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Title: Automated Sample Multiplexing by Using Combined Precursor Isotopic Labeling and Isobaric Tagging (combined precursor isotopic labelling and isobaric tagging)

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. When you can, please upload the screen capture video for 2.3.3 to https://www.jove.com/account/file-uploader?src=18709358

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current protocol length: **23 steps, 54 shots**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Renã AS Robinson:** Our updated and automated cPILOT protocol makes it easier for researchers to analyze proteomics samples in a high-throughput way. This is significant because it can allow for faster access to biological information about various diseases or conditions that are studied in clinical samples.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Albert B Arul:** The main advantage of this technique is that it allows us to process multiple samples in parallel, thereby reducing experimental errors and increasing sample through-put.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Brandon:** Our laboratory is interested in applications related to aging and Alzheimer's disease, but this technique can be used broadly to study any disease, or challenge for which there are biological tissues.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Videographer: This statement is optional. If you don't have time, don't film it.*

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh.

Protocol

2. Protein Extraction and Sample Preparation

2.1. Begin by turning on the positive pressure apparatus, the heating and cooling device, and the vacuum pumps **[1]**. Connect all accessories with the liquid handler **[2]**, which will show a blue light once it is connected to the computer and ready to operate **[3]**.

2.1.1. WIDE: Establishing shot of talent turning on the accessories.

2.1.2. Talent connecting one of the accessories to the liquid handler.

2.1.3. Blue indicator light.

2.2. Aliquot 300 microliters of the liver homogenate into a 500-microliter tube **[1]** and place it on a 2-milliliter deep well plate. Store the sample at 4 degrees Celsius until the start of the protocol **[2]**.

2.2.1. Talent aliquoting the liver homogenate.

2.2.2. Talent putting the sample plate into the fridge and closing the door.

2.3. Open the method in the software **[1]** and place the required tips and labware into their positions **[2]**. Then, cross check with the final deck layout and click **Next** to continue the protocol **[3]**.

2.3.1. Talent at the computer opening the method.

2.3.2. Talent placing the tips and labware in position.

2.3.3. SCREEN: 2.3.3.mp4. Talent clicking **Next** in the software.

2.4. Load 230-microliter tips and aspirate 90 microliters of 8 molar urea from the reservoir **[1]**, then dispense it to row 1 of the black 2-milliliter deep well plate **[2]**. Unload the tips at TL1 and repeat this step until urea has been added to all the wells **[3]**. *Videographer: This step is important!*

2.4.1. Tips being loaded and urea aspirated on the device. NOTE: 2.4.1, 2.4.2, and 2.4.3 and 2.5 Combined, unslated

2.4.2. Urea dispensed.

2.4.3. Tips being unloaded.

2.5. After adding the denaturation buffer, use 90-microliter tips to transfer 10 microliters of mouse liver homogenate to the deep well plate **[1]**. Unload the tips, then load one row of 90-microliter tips and aspirate 3 microliters of DTT from reagent plate 1 **[2]**. Dispense DTT to rows 1 and 2 of the deep well plate **[3]**.

2.5.1. Talent at the computer controlling the tip loading and homogenate transfer.

2.5.2. Tips being loaded and DTT aspirated.

2.5.3. DTT being dispensed.

2.6. Seal the sample plate with aluminum foil **[1]** and incubate it at 37 degrees Celsius for 600 seconds while rotating at 300 rpm **[2]**. After the incubation, load one row of 90-microliter tips, aspirate 6 microliters of IAM from reagent plate 1 at row 3 **[3]** and dispense it to row 1 of the sample plate **[4]**. *Videographer: This step is important!*

2.6.1. Talent sealing the plate.

2.6.2. Plate incubating while rotating. *Videographer: Obtain multiple usable takes because this will be reused in 2.7.1 and 2.10.4.*

2.6.3. Tips loaded and IAM aspirated.

2.6.4. IAM dispensed.

2.7. Seal the sample plate and incubate it at 4 degrees Celsius for 30 minutes while rotating at 300 rpm on the cooling device **[1]**. Then, unseal the sample plate and load one row of 90 microliter tips **[2]**. *Videographer: This step is important!*

2.7.1. *Use 2.6.2.*

2.7.2. Tips loaded.

2.8. Aspirate 5 microliters of cysteine from the reagent plate 1 at row 2 **[1]** and dispense it to rows 1 and 2 of the sample plate. Incubate the plate at room temperature for 30 minutes **[2]**.

2.8.1. Cysteine aspirated.

2.8.2. Cysteine dispensed to the sample plate.

2.9. After the incubation, perform a timed shake of 1800 rpm for 30 minutes **[1]**. Then, add 800 microliters of 20 millimolar Tris buffer with 10 millimolar calcium chloride to each well on the sample plate to dilute the urea concentration to 2 molar **[2]**.

2.9.1. Plate shaking.

2.9.2. Buffer being dispensed to the sample plate.

2.10. Add 20 microliters of trypsin to the first row of a 96-well plate **[1]** and place the plate at a specified location on the deck **[2]**. After adding the trypsin to the sample plate, seal it and incubate it **[3]** for 15 hours at 37 degrees Celsius and 600 rpm on the heating and cooling device **[4]**. *Videographer: This step is important!*

2.10.1. Talent adding trypsin to the plate.

2.10.2. Talent placing the plate on the deck.

2.10.3. Talent sealing the plate.

2.10.4. *Use 2.6.2.*

2.11. Stop the digestion by aspirating 150 microliters of 5% formic acid from row 3 of the formic acid plate and dispensing it to the sample plate at rows 1 and 2 **[1]**.

2.11.1. Formic acid being dispensed.

3. Desalting Step 1

3.1. Use 1070 microliter tips to aspirate 600 microliters of acetonitrile **[1]**, then dispense it in rows 1 and 2 of the solid-phase extraction, or SPE, plate **[2]**. Place the plate on positive pressure apparatus and apply pressure **[3]**. *Videographer: This step is important!*

3.1.1. Tips being loaded and acetonitrile aspirated.

3.1.2. Acetonitrile dispensed to the SPE plate.

3.1.3. Pressure applied to plate on the PPA. *Videographer: Obtain multiple usable takes because this will be reused in 3.2.3.*

3.2. Next, load 2 rows of tips, aspirate 534 microliters of the digested samples, and dispense them to rows 1 and 2 of the SPE plate **[1]**. Apply pressure to the plate and repeat this step until all the samples are loaded **[2]**.

3.2.1. Sample aspirated and dispensed onto the SPE plate.

3.2.2. *Use 3.1.3.*

3.3. Aspirate 600 microliters of ACN-water solution **[1]** and dispense it to rows 1 and 2 of the SPE plate **[2]**, then place the plate on top of a collection plate to elute the peptides using the positive pressure apparatus **[3]**.

3.3.1. Talent at the computer controlling the loading of the tips and aspiration of ACN-water.

3.3.2. ACN-water being dispensed.

3.3.3. Pressure applied to plate and peptides eluted.

4. Dimethylation Labeling and Isobaric Tagging

4.1. After setting up the required tips and labware in the software, cross check with the deck layout and click **Next** to continue the protocol **[1]**.

4.1.1. SCREEN: 4.1.1.mp4. Talent at the computer, setting up the tips and labware in the software and then clicking **Next**.

4.2. To perform dimethylation labeling, load 90-microliter tips and aspirate 16 microliters of 60 millimolar light formaldehyde from the second row of reagent plate 2 **[1]**. Dispense it to row 1 of the sample plate **[2]**, then unload the tips **[3]**. *Videographer: This step is important!*

4.2.1. Light Formaldehyde aspirated.

4.2.2. Light Formaldehyde dispensed.

4.2.3. Tips unloaded.

4.3. Load 1 row of 90 microliter tips and aspirate 16 microliters of 60 millimolar heavy formaldehyde from row 3 of reagent plate 2. Dispense it to row 2 of the sample plate and unload the tips **[2]**. *Videographer: This step is important!*

~~4.3.1. Talent controlling the loading of the tips and paraformaldehyde aspiration through the software.~~

4.3.2. Heavy formaldehyde dispensed.

4.4. Load 2 rows of 90-microliter tips and aspirate 16 microliters of 24 millimolar sodium cyanoborohydride and 24 millimolar sodium cyanoborodeutaride from rows 1 and 2 of reagent plate 3, then dispense the reagents to rows 1 and 2 of the sample plate **[1]**.

4.4.1. Reagents aspirated and dispensed.

4.5. Unload the tips and use the orbital shaker to perform a timed shake for 15 minutes at 1800 rpm. Then, load 2 rows of 90-microliter tips and aspirate 32 microliters of 1% ammonia from rows 3 and 4 of reagent plate 3. Dispense it into rows 1 and 2 of sample plate 2 **[1]**. *Videographer: This step is important!*

4.5.1. Plate shaking.

~~4.5.2. Talent controlling the tip loading, then reagent aspiration and dispensing on the software.~~

4.6. Combine equal volumes of light and heavy dimethylated peptides in a new 2-milliliter deep well plate for desalting **[1]**. *Videographer: This step is difficult and important!*

4.6.1. Talent combining the peptides.

4.7. To perform isobaric tagging, reconstitute the peptides with 230 microliters of 100 millimolar triethylamonium bicarbonate buffer on an orbital shaker **[1-TXT]**. Then, use 90-microliter tips to aspirate 25 microliters of the combined dimethylated peptides and dispense them to the first row of the TMT processing plate **[2]**.

4.7.1. Plate on an orbital shaker. NOTE: 4.7 combined and unslated **TEXT: 30 seconds at 1800 rpm**

4.7.2. Peptides aspirated and dispensed.

4.8. Load 1 row of 90-microliter tips and aspirate 10 microliters of TMT. Dispense it in the first row of the TMT processing plate and unload the tips **[1]**, then perform a timed shake for 1 hour at 1800 rpm **[2]**. *Videographer: This step is difficult and important!*

4.8.1. TMT aspirated and dispensed.

4.8.2. TMT plate shaking.

4.9. Aspirate 8 microliters of hydroxylamine from row 2 of the TEAB plate and dispense it to row 1 of the TMT processing plate **[1]**. After a 15-minute shake at 1800 rpm, transfer 30.5 microliters of the TMT labeled peptides to a 1.5-milliliter tube **[2]**. Then, proceed with data acquisition and analysis according to manuscript directions **[3]**.

4.9.1. Talent controlling the aspirating and dispensing of hydroxylamine on the software.

4.9.2. Talent transferring the sample from the TMT plate to a 1.5mL tube.

4.9.3. Talent loading the sample into the autosampler connected to the mass spectrometer.

Results

5. Results: Automated cPILOT Processing of 22 Samples

5.1. Representative MS data of a peptide identified in all 22 reporter ion channels from a 22-plex combined precursor isotopic labelling and isobaric tagging experiment is shown here. The sequence of the peptide corresponds to Betaine-homocysteine S-methyl transferase **[1]**.

5.1.1. LAB MEDIA: Figure 2.

5.2. The most intense fragment ions for both the light and heavy dimethylated peaks were further isolated for MS^3 *(pronounce 'M-S-3')* fragmentation. The reporter ion intensities are directly proportionate to the peptide abundance in the sample **[1]**.

5.2.1. LAB MEDIA: Figure 2. *Video Editor: Emphasize the 2 plots at the bottom.*

5.3. Overall, this combined precursor isotopic labelling and isobaric tagging experiment reduced the sample processing times and made it possible to visualize the protein expression across multiples channels or conditions **[1]**.

5.3.1. LAB MEDIA: Table 4.

5.4. The box plot of log10 abundance versus total reporter ion intensities across all the 22 channels shows lesser inter-well or inter-sample variability. Evaluation of the total automation was done by examining the error in reporter ion abundance across each protein in the 22 samples **[1]**.

5.4.1. LAB MEDIA: Figure 3.

5.5. Sample processing with the robotic platform resulted in very low coefficients of variation. Across the 3098 peptides, the average coefficient of variation in reporter ion abundance was 12.36 and 15.03 % for light and heavy dimethylated peptides, respectively **[1]**.

5.5.1. LAB MEDIA: Figure 4.

Conclusion

6. Conclusion Interview Statements

6.1. **Albert B Arul:** When attempting this protocol, it is important to keep in mind that isotopic and isobaric labelling are used in a single experiment. Hence, careful planning needs to be performed to designate each sample with the tags intended to be used in the experiment.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.6.1, 4.8.1, 4.8.2.*

6.2. **Renã AS Robinson:** This method can be readily integrated into other robotic platforms and can be applied to a variety of samples, such as cell lysates, tissue lysates, or biospecimens.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Videographer: This statement is optional. If you don't have time, don't film it.*