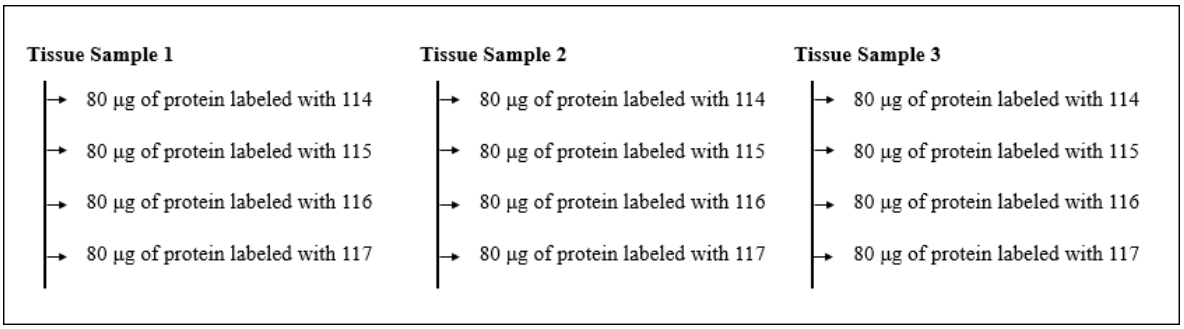

Table 1. Strategy of iTRAQ labeling

	iTRAQ Reagents			
Reaction	**114**	**115**	**116**	**117**
I	Tissue 1	Tissue 2	Tissue 3	Tissue 4
II	Tissue 2	Tissue 3	Tissue 4	Tissue 1
III	Tissue 3	Tissue 4	Tissue 1	Tissue 2
IV	Tissue 4	Tissue 1	Tissue 2	Tissue 3

Supplementary file 4

Parameters for data analysis

A. Processing workflow:

Spectrum Files RC.

1. Search Settings:

Protein Database: Human_Proteome_22082019.fasta

Enzyme Name: Trypsin (Full)

Precursor Mass Tolerance: 20 ppm

Fragment Mass Tolerance: 0.5 Da

Static Modification: Carbamidomethyl / +57.021 Da (C)

Mascot

1. Input Data:

Instrument: Default

Protein Database: Human_22082019

Enzyme Name: Trypsin

Maximum Missed Cleavage Sites: 2

Taxonomy: All entries

2. Tolerances:

Fragment Mass Tolerance: 0.05 Da

Precursor Mass Tolerance: 10 ppm

Use Average Precursor Mass: False

4. Dynamic Modifications:

Show All Modifications: False

1. Dynamic Modification: Oxidation (M)

2. Dynamic Modification: Phospho (ST.)

3. Dynamic Modification: Phospho (Y)

4. Dynamic Modification: Acetyl (Protein N-term)

5. Static Modifications:

Static Modification: Carbamidomethyl (C)

Percolator

1. Input Data:

Maximum Delta Cn: 0.05

Maximum Rank: 0

2. Decoy Database Search:

Target FDR (Strict): 0.01

Target FDR (Relaxed): 0.05

Validation based on: q-Value

Sequest HT

1. Input Data:

Protein Database: Human_Proteome_22082019.fasta

Enzyme Name: Trypsin (Full)

Max. Missed Cleavage Sites: 2

Min. Peptide Length: 6 for LFQ and 7 for iTRAQ

Max. Peptide Length: 144

Max. Number of Peptides Reported: 10

2. Tolerances:

Precursor Mass Tolerance: 10 ppm

Fragment Mass Tolerance: 0.05 Da

Use Average Precursor Mass: False

Use Average Fragment Mass: False

3. Dynamic Modifications:

Max. Equal Modifications Per Peptide: 3

Max. Dynamic Modifications Per Peptide: 4

i. Dynamic Modification: Oxidation / +15.995 Da (M)

ii. Dynamic Modification: Phospho / +79.966 Da (S, T, Y)

iii. Dynamic Modification (only for iTRAQ study): iTRAQ 4-plex / +144.102 Da (K)

4. Dynamic Modifications (protein terminus):

N-Terminal Modification: Acetyl / +42.011 Da (N-Terminus)

5. Dynamic Modifications (peptide terminus):

N-Terminal Modification (only for iTRAQ study): iTRAQ 4-plex / +144.102 Da (N-307Terminus)

6. Static Modifications:

Static Modification: Carbamidomethyl / +57.021 Da (C)

Minora Feature Detector

1. Peak & Feature Detection:

Min. Trace Length: 513

Min. # Isotopes: 2 Peaks

Max. ΔRT of Isotope Pattern Multiplets [min]: 0.2

2. Feature to ID. Linking:

PSM Confidence At Least: High

B. Consensus workflow:

MSF Files

1. Storage Settings:

Spectra to Store: Identified or Quantified

Feature Traces to Store: All

2. Merging of Identified Peptide and Proteins:

Merge Mode: Globally by Search Engine Type

File Limit for Automatic Merge.: 10

3. FASTA Title Line Display:

Reported FASTA Title Lines: Best match

Title Line Rule: standard

4. PSM Filters:

Maximum Delta Cn: 0.05

Maximum Rank: 0

Maximum Delta Mass: 0 ppm

PSM Grouper

1. Peptide Group Modifications: Site Probability Threshold: 75

Peptide Validator

1. General Validation Settings:

Validation Mode: Only PSM level FDR Calculation based on score

Target FDR (Strict) for PSMs: 0.01

Target FDR (Relaxed) for PSMs: 0.05

Target FDR (Strict) for Peptides: 0.01

Target FDR (Relaxed) for Peptides: 0.0514

2. Specific Validator Settings:

Validation Based on: q-Value

Use Concatenated FDR Calculation for PSM Level FDR Calculation Based on Score:

True

Reset Confidences for Nodes without Decoy Search (Fixed score thresholds): False

Processing node 3: Peptide and Protein Filter

1. Peptide Filters:

Peptide Confidence At Least: High

Keep Lower Confident PSMs: False

Minimum Peptide Length: 6

Remove Peptides Without Protein Reference: False

2. Protein Filters:

Minimum Number of Peptide Sequences: 1

Count Only Rank 1 Peptides: False

Count Peptides Only for Top Scored Protein: False

Protein FDR Validator

1. Confidence Thresholds:

Target FDR (Strict): 0.01

Target FDR (Relaxed): 0.05

Protein Marker

1. Contaminant Database:

Protein Database: contaminants_26042018.fasta

2. Annotate Species:

As Species Map: False

As Species Names: False

Feature Mapper

1. Chromatographic Alignment:

Perform RT Alignment: True

Maximum RT Shift [min]: 5

Mass Tolerance: 10 ppm

Parameter Tuning: Coarse

2. Feature Linking & Mapping:

RT Tolerance [min]: 0

Mass Tolerance: 0 ppm

Min. S/N Threshold: 5

Precursor Ions Quantifier

1. General Quantification Settings:

Peptides to Use: Unique + Razor

Consider Protein Groups for Peptide Uniqueness: True

Reject Quan Results with Missing Channels: False

2. Precursor Quantification

Precursor Abundance Based On: Area

Min. # Replicate Features [%]: 0

3. Normalization and Scaling:

Normalization Mode: Total Peptide Amount

Scaling Mode: On All Average

Display Settings

1. General:

Filter Set: Filter Set Master Protein Filter

Row Filter for Target Protein: Master is equal to Master

Layout Definition: (not specified)