FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 62577
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Project Page Link: https://www.jove.com/account/file-uploader?src=19086033

Title: Identification of Antibacterial Immunity Proteins in *Escherichia coli* using MALDI-TOF-TOF-MS/MS and Top-Down Proteomic Analysis

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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 something similar? **No**

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

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, then the interviewee removes the mask for line delivery only. When the take is captured, 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? **Yes**

   If **Yes**, how far apart are the locations? 100 feet

Current Protocol Length

Number of Steps: 19
Number of Shots: 43

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Clifton Fagerquist:** The protocol demonstrates a rapid method for the identification of bacterial proteins using antibiotic induction and mass spectrometry.

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

1.2. **Clifton Fagerquist:** The main advantage of the technique is its speed and simplicity. Sample preparation is uncomplicated.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.2*

OPTIONAL:

1.3. **Clifton K. Fagerquist:** The technique can be extended to the characterization of any pathogenic bacteria, such as virulence factors or antibiotic resistance, to better inform appropriate treatment for a bacterial infection.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.1*

***Videographer**: Please film the screen for the shots labeled as **SCREEN**.*

Video editor**: Some shots are labeled as SCREEN, and the authors have provided the screenshot images for the same (Placed as LAB MEDIA with **OR** in the shot). **Please use either filmed screen or images which fits best.

Protocol

2. Mass Spectrometry

2.1. To begin, use a sterile 1 microliter loop to harvest bacteria from single colonies grown on LB *(L-B)* agar plate **[1]** and transfer to a 2-milliliter O-ring-lined screw-cap microcentrifuge tube containing 300 microliters of HPLC *(H-P-L-C)*-grade water **[2]**. Then, briefly vortex the tube **[3]** and pellet the cells by centrifugation **[4-TXT]**.

2.1.1. WIDE: Talent harvesting the cells from colonies. *Videographer: This shot is important!*

2.1.2. Talent transferring the cells in the tube containing water. *Videographer: This shot is important!*

2.1.3. Talent vortexing the tube. *Videographer: This shot is important!*

2.1.4. Talent placing the tube in a centrifuge. **TEXT: Centrifuge: 11,337 x *g*, 2 min; RT** *Videographer: This shot is important!*

2.2. Add 0.75 microliters aliquot of the sample supernatant on the stainless steel MALDI *(Mal-dee)* target and allow it to dry **[1]**. Overlay the dried sample spot with 0.75 microliters of a saturated solution of sinapinic acid prepared in 33% acetonitrile, 67% water, and 0.2% trifluoroacetic acid, and allow the spot to dry **[2]**.

2.2.1. Talent adding a drop of sample on MALDI target. *Videographer: This shot is important!*

2.2.2. Talent adding sinapinic acid to the dried sample spot. *Videographer: This shot is important!*

2.3. Once the spot is dried, load the target in the mass spectrometer **[1]**. Open the acquisition software and click MS *(M-S)* linear mode acquisition **[2]**. For mass-to-charge range, enter mass-to-charge of the lower and upper bounds into their respective fields **[3]**. Click on the sample spot to be analyzed on the MALDI target template **[4]**.

2.3.1. Talent placing the target in the mass spectrometer.

2.3.2. Talent at the computer, opening the software and clicking MS linear mode acquisition option with monitor visible in the frame.

2.3.3. LAB MEDIA: 62577_SCREEN SHOT_#2.png *Video editor: Please emphasize the red box.*

2.3.4. LAB MEDIA: 62577_SCREEN SHOT_#3.png *Video editor: Please emphasize the red ellipse.*

2.4. Then, depress the left mouse button **[1]** and drag the cursor over the sample spot to specify the rectangular region to be sampled for laser ablation or ionization **[2]**. Release the mouse button **[3]** to initiate the acquisition and collect 1,000 laser shots for each sample spot **[4]**.

2.4.1. Talent pressing the mouse button.

2.4.2. SCREEN: The cursor being dragged over the sample spot **or** LAB MEDIA: 62577_SCREEN+SHOT_#4.png

2.4.3. Talent releasing the button.

2.4.4. SCREEN: Acquisition started, and laser shots being collected.

2.5. If ions are not detected, increase the laser intensity by adjusting the **Sliding Scale Bar** under **Laser Intensity** until the protein ion signal is detected **[1]**. After completion of the MS linear mode acquisition, click the MS/MS *(M-S-M-S)* reflectron mode acquisition **[2]**. Enter the precursor mass to be analyzed into the **Precursor Mass** field **[3]**.

2.5.1. LAB MEDIA: 62577_SCREEN SHOT_#5.png *Video editor: Please emphasize the red rectangle.*

2.5.2. SCREEN: The MS/MS reflectron mode acquisition being clicked **OR** LAB MEDIA: 62577_SCREEN SHOT_#6.png

2.5.3. LAB MEDIA: 62577_SCREEN SHOT_#7.png *Video editor: Please emphasize the red rectangle.*

2.6. Next, enter an isolation width in Daltons in the **Precursor Mass Window** for the low and high mass side of the precursor mass **[1]**. Click the **CID** *(C-I-D)* **Off** button, then click the **Metastable Suppressor ON** button **[2]**. Adjust the laser intensity to at least 90% of its maximum value by adjusting the **sliding scale bar** as demonstrated **[3]**.

2.6.1. LAB MEDIA: 62577_SCREEN SHOT_#8.png *Video editor: Please emphasize the red rectangle.*

2.6.2. LAB MEDIA: 62577_SCREEN SHOT_#9.png *Video editor: Please emphasize the red rectangle.*

2.6.3. SCREEN: Laser intensity being adjusted **OR** LAB MEDIA: 62577_SCREEN SHOT_#10.png *Video editor: Please emphasize the red rectangle.*

2.7. Click on the sample spot to be analyzed on the MALDI target template **[1].** Then, depress the left mouse button **[2]** and drag the cursor over the sample spot to specify the rectangular region to be sampled for laser ablation or ionization **[3]**.

2.7.1. LAB MEDIA: 62577_SCREEN SHOT_#11.png *Video editor: Please emphasize the red ellipse.*

2.7.2. Talent pressing the mouse button.

2.7.3. SCREEN: The cursor being dragged over the sample spot **OR** LAB MEDIA: 62577_SCREEN SHOT_#12.png *Video editor: Please emphasize the green square in the lower panel at the left side.*

2.8. Release the mouse button **[1]** to initiate the acquisition and collect 10,000 laser shots for each sample spot, as demonstrated **[2]**.

2.8.1. Talent releasing the button.

2.8.2. LAB MEDIA: 62577_SCREEN SHOT_#13.png *Video editor: Please emphasize the graph at the bottom right of the image and highlight the label (Red fonts) at the top of the graph*.

3. Operating Protein Biomarker Seeker software

3.1. Double click on the **Protein Biomarker Seeker** executable file, and the graphical user interface window will appear **[1]**. Enter the mass of the protein biomarker in the **Mature Protein Mass** field and the mass measurement error in the **Mass Tolerance** field **[2]**.

3.1.1. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 00:07 to 00:13 *Video editor: Please trim the background out or zoom in on the window of the software operated by the author.*

3.1.2. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 00:37 to 00:54.

3.2. Optionally, click on the **Complementary b/y** *(b-y)* **ion Protein Mass Calculator** button to calculate the protein mass from a putative complementary fragment ion pair and the pop-up window of the protein mass calculator tool will appear **[1]**.

3.2.1. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 01:17 to 01:34 *Video editor: Please speed up the video.*

3.3. Enter the ***m/z*** *(mass-to-charge ratio)* of the putative complementary fragment ion pair, click on the **Add Pair** button, and the calculated protein mass will appear **[1]**.

Copy-paste this value in the **Mature Protein Mass** field and close the **Protein Mass Calculator Tool** window **[2]**.

3.3.1. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 01:35 to 01:56

3.3.2. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 02:10 to 02:17 and 02:32 to 02:36.

3.4. Click on the **Set Residue Restriction** box, select the **N-terminal Signal Peptide Length**, and the pop-up with a sliding scale and cursor will appear and move the cursor to the desired signal peptide length. If the signal peptide length is not selected, an unrestricted sequence truncation will be performed by the software **[1]**.

3.4.1. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 02:56 to 03:11 and 03:39 to 03:42.

3.5. Under the **Fragment Ion Condition [1]**, select the residues for polypeptide backbone cleavage by clicking on the boxes of one or more residues- D, E, N, and/or P and then click on the **Enter Fragment Ions (+1)** *(Plus-1)* **To Be Searched** button, and the pop-up **Fragment Page** will appear **[2]**.

3.5.1. Talent at the computer, looking at screen and moving the mouse.

3.5.2. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 03:59 to 04:10 and 04:24 to 04:29.

3.6. Next, click on the **Add Fragment Ion** button, and a dropdown field will appear for each fragment ion to be entered **[1]**. Enter the mass-by-charge ratios of the fragment ions and their associated mass-by-charge tolerance, then click on the **Save and Close** button **[2]**.

3.6.1. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 04:30 to 04:36.

3.6.2. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 05:11 to 05:41 and 07:15 to 07:17. *Video editor: Please speed up the video.*

3.7. In the box right to the **How Many Fragment Ions Need to be Matched**, scroll **[1]** to select the minimum number of fragment ions that must be matched for identification and select the cysteine residues in their oxidized state **[2]**. If the proteins are not identified after the search, repeat the search with cysteines in their reduced state **[3]**.

3.7.1. Talent rolling the scroll wheel of the mouse.

3.7.2. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 07:28 to 07:35. *Video editor: Please highlight the oxidized option just below the cysteine oxidation state.*

3.7.3. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 08:17 to 08:23.

3.8. Under the **File Setup**, click on the **Select FASTA File** icon to browse and select the FASTA file containing the *in silico* protein sequences of the bacterial strain previously constructed **[1]**. Then select an output folder and create an output file name **[2]**.

3.8.1. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 09:13 to 09:33.

3.8.2. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 09:42 to 10:03. *Video editor: Please speed up the video.*

3.9. Click on the **Run Search on File Entries** icon, a pop-up window will appear entitled **Confirm Search Parameters** displaying the search parameters before the search is initiated and if the search parameters are correct, click **Begin Search**, and if the parameters are incorrect, click **Cancel** and re-enter the correct parameters **[1]**.

3.9.1. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 10:04 to 10:39

3.10. Once the search is initiated, the parameter window will close, and a new pop-up window with a progress bar will appear, showing the progress of the search and a running tally of the number of identifications found **[1]**.

3.10.1. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 10:34 to 10:38.

3.11. Upon completion of the search, the progress bar will be closed automatically, and a summary of the search will be displayed in the log field of the graphic user interface along with a new pop-up window displaying the protein identifications found **[1]**.

3.11.1. LAB MEDIA: 62577_Video_2021-06-08 07-01-29.mp4: 10:39 to 10:47 and 11:34 to 11:37

Results

4. Results: Analysis of Bacterial Proteins Using Mass Spectrometry

4.1. In the current study, bacterial cultures were grown in LB with two different concentrations of mitomycin-C. In the mass spectrum of bacterial culture grown with mitomycin-C concentration **[1]**, cold-shock protein-C, cold-shock protein-E, and a plasmid-borne protein of unknown function were identified **[2]**.

4.1.1. LAB MEDIA: Figure 3 *Video editor: Please emphasize the top panel.*

4.1.2. LAB MEDIA: Figure 3 *Video editor: Please emphasize the peaks of CspC, CspE, and Plasmid protein in the mass spectrum.*

4.2. The unknown protein ion at 9780 was analyzed by MS/MS, and the precursor ion was isolated with a TIS *(timed ion selector)* window of approximately 100 Daltons **[1]**. The fragment ion at mass-to-charge 2675.9 is spillover from the dissociation of the metastable protein ion at 9655 **[2]**.

4.2.1. LAB MEDIA: Figure 3 *Video editor: Please emphasize the top panel.*

4.2.2. LAB MEDIA: Figure 3 *Video editor: Please emphasize the peak at 2675.9 with an asterisk.*

4.3. The sequence of the immunity protein for colicin E3 **[1]** contains basic residues-possible sites of ionization **[2]**, the fragment ions detected from polypeptide backbone cleavage **[3]** when the reduced form of cysteine was used during the search **[4]**. The N-terminal methionine is removed as a post-translation modification **[5]**.

4.3.1. LAB MEDIA: Figure 3 *Video editor: Please emphasize the protein sequence.*

4.3.2. LAB MEDIA: Figure 3 *Video editor: Please emphasize blue alphabets in the sequence.*

4.3.3. LAB MEDIA: Figure 3 *Video editor: Please emphasize red alphabets with an asterisk and their corresponding peak in the graph.*

4.3.4. LAB MEDIA: Figure 3 *Video editor: Please emphasize the C alphabet in a black box in the sequence.*

4.3.5. LAB MEDIA: Figure 3 *Video editor: Please emphasize the M alphabet underline in the sequence.*

4.4. When the bacterial culture was grown at a high mitomycin-C concentration, the immunity protein of bacteriocin was identified **[1]**. The unknown peak at 9651 was

analyzed by MS/MS, and the precursor ion was isolated with a narrower and asymmetric TIS window of minus 75/plus 60 Daltons **[2]**.

4.4.1. LAB MEDIA: Figure 4 *Video editor: Please emphasize the Im3 peak at 9778 m/z in the top panel.*

4.4.2. LAB MEDIA: Figure 4 *Video editor: Please emphasize the peak at 9651 m/z in the top panel.*

4.5. The sequence of the immunity protein of bacteriocin **[1]** contains basic residues - possible sites of ionization **[2]**, the fragment ions detected from polypeptide backbone cleavage **[3]** when the reduced form of cysteine was used during the search **[4]**.

4.5.1. LAB MEDIA: Figure 4 *Video editor: Please emphasize the protein sequence.*

4.5.2. LAB MEDIA: Figure 4 *Video editor: Please emphasize blue alphabets in the sequence.*

4.5.3. LAB MEDIA: Figure 4 *Video editor: Please emphasize red alphabets with an asterisk and their corresponding peak in the graph.*

4.5.4. LAB MEDIA: Figure 4 *Video editor: Please emphasize the C alphabet in a black box in the sequence.*

4.6. With the different concentrations of antibiotics, relative protein abundance may vary, and these were analyzed using mass spectrometry **[1]**.

4.6.1. LAB MEDIA: Figure 3 and 4

Conclusion

5. Conclusion Interview Statements

5.1. **Clifton K. Fagerquist:** When harvesting bacterial cells, observe colony morphology and bacterial growth with respect to antibiotics and concentration. A one microliter loop of cells is necessary to detect proteins.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.1.1 and 2.1.2*