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Enhanced Sample Multiplexing of Tissues using Combined Precursor Isotopic Labeling and Isobaric Tagging (cPILOT)

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Abstract:	There is an increasing demand to analyze many biological samples for disease understanding and biomarker discovery. Quantitative proteomics strategies that allow simultaneous measurement of multiple samples have become widespread and greatly reduce experimental costs and times. Our laboratory developed a technique called combined precursor isotopic labeling and isobaric tagging (cPILOT), which enhances sample multiplexing of traditional isotopic labeling or isobaric tagging approaches. Global cPILOT can be applied to samples originating from cells, tissues, bodily fluids, or whole organisms and gives information on relative protein abundances across different sample conditions. cPILOT works by 1) using low pH buffer conditions to selectively dimethylate peptide N-termini and 2) using high pH buffer conditions to label primary amines of lysine residues with commercially-available isobaric reagents (see table of materials/reagents). The degree of sample multiplexing available is dependent on the number of precursor labels used and the isobaric tagging reagent. Here, we present a 12-plex analysis using light and heavy dimethylation combined with six-plex isobaric reagents to analyze 12 samples from mouse tissues in a single analysis. Enhanced multiplexing is helpful for reducing experimental time and cost and more importantly, allowing comparison across many sample conditions (biological replicates, disease stage, drug treatments, genotypes, or longitudinal time-points) with less experimental bias and error. In this work, the global cPILOT approach is used to analyze brain, heart, and liver tissues across biological replicates from an Alzheimer's disease mouse model and wild-type controls. Global cPILOT can be applied to study

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Cover Letter

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September 19, 2016

Nandita Singh
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Dear Dr. Singh,

Thank you for the invitation to submit revisitions to our manuscript entitled "**Enhanced Sample Multiplexing of Tissues using Combined Precursor Isotopic Labeling and Isobaric Tagging (cPILOT)**". We have fully addressed all of the Editorial concerns to the best of our ability and hope that these are adequate for continued consideration of this manuscript for publication in *JOVE.*

We look forward to your review of our revised manuscript and thank you for its consideration.

Sincerely,

Renã A. S. Robinson
Assistant Professor

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TITLE:
Enhanced Sample Multiplexing of Tissues using Combined Precursor Isotopic Labeling and Isobaric Tagging (cPILOT)

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Quantitative proteomics, cPILOT, TMT, isotopic labeling and isobaric tagging, multiplexing, tissues

SHORT ABSTRACT
Combined precursor isotopic labeling and isobaric tagging (cPILOT) is a quantitative proteomics strategy that enhances sample multiplexing capabilities of isobaric tags. This protocol describes the application of cPILOT to tissues from an Alzheimer's disease mouse model and wild-type controls.

LONG ABSTRACT
There is an increasing demand to analyze many biological samples for disease understanding and biomarker discovery. Quantitative proteomics strategies that allow simultaneous measurement of multiple samples have become widespread and greatly reduce experimental costs and times. Our laboratory developed a technique called combined precursor isotopic labeling and isobaric tagging (cPILOT), which enhances sample multiplexing of traditional isotopic labeling or isobaric tagging approaches. Global cPILOT can be applied to samples originating from cells, tissues, bodily fluids, or whole organisms and gives information on relative protein abundances

across different sample conditions. cPILOT works by 1) using low pH buffer conditions to selectively dimethylate peptide N-termini and 2) using high pH buffer conditions to label primary amines of lysine residues with commercially-available isobaric reagents (see table of materials/reagents). The degree of sample multiplexing available is dependent on the number of precursor labels used and the isobaric tagging reagent. Here, we present a 12-plex analysis using light and heavy dimethylation combined with six-plex isobaric reagents to analyze 12 samples from mouse tissues in a single analysis. Enhanced multiplexing is helpful for reducing experimental time and cost and more importantly, allowing comparison across many sample conditions (biological replicates, disease stage, drug treatments, genotypes, or longitudinal time-points) with less experimental bias and error. In this work, the global cPILOT approach is used to analyze brain, heart, and liver tissues across biological replicates from an Alzheimer's disease mouse model and wild-type controls. Global cPILOT can be applied to study other biological processes and adapted to increase sample multiplexing to greater than 20 samples.

INTRODUCTION:

Proteomics often involves the analysis of many samples used to better understand disease processes, enzyme kinetics, post-translational modifications, response to environmental stimuli, response to therapeutic treatments, biomarker discovery, or drug mechanisms. Quantitative methods can be employed to measure relative differences in protein levels across the samples and can be label-free or involve isotopic labeling (metabolic, chemical, or enzymatic). Stable isotope labeling methods have grown in popularity because they allow many samples to be analyzed simultaneously and are suitable for samples from different cells, tissues, bodily fluids, or whole organisms. Isotope labeling methods[1-7] increase experimental throughput, while reducing acquisition time, costs, and experimental error. These methods use precursor mass spectra to measure relative abundances of proteins from peptide peaks. In contrast, isobaric tagging reagents[8-10] generate reporter ions that are either detected in MS/MS or MS^3 [11] spectra and these peaks are used to report on relative abundances of proteins.

The current state-of-the-art in proteomics multiplexing is either a 10-plex[12] or 12-plex isobaric tag analysis[13]. Enhanced sample multiplexing (i.e. >10 samples) methods have been developed by our laboratory for tissues[14-17], and by others for the analysis of cells[18-20], tissues [21], or targeted peptides.[22] We developed an enhanced multiplexing technique called combined precursor isotopic labeling with isobaric tagging (cPILOT). Global cPILOT is useful for getting information about the relative concentrations of all proteins across different sample conditions ($\geq$ 12).[14] Figure 1 shows a general cPILOT workflow. Tryptic or Lys-C peptides are selectively labeled at the N-terminus with dimethylation using low pH^2 and at lysine residues with 6-plex reagents using high pH. This strategy doubles the number of samples that can be analyzed with isobaric reagents which helps to reduce experimental costs and additionally, reduces experimental steps and time.

cPILOT is flexible as we have developed other methods to study oxidative post-translational modifications, including 3-nitrotyrosine-modified proteins[14] and cysteine containing peptides with S-nitrosylation (oxcyscPILOT).[23] We have also developed an amino acid selective approach, cysteine cPILOT (cyscPILOT).[17] MS^3 acquisition with a top-ion[11] or selective-y_1-ion method[15] can help reduce reporter ion interference and improve quantitative accuracy of

cPILOT. The use of MS^3 in the acquisition method requires a high-resolution instrument with an orbitrap mass analyzer although low resolution ion trap instruments may also work.[24]

Previously, cPILOT has been used to study liver proteins[16] from an Alzheimer's disease mouse model. Here, we describe how to perform global cPILOT analysis using brain, heart, and liver homogenates to study the role of the periphery in Alzheimer's disease. This experiment incorporates biological replication. Because of the versatility of cPILOT, interested users can use the technique to study other tissues for a range of biological problems and systems.

PROTOCOL:

Ethics Statement: Mice were purchased from an independent, non-profit biomedical research institution and housed in the Division of Laboratory Animal Resources at the University of Pittsburgh. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

1. Protein Extraction And Generation Of Peptides For Chemical-Tagging

1.1. Extract protein from tissue, cells, or bodily fluids.

1.1.1. Homogenize 60 – 90 mg of tissue (e.g. brain, heart, and liver) in phosphate buffer saline (1X PBS) with 8 M urea (500 µL) using a mechanical homogenizer. Use the following parameters for the homogenizer: lysing matrix A beads, 4 m/s, 20 s. Heart tissue may require up to 9 cycles of homogenization.

Note Protease or phosphatase inhibitors may be added to the buffer if necessary. They were not used in this study.

1.1.1.1 Remove tissue homogenate from the lysing tube and transfer to a micro-centrifuge tube. Rinse lysing tubes with 100 – 500 µL of PBS with 8 M urea and combine the rinse solution with tissue homogenate.

1.1.2. Centrifuge the homogenized tissues (9,447 × g, 4 ^{o}C, 15 min) and collect the supernatant.

1.2. Determine the protein concentration using a bicinchoninic acid (BCA) assay according to manufacturer's instructions.

Note: Further dilution with buffer may be necessary if the protein concentration is too high. The resulting concentrations for samples from these tissues were in between 6 – 13 µg/µL.

1.2.1 Optional: Add an internal quality control standard (e.g. bovine alpha casein or other exogenous protein) with the ratio 1 µg standard:100 µg protein sample.

2. Sample Digestion

2.1. Add 100 µg (100×10^{-6}) of protein from each sample to individually labeled micro-centrifuge tubes. The volume is dependent on the protein concentration (see step 1.2).

2.2. Add dithiothreiotol (DTT) (40:1 reagent to sample mol ratio) to each sample. Incubate at 37 ^{o}C for 2 h. The calculation for the molar ratio is based off the protein mass of bovine serum albumin (BSA), which is 66.5 × 10^3 g/mol.

2.3. Add iodoacetamide (IAM) (80:1 reagent to sample mol ratio) to each sample. Incubate on ice in the dark for 2 h. This reaction is performed on ice for 2 h to prevent side reactions.

2.4. Add L-cysteine (40:1 reagent to sample mol ratio) to each sample. Incubate at room temperature for 30 min.

2.5. Add 20 mM Tris buffer with 10 mM $CaCl_2$ (pH 8.2) to dilute urea to a final concentration of 2 M.

2.6. Add L-1-tosylamido-2 phenylethyl cholormethyl ketone (TPCK)-treated trypsin (50:1 substrate to enzyme mol ratio) to each sample and incubate at 37 ^{o}C for 24 h.

2.7. Quench the protein digestion by flash-freezing the sample in liquid nitrogen and store at -80 ^{o}C until further processing.

3. Sample Desalting

3.1. Re-acidify the samples by adding formic acid (FA). Add enough FA to obtain a final concentration of 0.1%. If samples are originally at -80 ^{o}C, thaw samples on ice before re-acidification.

3.2. De-salt the peptides using C_{18} hydrophilic lipophilic balanced (HLB) 10 mg cartridges as previously described[16].

3.3. Dry the samples by vacuum centrifugation (5 torr, 45 ^{o}C, 77 × g) to a volume of ~ 10 µL.

4. Dimethylation Labeling (N-termini)

4.1. Reconstitute peptides in 1% acetic acid (0.25 µg/µL) dissolved in high performance liquid chromatography (HPLC) or nano-pure grade water. Remove a 50 µg portion to a new micro-centrifuge tube (1.5 mL) and store the remainder at -80 ^{o}C.

4.2. Add 8 µL of 60 mM (4%) CH_2O (37% wt/v) or 60 mM (4%) $^{13}CD_2O$ (20% wt/v) to the samples for labeling with light or heavy dimethylation, respectively. Typically, 4 µL of reagent is added per 25 µg of peptides (Steps 4.2 – 4.3, 4.6).

4.3. Add 8 µL of 24 mM $NaBH_3CN$ or 24 mM $NaBD_3CN$ to the samples labeled with light or heavy dimethylation, respectively.

4.4. Vortex and shake on a tube shaker for ~10 min at room temperature.

4.5. Quench the reactions by adding 16 µL of 1% ammonia (~28 – 30% v/v) for 5 min. Typically, 8 µL of reagent is added per 25 µg of peptides.

4.6. Re-acidify the reaction mixtures by adding 8 µL of 5% FA (98% v/v).

4.7. Combine the light and heavy dimethylated peptides (Figure 1) to generate a total of six samples. See Table 1 for a description of sample tagging and pooling used for this study.

4.8. Perform sample desalting according to steps 3.2 and 3.3.

5. Isobaric Tagging (Lys residues)

5.1. Reconstitute 100 µg of dimethylated peptides (1 µg/µL) in tri-ethyl ammonium bicarbonate (TEAB) buffer (pH ~ 8.5).

5.2. Prepare isobaric reagents as stated in the manufacturer's protocol (see table of materials/reagents).

5.3. Add solubilized isobaric reagents (41 µL) to peptides and vortex for ~10 s. Shake the samples on a micro-centrifuge tube shaker for ~1 h. Quench the reactions with 8 uL hydroxylamine (10% w/v) and incubate the samples for 15 min at room temperature.

5.4. Pool the six labeled samples into a single mixture and desalt (Steps 3.1 – 3.3) or store at -80 ^{o}C until further use.

Note: For improving proteome coverage and depth, a multi-dimensional separation strategy is suggested such as strong cation exchange (SCX).

6. Strong Cation Exchange

6.1. Perform SCX as per the manufacturer's protocol (see table of materials/reagents).

6.1.1. Prepare ~ 1 mL of ammonium formate solutions (20 mM, 40 mM, 60 mM, 80 mM, 100 mM, 150 mM, 250 mM, & 500 mM) with 10% acetonitrile (ACN), 0.1% FA (pH 3).

6.1.2. Remove the red cap from the top of the pipette tip and tap the pipette tip with packing slurry to ensure that the packing material is towards the bottom of the pipette tip.

Note: Critical: Do not throw away the pipette tip until the end of the protocol.

6.1.3. Place the centrifuge adapter onto the top of a micro-centrifuge tube (2 mL) and secure the pipette tip inside.

6.1.4. Add 150 µL of SCX reconstitution buffer to pipette tips.

6.1.5. Centrifuge the pipette tips for 6 min at room temperature (4,000 × g). Repeat this step two more times and discard the waste.

6.1.6. Dissolve the peptides in 150 µL of SCX reconstitution buffer. Ensure that the pH is 3.

6.1.7. Add peptides to the pipette tips, centrifuge (see 6.1.5.), and keep the eluent.

6.1.8. Transfer the filter and pipette to a new micro-centrifuge tube (2 mL) and add 150 µL of 20 mM ammonium formate solution. Centrifuge for 6 min at room temperature (4,000 × g) and keep the eluent.

6.1.9. Repeat step 6.1.8 an additional seven times, using successive concentrations (i.e. 40 mM, 60 mM, 80 mM, 100 mM, 150 mM, 250 mM and 500 mM) of ammonium formate.

6.2. Transfer the eight SCX fractions to micro-centrifuge tubes (1.5 mL) and concentrate them by using centrifugal evaporation (see step 3.3).

7. Liquid Chromatography – Tandem Mass Spectrometry and Triple Staged Mass Spectrometry (LC-MS/MS & MS^3)

7.1. Reconstitute the peptides in mass spectrometry grade water with 0.1% FA, filter, and place into an auto-sampler vial. Filter peptides as follows:

7.1.1. Add reconstituted peptides to a micro-centrifuge tube containing a 0.65 µm filter (see table of materials/equipment).

7.1.2. Centrifuge peptides at 12,000 × g for 1 min. Remove filter, discard, and add filtered peptides into an auto-sampler vial.

7.2. Prepare the mobile phase buffers as follows: 3% (v/v) ACN with 0.1% FA (A) and 100% ACN with 0.1% FA (B).

7.3. Inject 6 µl of each SCX fraction sample onto a trap column packed to 2 cm with C_{18} material (5 µm, 200 Å pore size).

Note: Sample cleaning on the trap is as follows: 3 min, 0% B; 3 µL/min using a 2D liquid chromatography system (see table of materials/reagents).

7.4. Run the analytical separation method. Use a 75 µm i.d. × 13.2 cm laser pulled-tip fused silica capillary column packed with C_{18} material (5 µm, 100 Å). The gradient is: 0–5 min, 10% B; 5–40 min, 10%–15% B; 40–90 min, 15%–25% B; 90–115 min, 25%–30% B; 115–130 min, 30%–60% B; 130–135 min, 60%–80% B; 135–145 min, 80% B, 145–150 min, 80%–10% B; 150–180 min, 10% B; 300 nL/min, 180 min.

7.5. Run the data acquisition for the mass spectrometer while the analytical separation method is running.

Note: The parameters for the MS survey scan are as follows: 400 – 1600 *m/z*, 60,000 resolution, automatic gain control (AGC) target 1 x 10^6 ions, maximum injection time 500 ms. Parameters for CID – MS/MS are as follows: Top 1-7 or Top 8-14 data dependent acquisition (DDA), 3 *m/z* isolation width, 500 minimum signal, 35% normalized collision energy (NCE), 0.25 activation q, 10 ms activation time, 3 x 10^4 AGC, 50 ms maximum injection time. Parameters for HCD – MS^3 are as follows: 200 minimum signal, 4 *m/z* isolation width, 60% NCE, 0.1 activation time, 3 x 10^5 AGC, 250 ms IT, 300 – 1300 *m/z* MS/MS selection range, exclude un-fragmented parent ion.

7.6. Perform each analysis in duplicate for both the top 1 – 7 and top 8 – 14 runs.

Note: For samples run on a newer model of an instrument containing an orbitrap or a tribrid mass spectrometer, DDA parameters will vary and can be optimized to include more MS/MS and MS^3 scans. Additionally, for tribrid instruments, synchronous precursor selection (SPS) – MS^3 should be employed.

8. Data Analysis[16]

8.1. Search the RAW files using protein analysis software against an appropriate database, such as mouse Uniprot.

Note: It is important to create two workflows for each RAW file in order to search for light and heavy dimethylated peptides.

8.2. Search the RAW files using the following parameters: trypsin with two missed cleavages, peptide mass range 300 – 6000 Da, 15 ppm parent mass tolerance, 1 Da fragmentation tolerance. Static modifications: light dimethylation (+28.031, peptide N-terminus) or heavy dimethylation (+36.076 Da, peptide N-terminus), carbamidomethyl (+57.021 Da, C).

Note: Sometimes the heavy dimethylated peak is ~7 Da (+35.069 Da, peptide N-terminus) is from the light dimethylated peak and thus should be incorporated into the search workflow for heavy dimethylated peptides. Dynamic modifications: oxidation (+15.995 Da, M), 6-plex isobaric tag (229.163 Da, K). Include a decoy database search to yield false discovery rates (e.g. 1 and 5%) and a quantitation node to search for the reporter ion intensities of isobaric tags.

8.3. Statistics

8.3.1. Export data into an electronic spreadsheet program in order to normalize protein reporter ion values. For each protein, divide either the median reporter ion ratios or raw reporter ion intensities by the median ratio of the internal standard or raw reporter ion intensities of the internal standard, respectively. Use the appropriate statistical software such as an electronic spreadsheet program, Perseus, or R to determine significant changes among sample conditions.

REPRESENTATIVE RESULTS:

cPILOT uses amine-based chemistry to chemically label peptides at the N-terminus and lysine residues and enhances sample multiplexing capabilities. **Figure 2** shows representative MS data that is obtained from a 12-plex cPILOT analysis of brain, heart, and liver tissues from an Alzheimer's disease mouse model and wild-type controls. As shown in **Table 1**, two biological replicates for the Alzheimer's disease and wild-type mice are included in this 12-plex analysis. **Figure 2A** shows a doubly-charged peak pair that is separated by *m/z* spacing of 4 indicating a single dimethyl group was incorporated into the peptide. Both the light and heavy dimethylated peaks in this pair are independently isolated and fragmented with CID. The MS/MS data for each of the dimethylated peptides is shown in **Figures 2B** and **C**. Search results indicate this pair of peaks belongs to the T(dimethyl)ELNYFAK(isobaric-tag[6]) peptide of the protein phosphoglycerate kinase 1. The most intense fragment ion is y^{3+} which is similar for both the light and heavy dimethylated peaks. These peaks are isolated further for HCD-MS^3 and the reporter ions (*m/z* 126-131) are observed as shown in **Figures 2D and E**. Both sets of MS^3

spectra are necessary to get information about the 12 samples. In this example, the reporter ion ratios (AD/WT) (Alzheimer's disease/Wild-type control) for brain, liver, and heart tissues are similar across the two biological replicates. The fold-change values for each comparison suggest that phosphoglycerate kinase 1 levels in brain and heart are higher in AD mice, whereas in liver the levels are lower.

Figure Legends:

Figure 1. Proteomics workflow using cPILOT. As an example, this workflow outlines the analysis of 12 individual samples. Proteins from tissues, cells, or bodily fluids are extracted and a suitable protein standard (e.g. bovine alpha-casein) is added. Proteins are digested using trypsin. Peptides are labeled at the N-terminus by using light or heavy dimethylation (pH ~2.5) and at lysine residues by using TMT^6-plex (pH ~8.5). Labeled peptides are pooled into a single mixture and subject to SCX RP-LC-MS/MS and MS^3.

Figure 2. Example cPILOT data of peptides from brain, heart, and liver tissues of an Alzheimer's disease mouse model and wild-type controls. Precursor data A) shows light and heavy dimethylated peptides, represented by the peaks at *m/z* 643.854 and 647.875. These peptides were selected, isolated, and fragmented, thus generating CID – MS/MS spectra B) and C), which provided peptide identification. An additional selection, isolation, and fragmentation of the most intense fragment ion of the light and heavy dimethylated peptides at the MS/MS stage-generated HCD – MS^3 spectra D) and E), respectively. The peptide sequence is T(dimethyl)ELNYFAK(isobaric-tag^6) and belongs to phosphoglycerate kinase 1.

Table 1: cPILOT grouping of AD and WT Brain, Heart, and Liver Tissues.

DISCUSSION:

cPILOT allows for the simultaneous measurement of more than 12 unique samples. In order to ensure successful tagging at both the N-terminus and lysine residues of peptides, it is imperative to have the correct pH for each set of reactions and to perform the dimethylation reaction first for peptide labeling. Selective dimethylation at the N- terminus is performed by having a pH at ~2.5 (± 0.2). This is achieved by exploiting the differences of the pKA's of the amino groups on lysine and the N-terminus. At pH 2.5, lysine is inactive (pKA ~10.5); however, if the pH is mildly acidic (i.e. pH 5 – 7) or basic, both the N-termini and lysine residues will be dimethylated. In addition, if isobaric tagging is performed first at a low pH, the N-termini will have the isobaric label and lysine residues will be dimethylated. This may result in less fragments being selected for MS^3 as b-ions would need to be selected. The relative costs of the dimethylation reactions are inexpensive compared to the commercial isobaric reagents. While one can use an entire isobaric reagent vial per 100 µg, we have also had success using half of the reagent vial per 100 µg with comparable labeling efficiency. This helps to significantly reduce the costs for individual cPILOT experiments and allows more samples to be analyzed. Also it is important to note that other isobaric tagging reagents can be used in place of the isobaric reagent used in this protocol as we have previously demonstrated.[14] To ensure high labeling and tagging efficiencies, it is important to add reagents to samples quickly. This will allow for samples to have approximately the same reaction time. For quantitative purposes, each sample should be treated identically especially prior to pooling samples at the dimethylation and isobaric labeling

steps. Finally, it should be noted that working with many samples simultaneously in the initial steps requires careful user skill and attention to sample handling.

The most ideal scenario is to obtain reporter ions for every protein in the mixture across the 12 samples. However, this is not the case for a large number of proteins. The number of peptides detected with quantitation information for cPILOT and other enhanced multiplexing approaching depends on several factors including sample type, sample fractionation and processing steps, MS data acquisition methods, and instrument type. Although both dimethylation and isobaric tagging steps have a high peptide labeling efficiency of ~95-99%, there is still ~20% of the MS^3 data which will not contain reporter ion information. This is due in part to using trypsin which generates arginine-terminated peptides that result in no incorporation of isobaric reagent. This can be solved using other enzymes, such as LysC, with a potential tradeoff in protein identifications.[25] Also for heavy dimethylated peptides, the peak selected for fragmentation can be the M or M-1 peak, which have different intensities and will affect the reporter ion intensities observed in the MS^3 stage. Thus there may be some low intensity reporter ions that are not detected for some samples. Such situations are often commonly observed for low intensity MS/MS fragments that are isolated for MS^3 steps. A great solution to this issue has been incorporated into tribrid mass spectrometers, which instead of using single – notch selection for MS^3 use multi-notch or multiple MS/MS fragments. [26]

There is a lot of versatility in the cPILOT approach especially with regards to the number of samples that can be compared in a single analysis (i.e., up to 20 with commercial isobaric reagents) and the types of tissues used. We demonstrated that it is easy to obtain accurate quantitative information from brain, heart, and liver tissues in the same analysis in the context of a disease. This method, similarly to other multiplexing methods, allows for the analysis of multiple samples at once, and is applicable to samples originating from cells, tissues, bodily fluids, or whole organisms. In addition, cPILOT labeled peptides are able to be analyzed using either a low[24] or high resolution (60,000) instrument In comparison, obtaining successful measurement using other multiplexing methods may be limited to a specific type of sample (i.e. cells)[18], may require an instrument with very high resolution[20], access to stable isotope labels, or funds available for high cost of labels. cPILOT is great for users interested in disease understanding, biomarker discovery, response to a drug or therapeutic intervention, or longitudinal changes across many time points. Furthermore, for large shotgun proteomics analyses of clinical samples (where N is large, hundreds to thousands), cPILOT may also be suitable to help reduce experimental costs and time. In the future, cPILOT will be expanded to multiplex a larger number of samples by using existing and novel isotopic and isobaric labels.

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DISCLOSURES:
The authors have no competing interests.

REFERENCES:

1 Ong, S.-E. *et al.* Stable Isotope Labeling by Amino Acids in Cell Culture, SILAC, as a Simple and Accurate Approach to Expression Proteomics. *Mol Cell Proteomics.* **1** (5), 376-386, doi:10.1074/mcp.M200025-MCP200 (2002).
2 Koehler, C. J., Arntzen, M. Ø., de Souza, G. A. & Thiede, B. An Approach for Triplex-Isobaric Peptide Termini Labeling (Triplex-IPTL). *Anal. Chem.* **85** (4), 2478-2485, doi:10.1021/ac3035508 (2013).
3 Langen, H. F., M.; Evers, S.; Wipf, B.; Berndt, P. *From Genome to Proteome, 3rd Siena 2D Electrophoresis Meeting*. Wiley-VCH, Weinheim, Germany, Siena. (1998).
4 Yao, X., Freas, A., Ramirez, J., Demirev, P. A. & Fenselau, C. Proteolytic 18O Labeling for Comparative Proteomics: Model Studies with Two Serotypes of Adenovirus. *Anal. Chem.* **73** (13), 2836-2842, doi:10.1021/ac001404c (2001).
5 Reynolds, K. J., Yao, X. & Fenselau, C. Proteolytic 18O Labeling for Comparative Proteomics: Evaluation of Endoprotease Glu-C as the Catalytic Agent. *J. Proteome Res.* **1** (1), 27-33, doi:10.1021/pr0100016 (2002).
6 Gygi, S. P. *et al.* Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotech.* **17** (10), 994-999, doi:doi:10.1038/13690 (1999).
7 Schmidt, A., Kellermann, J. & Lottspeich, F. A novel strategy for quantitative proteomics using isotope-coded protein labels. *PROTEOMICS.* **5** (1), 4-15, doi:10.1002/pmic.200400873 (2005).
8 Thompson, A. *et al.* Tandem Mass Tags: A Novel Quantification Strategy for Comparative Analysis of Complex Protein Mixtures by MS/MS. *Anal. Chem.* **75** (8), 1895-1904, doi:10.1021/ac0262560 (2003).
9 Ross, P. L. *et al.* Multiplexed Protein Quantitation in Saccharomyces cerevisiae Using Amine-reactive Isobaric Tagging Reagents. *Mol Cell Proteomics.* **3** (12), 1154-1169, doi:10.1074/mcp.M400129-MCP200 (2004).
10 Xiang, F., Ye, H., Chen, R., Fu, Q. & Li, L. N,N-Dimethyl Leucines as Novel Isobaric Tandem Mass Tags for Quantitative Proteomics and Peptidomics. *Anal. Chem.* **82** (7), 2817-2825, doi:10.1021/ac902778d (2010).
11 Ting, L., Rad, R., Gygi, S. P. & Haas, W. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. *Nat Meth.* **8** (11), 937-940, doi:doi: 10.1038/nmeth.1714 (2011).
12 McAlister, G. C. *et al.* Increasing the Multiplexing Capacity of TMTs Using Reporter Ion Isotopologues with Isobaric Masses. *Anal. Chem.* **84** (17), 7469-7478, doi:10.1021/ac301572t (2012).
13 Frost, D. C., Greer, T. & Li, L. High-Resolution Enabled 12-Plex DiLeu Isobaric Tags for Quantitative Proteomics. *Anal. Chem.* **87** (3), 1646-1654, doi:10.1021/ac503276z (2015).
14 Robinson, R. A. S. & Evans, A. R. Enhanced Sample Multiplexing for Nitrotyrosine-Modified Proteins Using Combined Precursor Isotopic Labeling and Isobaric Tagging. *Anal. Chem.* **84** (11), 4677-4686, doi:10.1021/ac202000v (2012).
15 Evans, A. R. & Robinson, R. A. S. Global combined precursor isotopic labeling and isobaric tagging (cPILOT) approach with selective MS3 acquisition. *PROTEOMICS.* **13** (22), 3267-3272, doi:10.1002/pmic.201300198 (2013).
16 Evans, A. R., Gu, L., Guerrero, R. & Robinson, R. A. S. Global cPILOT analysis of the APP/PS-1 mouse liver proteome. *PROTEOMICS – Clin Appl.* **9** (9-10), 872-884, doi:10.1002/prca.201400149 (2015).

17 Gu, L., Evans, A. R. & Robinson, R. A. S. Sample Multiplexing with Cysteine-Selective Approaches: cysDML and cPILOT. *J. Am. Soc Mass Spectrom.* **26** (4), 615-630, doi:10.1007/s13361-014-1059-9 (2015).
18 Dephoure, N. & Gygi, S. P. Hyperplexing: A Method for Higher-Order Multiplexed Quantitative Proteomics Provides a Map of the Dynamic Response to Rapamycin in Yeast. *Sci Signal.* **5** (217), rs2-rs2, doi:doi: 10.1126/scisignal.2002548 (2012).
19 Hebert, A. S. *et al.* Neutron-encoded mass signatures for multiplexed proteome quantification. *Nat Meth.* **10** (4), 332-334, doi:doi:10.1038/nmeth.2378 (2013).
20 Merrill, A. E. *et al.* NeuCode Labels for Relative Protein Quantification. *Mol Cell Proteomics.* **13** (9), 2503-2512, doi:10.1074/mcp.M114.040287 (2014).
21 Braun, C. R. *et al.* Generation of Multiple Reporter Ions from a Single Isobaric Reagent Increases Multiplexing Capacity for Quantitative Proteomics. *Analytical Chemistry.* **87** (19), 9855-9863, doi:10.1021/acs.analchem.5b02307 (2015).
22 Everley, R. A., Kunz, R. C., McAllister, F. E. & Gygi, S. P. Increasing Throughput in Targeted Proteomics Assays: 54-Plex Quantitation in a Single Mass Spectrometry Run. *Anal. Chem.* **85** (11), 5340-5346, doi:10.1021/ac400845e (2013).
23 Gu, L. & Robinson, R. A. S. High-throughput endogenous measurement of S-nitrosylation in Alzheimer's disease using oxidized cysteine-selective cPILOT. *Analyst.* **141** (12), 3904-3915, doi:10.1039/C6AN00417B (2016).
24 Cao, Z., Evans, A. R. & Robinson, R. A. S. MS3-based quantitative proteomics using pulsed-Q dissociation. *Rapid Commun Mass Spectrom.* **29** (11), 1025-1030, doi:10.1002/rcm.7192 (2015).
25 Swaney, D. L., Wenger, C. D. & Coon, J. J. Value of Using Multiple Proteases for Large-Scale Mass Spectrometry-Based Proteomics. *J. Proteome Res.* **9** (3), 1323-1329, doi:10.1021/pr900863u (2010).
26 McAlister, G. C. *et al.* MultiNotch MS3 Enables Accurate, Sensitive, and Multiplexed Detection of Differential Expression across Cancer Cell Line Proteomes. *Anal. Chem.* **86** (14), 7150-7158, doi:10.1021/ac502040v (2014).

Figure 1

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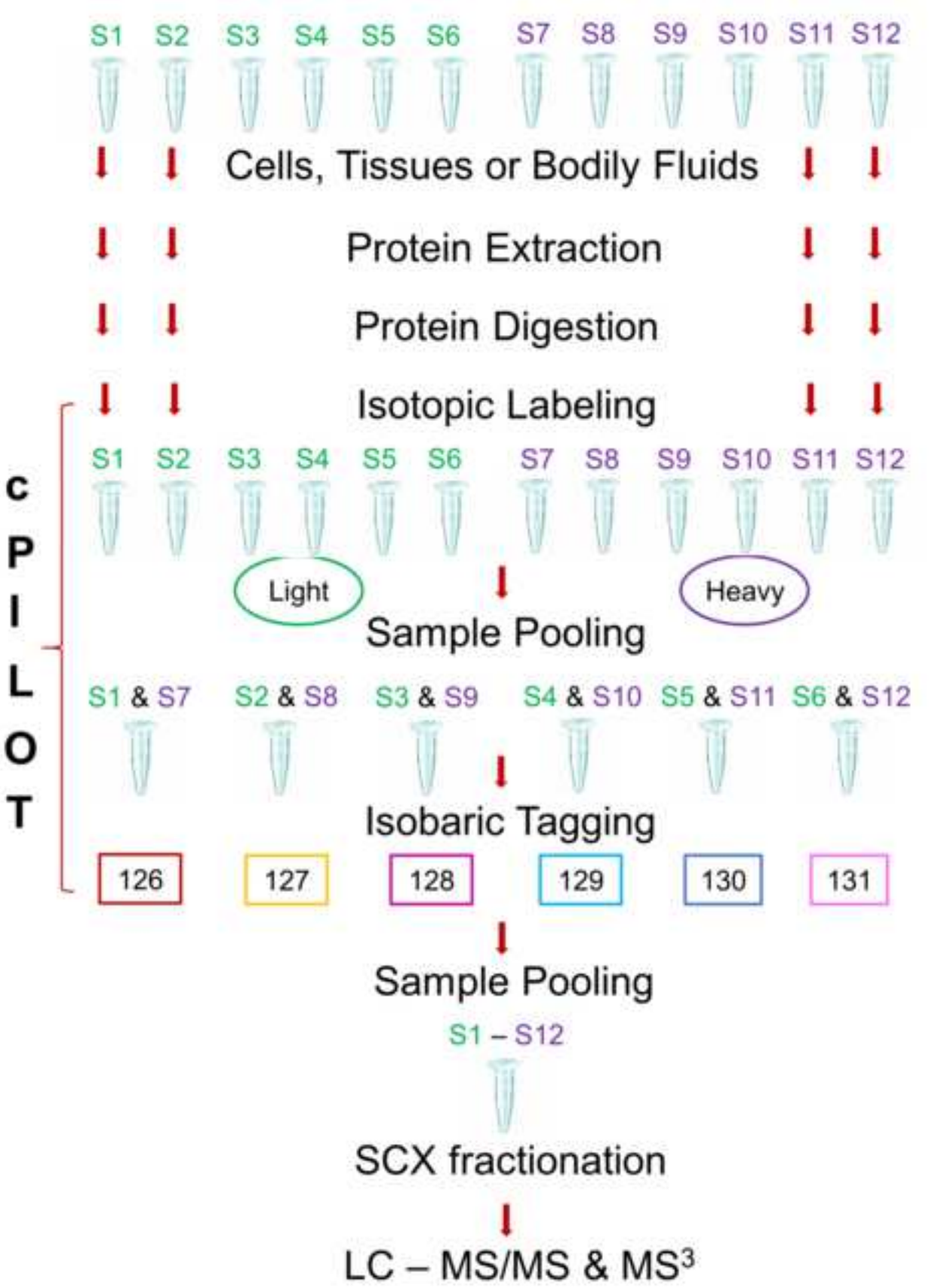

Figure 2 part 1

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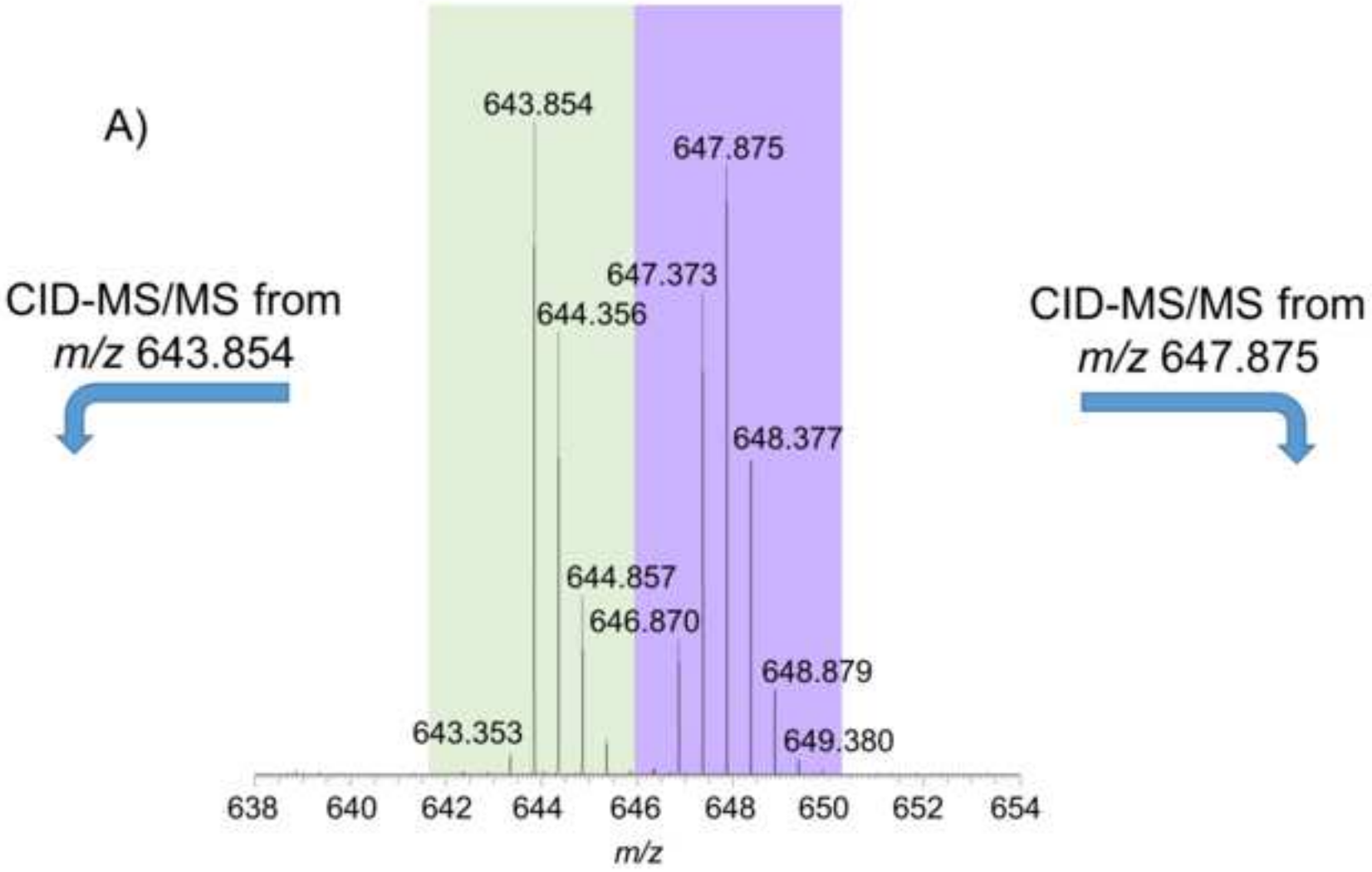

Figure 2 part 2

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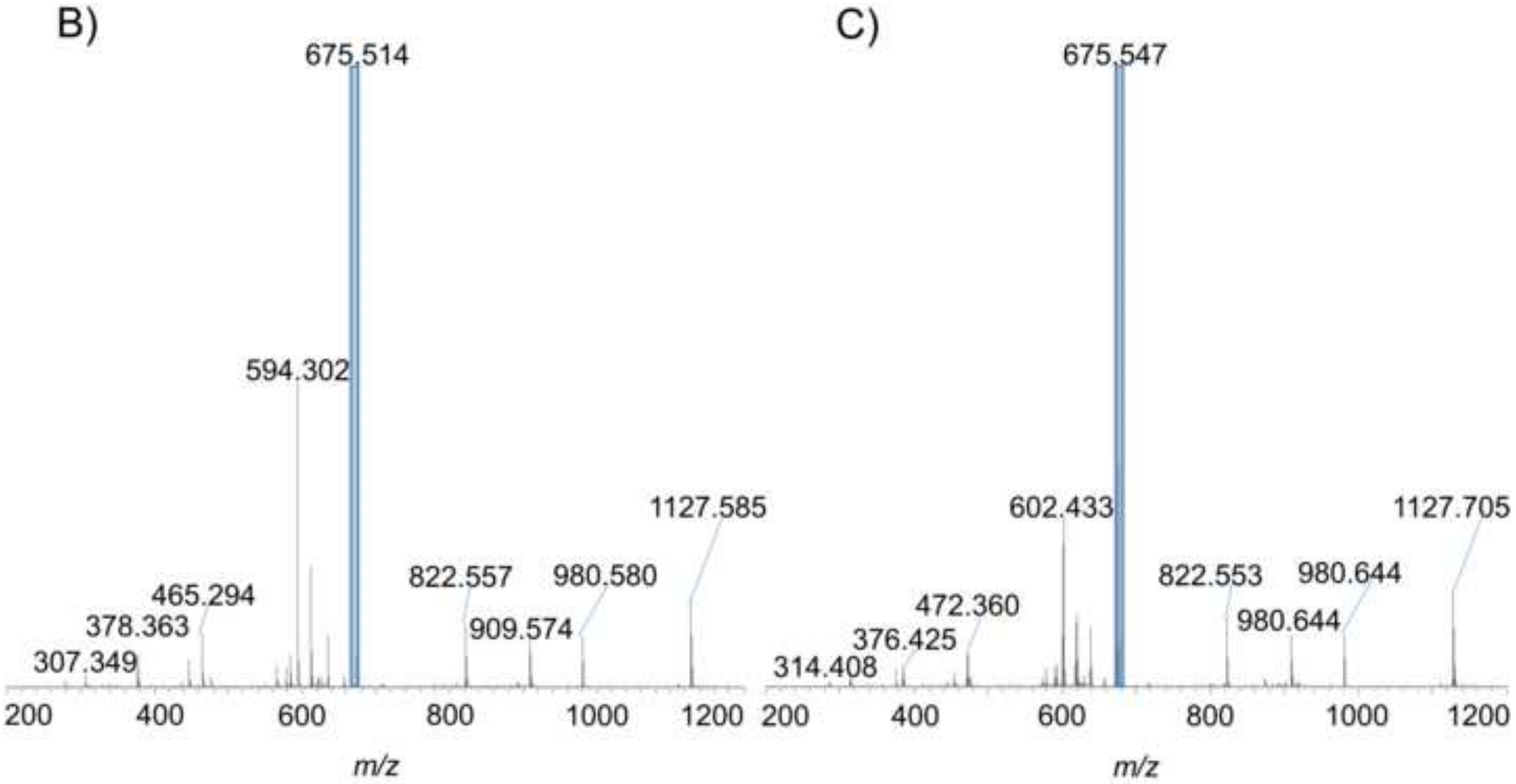

Figure 2 part 3

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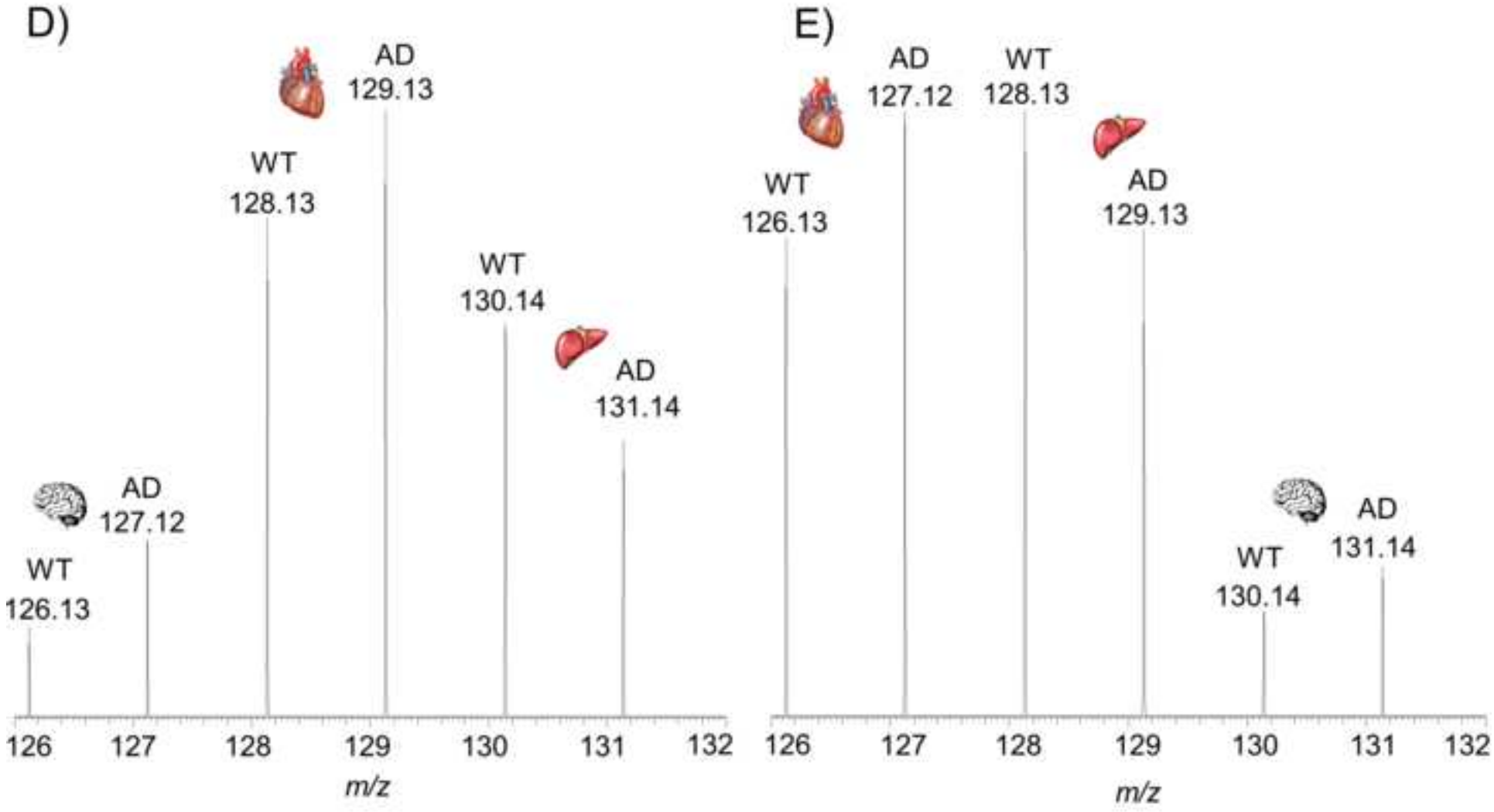

Click here to download Table Table 1_Jove_091516.xlsx

Table 1: cPILOT grouping of AD and WT Brain, Heart, and Liver Tissues

	Isobaric Reagent					
	126	**127**	**128**	**129**	**130**	**131**
Light Dimethylation	WT[a]	AD[b]	WT	AD	WT	AD
	brain		heart		liver	
Heavy Dimethylation	WT	AD	WT	AD	WT	AD
	heart		liver		brain	

The tissue is either from a wild-type control (WT)[a] or Alzheimers disease mouse (AD)[b].

Click here to download Excel Spreadsheet- Table of Materials/Equipment Table of specific

Name of the material/equipment	Company	Catalog Number	Comments/Description (optional)
Water - MS Grade	Fisher Scientific	W6-4	4 L quantity is not necessary
Acetonitrile - MS Grade	Fisher Scientific	A955-4	4 L quantity is not necessary
Acetic Acid	J.T. Baker	9508-01	
Ammonium hydroxide solution (28 - 30%)	Sigma Aldrich	320145-500ML	
Ammonium formate	Acros Organics	208-753-9	
Formic Acid	Fluka Analytical	94318-250ML-F	
BCA protein assay kit	Pierce Thermo Fisher Scientific	23227	
Urea	Biorad	161-0731	
Tris	Biorad	161-0716	
Dithiothreiotol (DTT)	Fisher Scientific	BP172-5	
Iodoacetamide (IAM)	Acros Organics	144-48-9	
L-Cysteine	Sigma Aldrich, Chemistry	168149-25G	
L-1-tosylamido-2 phenylethyl cholormethyl ketone (TPCK)-treated Trypsin from bovine pancreas	Sigma Aldrich, Life Science	T1426-100MG	
Formaldehyde (CH_2O) solution; 36.5 - 38% in H2O	Sigma Aldrich, Life Science	F8775-25ML	
Formaldehyde ($^{13}CD_2O$) solution; 20 wt % in D_2O, 98 atom % D, 99 atom % 13 C	Sigma Aldrich, Chemistry	596388-1G	
Sodium Cyanoborohydride; reagent grade, 95%	Sigma Aldrich	156159-10G	
Sodium Cyanoborodeuteride; 96 atom % D, 98% CP	Sigma Aldrich, Chemistry	190020-1G	
Strong Cation Exchange (SCX) spin tips sample prep kit	Protea BioSciences	SP-155-24kit	
Triethyl ammonium bicarbonate (TEAB) buffer	Sigma Aldrich, Life Science	T7408-100ML	
Isobaric Tagging Kit (TMT 6 plex) - 6 reactions (1 x 0.8 mg)	Thermo Fisher Scientific	90061	
Hydroxylamine hydrochloride	Sigma Aldrich, Chemistry	255580-100G	
Standard vortex mixer	Fisher Scientific	2215365	any mixer can be used

Oasis HLB 1cc (10 mg) extraction cartridges	Waters	186000383	These are C_{18} cartridges
Visiprep SPE vacuum manifold, DL (disposable liner), 24 port model	Sigma Aldrich	57265	A 12 port model is also sufficient
Speed-vac	Thermo Scientific	SPD1010	any brand of speed vac is sufficient
Water bath chamber	Thermo Scientific	2825/2826	Any brand of a water bath chamber with controlled temperatures is sufficient.
Mechanical Homogenizer (i.e. FastPrep-24 5G)	MP Biomedicals	116005500	
Eksigent Nano LC - Ultra 2D with Nano LC AS-2 autosampler	Sciex	-	This model is no longer available. Any nano LC with an autosampler is sufficient.
LTQ Orbitrap Velos Mass Spectrometer	Thermo Scientific	-	This model is no longer available. Other high resolution instruments (e.g. Orbitrap Elite, Orbitrap Fusion, or Orbitrap Fusion Lumos) can be used.
Protein software (e.g. Proteome Discoverer)	Thermo Scientific	IQLAAEGABSFAKJMAUH	
Analytical balance	Mettler Toledo	AL54	
Stir plate	VWR	12365-382	Any brand of stir plates are sufficient.
pH meter (Tris compatiable)	Fisher Scientific (Accumet)	13-620-183	Any brand of a ph meter is sufficient
pH 10 buffer	Fisher Scientific	06-664-261	Any brand of ph buffer 10 is sufficient
pH 7 buffer	Fisher Scientific	06-664-260	Any brand ph buffer 7 is sufficient
1.5 mL eppendorf tubes, 500pk	Fisher Scientific	05-408-129	Any brand of 1.5 mL eppendorf tubes are sufficient
0.6 mL eppendorf tubes, 500pk	Fisher Scientific	04-408-120	Any brand of 0.6 mL eppendorf tubes are sufficient
0.65µm Ultrafree MC DV centrifugal filter units	EMD Millipore	UFC30DV00	
2 mL microcentrifuge tubes, 72 units	Thermo Scientific	69720	

C_{18} packing material (5 µm, 100 Å)	Bruker	PM5/61100/000	This item is no longer available from Bruker. Alternative packing material with listed specifications will be sufficient.
C_{18} packing material (5 µm, 200 Å)	Bruker	PM5/61200/000	This item is no longer available from Bruker. Alternative packing material with listed specifications will be sufficient.

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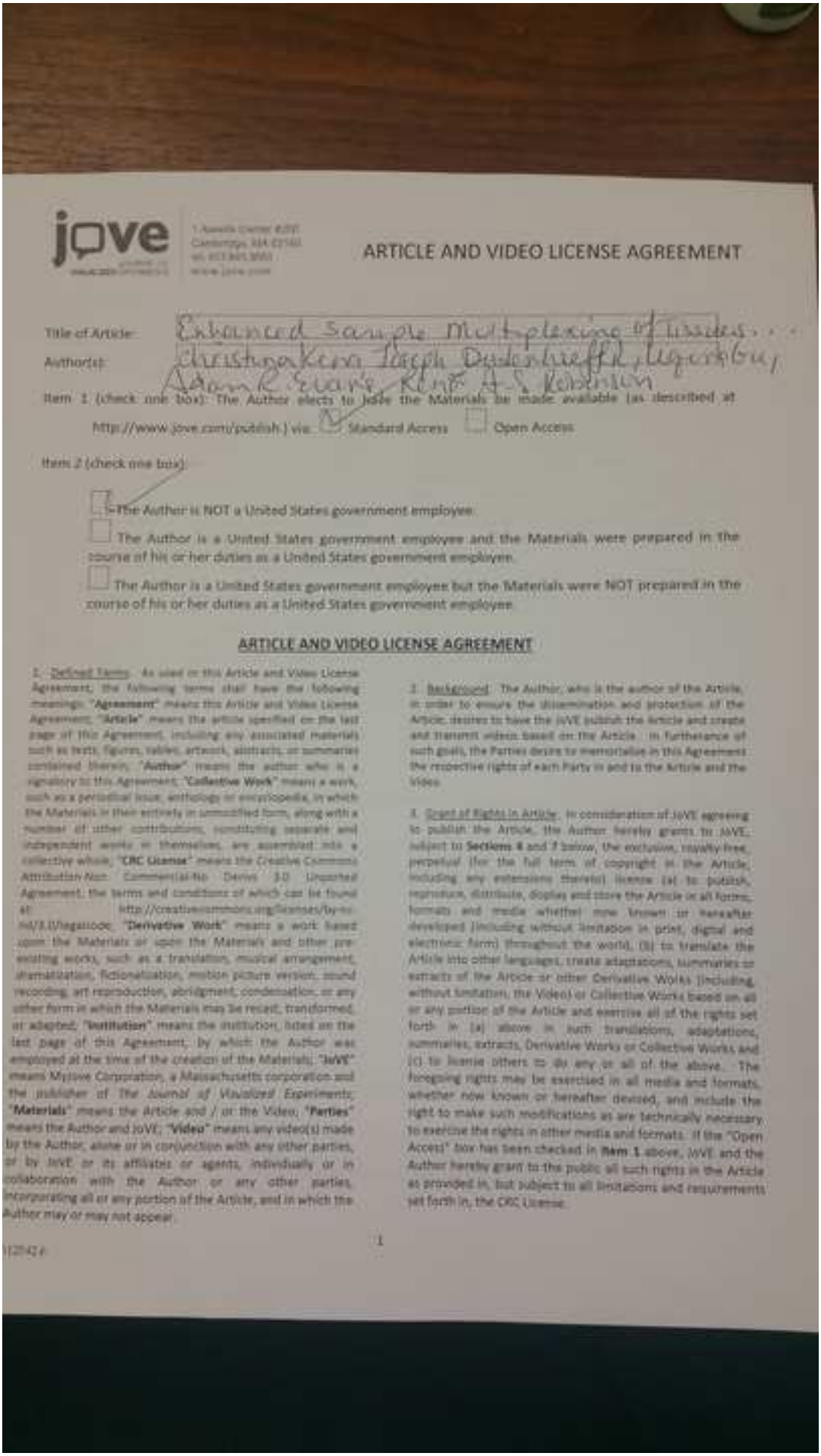

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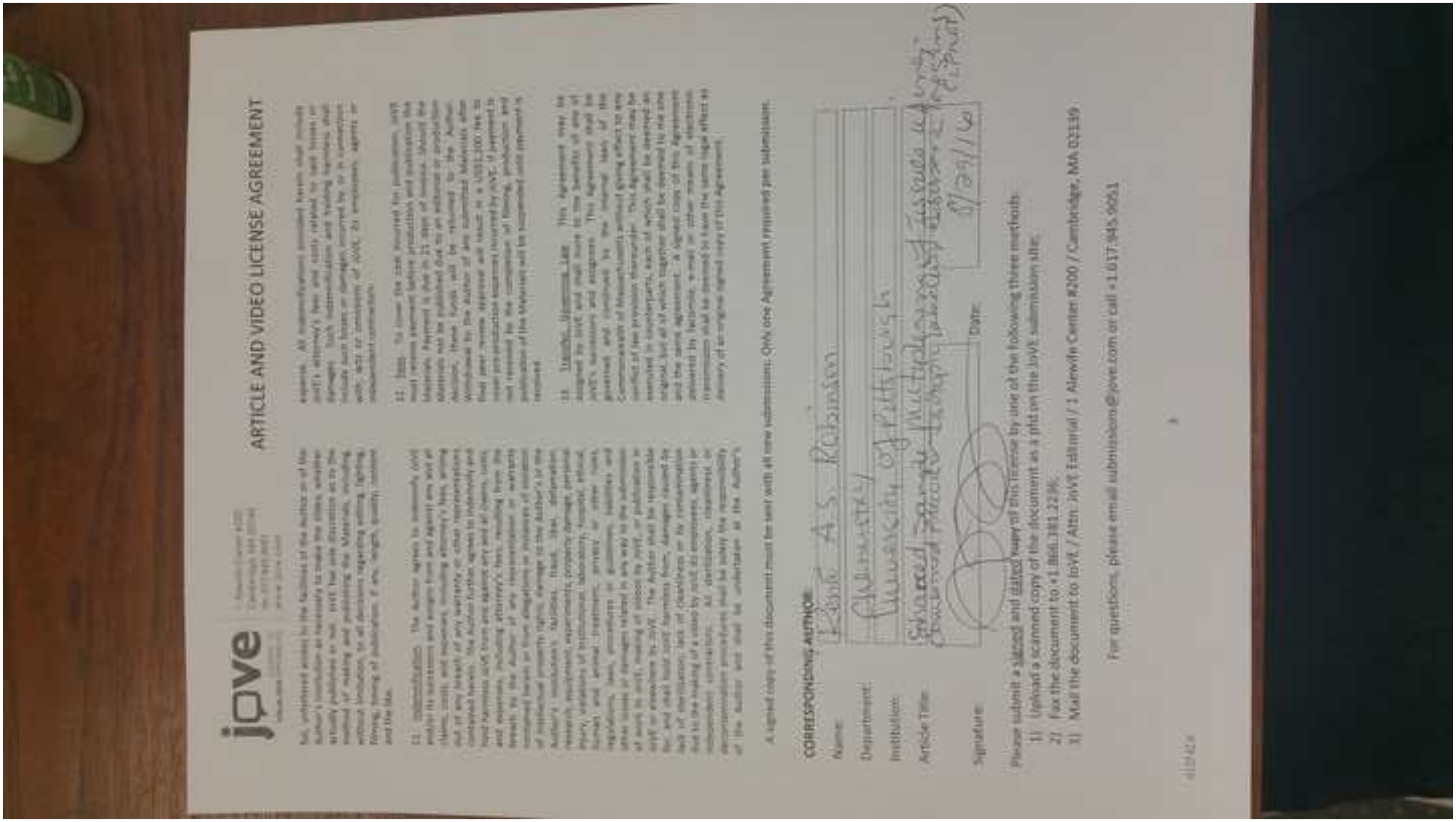

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We have removed all commercial sounding language from the introduction, protocol (see steps 1.1.1, 1.2, 5, 6, 7.6 and 8.3.1) , results, discussion, and figure 1.

• Please ensure that all items mentioned have been included in the Materials/Equipment list, and are accompanied by a catalog number. For e.g., FastPrep 24 system, etc.

All items mentioned in the manuscript are in the Materials/Equipment list and are accompanied by a catalog number.

• Please define all abbreviation before use. For e.g. ACN, etc.

We have defined abbreviations at the first use within the text. See steps 1.2, 3 and 6.1.1 in the protocol.

• Please describe centrifuge speeds as "x g" instead of the machine-dependent "rpm".

We have changed the centrifuge speeds from rpm to g. See steps 1.1.2, 3.3, 6.1.5, and 6.1.8 in the protocol.

• Please re-word the Short Abstract (10-50 words) to more clearly state the goal of the protocol. For example, “This protocol/manuscript describes…”

We have re-worded the short abstract to clearly state the goal of the protocol. Please see the revised short abstract:

“Combined precursor isotopic labeling and isobaric tagging (cPILOT) is a quantitative proteomics strategy that enhances sample multiplexing capabilities of isobaric tags. This protocol describes the application of cPILOT to tissues from an Alzheimer’s disease mouse model and wild-type controls.”

• Introduction 2nd paragraph- typo- should be “combined” precursor...cPILOT).

This typo has been fixed. Please see the second paragraph in the introduction.

• Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.). Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

The protocol section has been adjusted to reflect the imperative tense. Instances in which the imperative tense cannot be use have been made notes. Please see steps 7.4, 7.5, and 7.6 in the protocol.

• Step 2.1: In what volume?

We have clarified this by adding information in step 2.1 that references step 1.2. The added volume is dependent on the protein concentration.

• Step 3.1: How are the samples thawed?

We have updated the text in step 3.1 to describe how to thaw the samples.

• 6.2: Please provide the centrifugal evaporation details.

We have added the centrifugal evaporation details by referencing step 3.3 in the protocol.

• 7.4, and 7.5: Please re-write these steps in the imperative tense or add them as a Note.

These steps have been re-written in the imperative tense. Please see steps 7.4 and 7.5.

• 7.7 should be a Note. Ran should be run.

We have changed step 7.7 to a note and instance of "ran" have been changed to "run".

• Section 8: Please provide a reference for data analysis.

We have added a reference for the data analysis. The reference is below.
Evans, A. R., Gu, L., Guerrero, R. & Robinson, R. A. S. Global cPILOT analysis of the APP/PS-1 mouse liver proteome. *PROTEOMICS – Clin Appl.* 9 (9-10), 872-884, doi:10.1002/prca.201400149, (2015).

• After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages or less of text (which includes headings and spaces) to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

We have ensured that the protocol is within the 10-page limit. Please include all sections of the protocol except Sections 6.1, 6.2, and 8.3 for filming.

• Please disregard the comment below if all of your figures are original. If you are re-using figures from a previous publication, please obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]." We do not require the permission to re-use the figures until after internal and peer review. You can however, upload the permission to re-use email/file any time during the review process via your editorial manager account.

This comment is not applicable as both of the figures are original.

• Please ensure that your discussion covers the following in detail and in paragraph form:

1) modifications and troubleshooting,

This part of the discussion has been addressed and includes a discussion of how cPILOT can be modified with other isobaric tags and issues that may arise if the incorrect pH is used. Please see the first paragraph of the discussion section.

2) limitations of the technique,

This has been addressed in the discussion section. Please see the second paragraph of the discussion section.

3) significance with respect to existing methods,

We have added a sentence to the discussion section below.

"This method, similarly to other multiplexing methods, allows for the analysis of multiple samples at once, and is applicable to samples originating from cells, tissues, bodily fluids, or whole organisms."

4) future applications and

We have added the sentence below.

"In the future, cPILOT will be expanded to multiplex a larger number of samples by using existing and novel isotopic and isobaric labels."

5) critical steps within the protocol.

This part of the discussion has been addressed in the discussion section, whereby we highlight importance of using the correct pH, performing dimethylation prior to isobaric tagging, and give commentary on how to handle the samples. Please see paragraph one of this section.

• References: Please abbreviate all journal titles.
We have abbreviated all the journal titles. Please see the reference section.

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- The manuscript has been proofread.

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- DOIs have been added to missing references, except reference 3, in which one is not available.

•Grammar: 4.4 – "an tube"

- The phrase an tube has been changed to a tube.

•Visualization: The data analysis section does not contain enough information to be included in the video and should not be highlighted for filming.

- The data analysis section in the protocol has been un-highlighted.

•Results: What is the difference between panels D & E? Please specify in the figure legend.

- The difference between panels D and E have been specified. Please see figure caption (legend) 2.

•Discussion: Please discuss the advantages with respect to alternative multiplexing methods and include independent citations.

- Advantages of using cPILOT with respect to alternative multiplexing methods have been added to the discussion section. Please see lines 347 – 351.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this protocol, the authors described step-by-step methods for cPILOT, a quantitative proteomics strategy for multiplexing analysis. Overall, the experimental procedure has been described in great details and very easy to follow. It should serve as an important resource for the proteomics community who is interested in the application of cPILOT and multiplexing quantitative proteomics analysis. There are a few minor suggestions:

Major Concerns:
None

Minor Concerns:
1. Line 110, the author mentioned homogenizing tissues in PBS buffer with 8M urea. It would be better to note whether it's necessary to add some protease or phosphatase inhibitors.

- This issue has been addressed by talking about the addition of protease or phosphatase inhibitors. See lines 111 and 112.

2. Line 127, the author recommended adding DTT at a molar ratio of 40:1 to the sample, but on line 125, the author mentioned adding 100 µg of proteins. It would be hard to calculate how much DTT should actually be used.

- This issue has been addressed by prefacing how the calculations are made. Also, an example calculation was added. Please see lines 129 – 143.

3. Line 130, alkylation with iodoacetamide on ice. Why is it important to perform the reaction on ice?

- This has been addressed by stating the importance of doing this step on ice. Please see lines 145 and 146.

4. Line 140, the author mentioned adding 0.1% FA to re-acidify the sample. Does it actually mean adding FA till a final concentration of 0.1%?

- This has been re-phrased to state that the final concentration is 0.1% FA. Please see lines 156 and 157.

5. Line 194, the author mentioned a filtering step after reconstituting the peptides. It's necessary to provide some details about this step.

- Steps detailing how to filter reconstituted peptides have been added. As additional text was added, Line 194 has shifted. Please see lines 213 – 218.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The manuscript entitled "Enhanced Sample Multiplexing of Tissues using Combined Precursor Isotopic Labeling and Isobaric Tagging" describes a method to perform quantitative proteomics on multiple samples at once. The ability to multiplex samples through isotopic labeling and isobaric tagging decreases experimental time and costs. The manuscript is well written and the procedure and materials are adequately described. The authors also do a good job of describing multiple uses for the method.

Major Concerns:
N/A

Minor Concerns:
I recommend the manuscript be published with a minor revision. For figure 2D and E the authors label which peaks are from which tissue. It may also be useful, either on the figure or in the figure legend to label which peaks are from the WT and AD samples for each tissue.

- Identification markers indicating the origin of the peptide represented by the reporter ion intensity (either WT or AD) have been added. Please see figure 2D and 2E.

Additional Comments to Authors:
N/A