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Scriptwriter Name: Bridget Colvin
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Title: Quantitative Metabolomics of *Saccharomyces Cerevisiae* Using Liquid Chromatography Coupled with Tandem Mass Spectrometry

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

   Videographer: All screen capture files provided, do not film

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **33**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Karamat Mohammad**: This protocol has many advantages over the currently used yeast metabolomic methods in that it is more sensitive and that it can profile various classes of metabolites, including isobaric, isomeric, hydrophilic, and hydrophobic **[1]**.

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

REQUIRED:

1.2. **Karamat Mohammad**: The quenching method in this protocol stops all enzymatic reactions while significantly decreasing cellular metabolite leakage. To accurately annotate metabolites, a MS1 library is developed from several MS2 runs **[1]**.

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

Protocol

2. Cell Transfer and Culture

2.1. After overnight culture at 30 degrees Celsius and 200 revolutions per minute, determine the number of yeast cells per milliliter of culture **[1-TXT]** and add 5 milliliters of sterilized 20% stock solution of glucose to each of two Erlenmeyer flasks containing 45 milliliters of autoclaved YP (Y-P) medium per flask **[2-TXT]**.

2.1.1. WIDE: Talent counting cells, with yeast culture visible in frame **TEXT: See text for full yeast culture preparation details**

2.1.2. Talent adding solution to flask(s), with solution container visible in frame **TEXT: YP: yeast extract with bactopeptone**

2.2. Use a sterile transfer pipette to add 5 x 10^7 yeast cells to each flask **[1]** and grow the yeast cells for at least 24 additional hours at 30 degrees Celsius at 200 revolutions per minute **[2]**.

2.2.1. Talent adding cells to flask(s), with original culture flask also visible in frame

2.2.2. Talent placing flasks into shaking incubator

3. Membrane Integrity Assessment

3.1. The next day, after quenching the yeast cells, thoroughly wash the cells with 15 milliliters of ABC (A-B-C) buffer **[1-TXT]** and collect the cells by centrifugation **[2-TXT]**.

3.1.1. WIDE: Talent mixing cells, with buffer visible in frame **TEXT: See text for quenching details**

3.1.2. Talent placing tube into centrifuge **TEXT: 5 min, 3000 x g, 0 °C**

3.2. Resuspend the pellet in 1 milliliter of fresh ABC buffer **[1]** and add 500 microliters of the propidium iodide solution to the cells **[2]**.

3.2.1. Shot of pellet if visible, then buffer being added to tube, with buffer container visible in frame

3.2.2. Talent adding solution to tube, with solution container visible in frame

3.3. Vortex the sample three times for 10 seconds per vortex **[1]** and incubate the cells for 10 minutes on ice protected from light **[2]**.

3.3.1. Sample being vortexed
3.3.2. Talent placing tube on ice

3.4. At the end of the incubation, collect the cells by centrifugation **[1-TXT]** and wash the cells three times with 1 milliliter of fresh ABC buffer per wash **[2]**.

3.4.1. Talent placing tube into centrifuge **TEXT: 10 min, 13,400 x g, 4 °C**
3.4.2. Shot of pellet If visible, then buffer being added to tube

3.5. After the last wash, resuspend the pellet in 300 microliters of fresh ABC buffer **[1]** and add 10 microliters of the resulting cell suspension to a microscope slide **[2]**.

3.5.1. Shot of pellet, then buffer being added to tube *Videographer: Important step*
3.5.2. Talent adding cells to slide *Videographer: Important step*

3.6. Use a fluorescence microscope to capture differential interference contrast and fluorescence microscopy images of the cells with the filters set to an excitation wavelength of 593 nanometers and an emission wavelength of 636 nanometers **[1]**.

3.6.1. Talent at microscope, imaging cells, with monitor visible in frame *Videographer: Important step*

3.7. Then use an appropriate image analysis program to count the total cell number of cells in the brightfield and fluorescence images **[1]** and to determine the fluorescence intensity of staining for individual cells **[2]**.

3.7.1. SCREEN: step 3.7.1: 00:29-00:50 *Video Editor: please speed up*
3.7.2. SCREEN: step 3.7.2: 00:21-00:46 *Video Editor: please speed up*

4. Metabolite Extraction

4.1. For metabolite extraction, after culture, collect the yeast cells by centrifugation **[1-TXT]** and quickly remove the supernatant **[2]**.

4.1.1. WIDE: Talent placing tube(s) into centrifuge **TEXT: 3 min, 3000 x g, 0 °C**
4.1.2. Shot of pellet if visible, then supernatant being removed

4.2. Place the tube on dry ice **[1]** and add 2 milliliters of minus 20-degree Celsius chloroform, 1 milliliter of minus 20-degree Celsius methanol, 1 milliliter of ice-cold nano-pure water, and 200 microliters of 425-600-micron, acid-washed glass beads to the cells **[2]**.

4.2.1. Talent placing tube on dry ice *Videographer: Important/difficult step*
4.2.2. Talent adding chloroform, with chloroform, methanol, and bead containers visible in frame *Videographer: Important/difficult step*

4.3. When the beads have been added, place the tube, covered with aluminum foil, into a foam tube holder kit with a retainer **[1]** and vortex the sample for 30 minutes at medium speed at 4 degrees Celsius to facilitate metabolite extraction **[2]**.

4.3.1. Talent placing foil covered tube into holder kit *Videographer: Important/difficult step*
4.3.2. Talent vortexing sample *Videographer: Important/difficult step*

4.4. Next, incubate the tube for 15 minutes on ice **[1]** before centrifuging the sample to allow separation of the upper aqueous phase from the middle debris and protein and lower organic phases **[2-TXT]**.

4.4.1. Talent placing tube on ice
4.4.2. Talent placing tube into centrifuge **TEXT: 10 min, 3000 x g, 4 °C**

4.5. Use a micropipette to transfer approximately 400 microliters of the upper aqueous phase **[1]** to a washed and labeled 1.5-milliliter tube containing 800 microliters of minus 20-degree Celsius acetonitrile **[2]**.

4.5.1. Shot of phases, then upper phase being collected *Videographer: Important step*
4.5.2. Upper phase being added to ACN/ACN turning cloudy *Videographer: Important step*

4.6. Then centrifuge the sample **[1-TXT]** and transfer 800 microliters of the upper portion of the supernatant to a labeled mass spectrometry vial for zero-degree storage until liquid chromatography tandem mass spectrometry analysis **[2]**.

4.6.1. Talent placing tube into centrifuge **TEXT: 10 min, 13,400 x g, 4 °C**
4.6.2. Sample being collected/transferred, with labeled vial visible in frame

5. Extracted Metabolite Separation and Analysis

5.1. To separate the extracted metabolites, ultrasonicate the sample vial for 15 minutes **[1]** followed by three, 10-seconds vortexes at room temperature **[2]**.

5.1.1. WIDE: Talent ultrasonicating vial
5.1.2. Talent vortexing vial

5.2. After vortexing, place the vial into the well plate of the liquid chromatographer **[1]** and set the column to 45 degrees Celsius with a flow rate of 0.25 milliliters/minute and the well plate to zero degrees Celsius **[2]**.

5.2.1. Talent placing vial into well plate
5.2.2. Talent setting analysis parameters

5.3. Then use the table to set the liquid chromatography gradients for the analysis **[1]**.

5.3.1. LAB MEDIA: Table 1 Video Editor: please emphasize HPLC gradient program section

5.4. After separation, use 10 microliter-sample volumes of the injection in both the electrospray ionization-positive and -negative modes in a mass spectrometer equipped with heated electrospray ionization to identify and quantify the water-soluble metabolites **[1-TXT]**.

5.4.1. Talent injecting sample volume **TEXT: See text for LC analysis setting details**

5.5. At the end of the analysis, open the raw data in an appropriate compound analysis software program **[1]** and utilize the mass spectral fragmentation library to match the mass spectra to annotate the metabolites identified in the analysis **[2]**.

5.5.1. Talent opening software, with monitor visible in frame
5.5.2. SCREEN: step 5.5.2: 02:15-02:25

5.6. The exact masses of the MS1 (M-S-one) and isotope patterns can also be identified to allow annotation of the metabolites using online databases **[1]**.

5.6.1. SCREEN: step 5.6.1: 00:02-00:24

5.7. Then use the library of databases and spectra to search for the MS2 spectra of the raw data **[1-TXT]**.

5.7.1. SCREEN: step 5.7.1: 00:01-00:26 **TEXT: https://www.mzcloud.org**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
3.5., 3.6., 4.2., 4.3., 4.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
4.2., 4.3.

Results

6. Results: Representative Metabolic Intermediate and Product Identification and Quantification

6.1. The modified cell quenching method **[1]** causes significantly lower damage to the plasma membrane and cell wall **[2]** than the non-buffered 80% methanol at minus 40 degrees Celsius quenching method **[3]**.

6.1.1. LAB MEDIA: Figure 3
6.1.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize 2nd column of images*
6.1.3. LAB MEDIA: Figure 3 *Video Editor: please emphasize 4th column of images*

6.2. Indeed, almost all of the cells subjected to quenching using the modified method exhibit red fluorescence emission, which is characteristic of yeast cells in which the plasma membrane and cell wall are not damaged **[1]**.

6.2.1. LAB MEDIA: Figure 3 *Video Editor: please emphasize red signal in bottom image of 2nd column*

6.3. In contrast, almost all of the cells subjected to quenching using the non-buffered 80% methanol at minus 40 degrees Celsius method **[1]**, display green fluorescence emission, which is characteristic of yeast cells in which the plasma membrane and cell wall are significantly damaged **[2]**.

6.3.1. LAB MEDIA: Figure 3
6.3.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize green signal in bottom image of 4th column*

6.4. The modified cell quenching method **[1]** also causes significantly lower leakage of water-soluble metabolites from yeast cells **[2]** than the non-buffered 80% methanol at minus 40 degrees Celsius quenching method **[3]**.

6.4.1. LAB MEDIA: Figures 4 and 5
6.4.2. LAB MEDIA: Figures 4 and 5 *Video Editor: please emphasize Figure 4*
6.4.3. LAB MEDIA: Figures 4 and 5 *Video Editor: please emphasize Figure 5*

6.5. The retention time shift values of water-soluble metabolite standards **[1]** are significantly lower and the peak shapes are substantially sharper for the zwitterionic-phase column **[2]** compared to the reverse-phase column **[3]**.

6.5.1. LAB MEDIA: Supplemental Table 1

6.5.2. LAB MEDIA: Supplemental Table 1 *Video Editor: please emphasize Zwitterionic-phase columns*

6.5.3. LAB MEDIA: Supplemental Table 1 *Video Editor: please emphasize Reverse-phase Zorbax Eclipse Plus columns*

6.6. Another advantage of the liquid chromatography-tandem mass spectrometry method is the ability to use the zwitterionic-phase column to efficiently separate different water-soluble metabolites with diverse structural, physical, and chemical properties **[1]**.

6.6.1. LAB MEDIA: Figure 6 *Video Editor: please sequentially emphasize peaks from top to bottom row*

Conclusion

7. Conclusion Interview Statements

7.1. **Karamat Mohammad**: Be sure to incubate the cells in the dark when labeling with propidium iodide solution and to carefully transfer the upper phase containing the metabolites without disturbing the middle phase **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.3., 4.5.)

7.2. **Karamat Mohammad**: If any MS peak can't be annotated due to a lack of MS2 spectral match within the online spectral libraries, then NMR can be used to elucidate its structure **[1]**.

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