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Title: Ubiquitin Chain Analysis by Parallel Reaction Monitoring

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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, all set**

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

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Joseph Longworth:** As different ubiquitin chain topologies are associated with a broad range of biological effects, understanding changes in the abundance is relevant to a wide variety of biological states.

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

1.2. **Joseph Longworth:** Applying parallel reaction monitoring to ubiquitin chain analysis allows the specific identification and quantification of the ubiquitin chain topologies.

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

OPTIONAL:

1.3. **Marta Mendes:** Unlike typical PRM experiments, Analysis of the ubiquitin chain topology is restricted to specific modified peptides, many of which are not ideal for MS analysis.

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

1.4. **Marta Mendes:** Visual demonstration of this technique highlights the adjustments and expected results observed with these non-ideal peptides.

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

Protocol

2. Sample Preparation

2.1. Begin by preparing an ammonium bicarbonate solution, N-ethylmaleimide solution, and ubiquitin stabilization buffer as described in the text manuscript **[1]**. Resuspend the biological sample in the ubiquitin stabilization buffer and lyse the cells with an appropriate method **[2]**. *Videographer: This step is difficult!*

2.1.1. WIDE: Establishing shot of talent at the lab bench preparing buffers.
2.1.2. Talent resuspending the sample.

2.2. After cell lysis, centrifuge the sample at 18,000 *x g* for 10 minutes at 4 degrees Celsius **[1]** and transfer the supernatant to a fresh low protein binding microcentrifuge tube **[2]**. If necessary, store the samples at negative 20 degrees Celsius before proceeding **[3]**.

2.2.1. Talent putting samples into the centrifuge and closing the lid.
2.2.2. Talent transferring the supernatant to a fresh tube.
2.2.3. Talent putting the samples in the freezer.

2.3. To thaw the samples, mix them rapidly with a thermomixer and do not use temperatures above 50 degrees Celsius **[1]**.

2.3.1. Samples on a thermomixer.

2.4. Once thawed, centrifuge the samples at 18,000 *x g* for 10 minutes at 4 degrees Celsius **[1]** and transfer 20 micrograms to a fresh low-bind microcentrifuge tube **[2]**, then adjust the volume to 50 microliters with ubiquitin stabilization buffer **[3]**.

2.4.1. Talent putting the sample in the centrifuge and closing the lid.
2.4.2. Talent transferring sample to a fresh tube.
2.4.3. Talent adding ubiquitin stabilizing solution to the sample.

2.5. Prepare a negative control with a normalized volume of ubiquitin stabilization buffer **[1]** and a positive control with the buffer and commercially available chain types **[2]**. *Videographer: This step is important!*

2.5.1. Talent preparing the negative control.
2.5.2. Talent adding the chain types to the positive control.

2.6. Prepare a solution of 50 millimolar ammonium bicarbonate in water and adjust the pH to 8 with 1 molar sodium hydroxide **[1]**. Pellet an E. coli culture by centrifugation at 5,000 *x g* for 5 minutes **[2]**, wash the cells twice with PBS **[3]**, and resuspend them in ubiquitin stabilization buffer at 5 micrograms per milliliter **[4]**.

2.6.1. Talent adjusting the pH of the ammonium bicarbonate.
2.6.2. Talent putting a tube with the E. coli into the centrifuge and closing the lid.
2.6.3. Talent adding PBS to the cell pellet, with the PBS container in the shot.
2.6.4. Talent resuspending the cells in ubiquitin stabilization buffer.

2.7. Add 1 microgram of E. coli lysate to each sample and control **[1]**. Then, prepare 500 millimolar TCEP in MS grade water **[2-TXT]** and add it to each sample to a final concentration of 50 millimolar **[3]**. Vortex the samples briefly and incubate them for 30 minutes at room temperature **[4]**. *Videographer: This step is important!*

2.7.1. Talent adding E. coli lysate to a few samples.
2.7.2. Talent preparing TCEP. **TEXT: TCEP = tris(2-carboxyethyl)phosphine**
2.7.3. Talent adding TCEP to a few samples.
2.7.4. Talent vortexing a sample and setting it down on the lab bench.

2.8. Next, prepare a solution of 550 millimolar chloroacetamide in ammonium bicarbonate **[1-TXT]** and add it to the samples and controls to a final concentration of 55 millimolar **[2]**. Vortex the samples briefly and incubate them in the dark for 20 minutes **[3]**.

2.8.1. Talent preparing the CAA solution. **TEXT: Store in the dark**

2.8.2. Talent adding the solution to the samples.

2.8.3. Talent putting the samples in a dark place to incubate.

2.9. Add endopeptidase LysC *(pronounce 'lyse-C')* to the samples **[1-TXT]** and incubate them at 37 degrees Celsius for 3 hours **[2]**. After the incubation, dilute the samples with 200 microliters of 50 millimolar ammonium bicarbonate **[3]**, add trypsin, and incubate them for another 12 hours **[4-TXT]**. *Videographer: This step is difficult!*

2.9.1. Talent adding endopeptidase LysC to a sample. **TEXT: 1:25 (w/w) ratio to the protein content**

2.9.2. Talent putting the samples in the thermomixer and closing the lid.

2.9.3. Talent diluting the sample with the ammonium bicarbonate, with the 50 mM NH_4HCO_3 container in the shot and clearly labeled.

2.9.4. Talent adding trypsin to the samples. **TEXT: 1:25 (w/w) ratio to the protein content**

2.10. On the next day, add 10% formic acid solution to each sample and control at a 1 to 10 volume to volume ratio **[1]**. Verify that the pH is less than 3 **[2-added]** then add 0.5 microliters of heavy peptide standard to each sample **[3]**.

2.10.1. Talent adding formic acid to a few samples, with the formic acid container labeled and in the shot. This is performed in the chemical hood.

2.10.2. Added shot: pH verification

2.10.3. Talent adding the heavy peptide standard to a few samples.

2.11. After standard C18 sample cleanup, proceed with Mass Spectrometry analysis of the samples with a PRM based methodology. NOTE: Author added VO narration but no shots. Please add this to 2.10.3

3. Software Analysis

3.1. To export the isolation list, select **Isolation List** from the **Export Menu**, select the instrument type, and set the method to **Standard [1]**. Click **Ok**, which will open a prompt to save a CSV file that can be used to create a PRM method **[2]**.

3.1.1. SCREEN: 60702_screenshot_1.mkv. 0:00 – 0:11. *Video Editor: At around 0:10, emphasize the Method type selection (Standard) at the bottom of the small Export Isolation List window.*

3.1.2. SCREEN: 60702_screenshot_1.mkv. 0:11 – 0:17.

3.2. Next, import the scheduling run by selecting **File**, **Import**, and **Results**. Choose **Several** files to import simultaneously and click **Ok**, then select the files and wait for the import to complete **[1]**.

3.2.1. SCREEN: 60702_screenshot_2.mkv. 0:00 – 0:28. *Video Editor: You can speed through the import starting at about 0:15.*

3.3. When ready, review the identifications by clicking on the mass for each heavy peptide entry. Correct recognition of the peak is often determined automatically, but manual curation may be required **[1]**.

3.3.1. SCREEN: 60702_screenshot_2.mkv. 0:28 – 0:45.

3.4. Retention times selected for the heavy variants are also applied to the light versions, creating a schedule for the PRM. To modify the scheduling window, select the peak and click on **Settings** and **Peptide Settings**, then change the **Time Window** in the **Prediction** tab **[1]**.

3.4.1. SCREEN: 60702_screenshot_2.mkv. 0:45 – 1:03.

3.5. The schedule inclusion list can then be exported using **File**, **Export**, and **Isolation List**. Choose the appropriate instrument type, set method type to **Scheduled**, and click **Ok [1]**.

3.5.1. SCREEN: 60702_screenshot_2.mkv. 1:03 – 1:18.

3.6. To analyze the data, import all samples and perform curation, removing transitions with interference or poor signal to noise ratios **[1]**. Then, export data by either right clicking on relevant graphs and selecting **Copy data** or clicking on **File**, **Export**, and **Results [2]**.

3.6.1. SCREEN: 60702_screenshot_3.mkv. 0:20 – 1:02. *Video Editor: You can either speed this up, or show a few peaks being deleted and skip to the finished plot at 1:01.*

3.6.2. SCREEN: 60702_screenshot_3.mkv. 1:02 – 1:15.

Results

4. Results: Derivation of the Ubiquitin Topology-characteristic Peptide

4.1. Treatment with the proteasome inhibitor MG-132 prevents the degradation of ubiquitin-conjugated proteins, which results in an increase of the K48 chain **[1]** in the mouse melanoma cell line B16 **[2]**, as well as the two human cell lines A549 and HeLa **[3]**.

4.1.1. LAB MEDIA: Figure 3.

4.1.2. LAB MEDIA: Figure 3. Video Editor: Emphasize the B16 bars.

4.1.3. LAB MEDIA: Figure 3. Video Editor: Emphasize the A459 and HeLa bars.

4.2. Parallel reaction monitoring, or PRM, performs a full product ion scan after selection of the precursor ion, which means that product ions should be curated post run. The chromatogram for K48 is shown before and after curation **[1-TXT]**.

4.2.1. LAB MEDIA: Figure 4. *Video Editor: Label the first plot "***Before***" and the second plot "***After***".*

4.3. Product ions that have a signal with an inconsistent elution profile **[1]** and low intensity were removed **[2]**. The transitions selected during curation should be consistent between experiments, but may differ depending on chromatographic conditions, analysis settings, and the biological background of the sample **[3]**.

4.3.1. LAB MEDIA: Figure 4, just the left image. *Video Editor: Emphasize the y1 and y6 curves, just on the legend.*

4.3.2. LAB MEDIA: Figure 4 just the left image. *Video Editor: Emphasize the y7, b1, b6, and b7 curves, just on the legend.*

4.3.3. LAB MEDIA: Figure 4, just the right image.

4.4. Shown here are typical product ion chromatograms for each of the identified ubiquitin chain topologies in this experiment **[1]**.

4.4.1. LAB MEDIA: Figure 5.

4.5. When designing a PRM experiment, collision energy can be optimized to improve the signal **[1]**. When collision energies from 14 to 28 were applied, it was found that a higher collision energy of 26 was optimal for K63 **[2]** while a lower energy of 18 was ideal for M1 **[3]**.

4.5.1. LAB MEDIA: Figure 6.

4.5.2. LAB MEDIA: Figure 6. *Video Editor: Emphasize the bar at 26 on the K63 graph.*

4.5.3. LAB MEDIA: Figure 6. *Video Editor: Emphasize the bar at 18 on the M1 graph.*

4.6. Favourable collision energies were found for the topology-characteristic peptides in this experiment and a selection of unmodified ubiquitin fragments, but they may need to be optimized based on the mass spectrometer and fragmentation method used. **[1]**.

4.6.1. LAB MEDIA: Table 1.

Conclusion

5. Conclusion Interview Statements

5.1. **Joseph Longworth:** When attempting this procedure, it is important to remember that the identification of ubiquitin chain topology requires a balancing of conditions to ensure ubiquitin linkages are maintained whilst promoting its digestion into peptides.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.9.3.*

5.2. **Gunnar Dittmar:** This method has been vital to the understanding of ubiquitin signaling by providing accurate quantification of ubiquitin chain topology. It could be combined with a pulldown or organelle fractionation to address which ubiquitin chain topologies are associated with the targeted protein sample.

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