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Title: Separation and Fractionation of Culture Filtrate Proteins (CFPs) from *Mycobacterium tuberculosis*

Authors and Affiliations:

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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?

   NO

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

Current Protocol Length
Number of Steps: 14
Number of Shots: 45

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Ramalingam Bethunaickan:** This study aims to separate the culture filtrate proteins (CFPs) of Mycobacterium tuberculosis to find key protein fractions involved in immune response and disease, which may help in developing better diagnostics or vaccines. We are trying to find out which fractions are most immunogenic and how they contribute to the bacteria's ability to cause and sustain infection.

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

What are the most recent developments in your field of research?

1.2. **Ramalingam Bethunaickan:** Recent advancements in electrophoresis techniques now enable the separation of highly complex proteomes into simple fractions containing 2-4 proteins, allowing precise downstream proteomic and immunological analysis.

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

What are the current experimental challenges?

1.3. **Anbarasu Deenadayalan:** One major experimental challenge in proteome is the limited loading capacity during electrophoresis, loading too much protein say about 350ug of protein or more can lead to precipitation, affecting the quality of separation. Additionally, the electrophoresis process can denature proteins, making them unsuitable for further functional studies or assays that require proteins in their native state..

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.3.3.*

What significant findings have you established in your field?

1.4. **Madhavan Dhanapal:** Using our protein separation method, we successfully identified 27 new T cell antigens specific to *Mycobacterium tuberculosis*. These antigens show strong promise for the development of more accurate diagnostic tools and next-generation TB vaccines.

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

What research gap are you addressing with your protocol?

1.5. **Uma Devi Ranganathan:** This protocol addresses the gap in resolving complex proteomes by allowing separation into well-defined and simplified protein fractions. These fractions can be directly used for proteomic and immunological studies, helping in faster identification of disease-specific biomarkers without the need for further purification.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 4.*

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

Videographer's Note: For the protocol videos, all the natural light coming in either had green or blue coloured windows. Please balance the colour temperature closest to pure white, for the entire final video.
Panasonic LUT has been attached to this mail, if needed for editors.
I have shot 2 variations of images- 1 landscape & 1 portrait each for headshots of the authors. Please use them interchangeably for both #67679 & #67680.
#67679 has a lot of footage which has to be used for #67680.

2. Cultivation of *Mycobacterium tuberculosis* and Preparation of Culture Filtrate Proteins (CFPs)

Demonstrator: Madhavan Dhanapal

2.1. To begin, prepare Sauton's liquid medium by combining the given reagents in 950 milliliters of distilled water **[1-TXT]**. ~~Adjust the pH to 7.2 by adding approximately 5 milliliters per liter of 40% potassium hydroxide **[2].**~~ Make up the volume to 1 liter **[3]** and autoclave at 15 psi for 20 minutes **[4]**.

2.1.1. WIDE: Talent adding the reagents to a beaker or conical flask containing 950 milliliters of distilled water. **TXT: 30 mL Glycerol, 0.5 g KH_2PO_4, 0.5 g Magnesium sulfate, 2 g Citric acid, 0.5 g Casein hydrolysate, 0.05 g Ferric ammonium sulfate, 4 g Sodium glutamate, pH: 7.2**

2.1.2. ~~Talent adding potassium hydroxide to the solution.~~
Videographer's Note: Shot deleted by authors

2.1.3. Talent adding solution to make up the volume to 1 liter.

2.1.4. Talent placing the conical or beaker in an autoclave.

2.2. Now, transfer colonies of *Mycobacterium tuberculosis* grown on Lowenstein-Jensen or LJ *(L-J)* slopes into 2 milliliters of Sauton's liquid medium **[1]**. Using glass beads, vigorously shake the suspension under sterile conditions to break up mycobacterial clumps **[2]**.
Videographer's Note: Authors have added 2 extra shots. Total of 4 shots all slated as 2.2.1 on the clapboard

Added shot: Close up of the L-J slope

2.2.1. Talent transferring bacterial colonies into a microcentrifuge tube containing 2 milliliters of Sauton's liquid medium.

2.2.2. Talent adding glass beads to the tube and shaking the tube under a biosafety cabinet.

Added shot: Vigorous shaking of the suspension

2.3. To initiate mycobacterial growth, transfer the bacterial suspension into a McCartney bottle containing 10 milliliters of Sauton's liquid medium **[1]**. Incubate the bottle at 37 degrees Celsius for 2 weeks **[2]**.

2.3.1. Talent pipetting the bacterial suspension into a McCartney bottle containing 10 milliliters of fresh Sauton's liquid medium.
Added shot: Placing in beaker
Videographer's Note: The authors have added a shot which should come after 2.3.1. I have noted it as 2.3.1 - Shot 2 on the clapboard. Ok take is the final clip

2.3.2. Talent placing the McCartney bottle into an incubator.

2.4. Scale up the culture by transferring the grown culture to 200 milliliters of Sauton's medium in a 1-liter bottle **[1]**. Place the bottle on a shaker and incubate at 37 degrees Celsius for 4 weeks **[2]**.

2.4.1. Talent transferring the culture into a 1-liter bottle containing 200 milliliters of Sauton's medium.

2.4.2. Talent placing the bottle on a shaking incubator.

2.5. Transfer the log-phase culture into a 4-liter flask containing 2 liters of Sauton's medium **[1]**. Grow the culture at 37 degrees Celsius under stationary conditions for an additional 4 weeks **[2]** until a surface pellicle forms **[3]**.

2.5.1. Talent transferring culture into a 4-liter flask containing 2 liters of fresh Sauton's medium.

2.5.2. Talent placing the flask in an incubator.

2.5.3. CU: The formed surface pellicle.

2.6. Transfer the culture into centrifuge tubes **[1]** and harvest them by centrifuging at 1,000 to 1,500 *g* for 30 minutes at room temperature **[2]**. Filter the supernatant through a 0.45-micrometer vacuum filtration system **[3]**. Then, using a tangential flow filtration system with a 10 kilodalton cutoff, concentrate the filtrate containing culture filtrate proteins **[4]**.

2.6.1. Talent placing the flask culture into centrifuge tubes.
Added shot: 2.6.1 (Shot 2) - Tube into rotor & exit

Added shot: 2.6.1 (Shot 3) - Removal of gloves to prevent contamination
Videographer's Note: The authors have added 2 shots which should come after 2.6.1.

There are 3 shots in total in this step. I have noted it as 2.6.1 - Shot 2 on the clapboard & 2.6.1 - Shot 3 on the clapboard.

2.6.2. Talent loading the tube into a centrifuge.

2.6.3. Talent filtering the supernatant through a vacuum filtration system with a 0.45 micrometer filter.

2.6.4. Talent concentrating the filtered supernatant using a tangential flow filtration system.
Added shot: 2.6.4 - 2 - Filtration close-up of supernatant

Added shot: 2.6.4 - 3 - Concentrated supernatant
Videographer's Note: The authors have added 2 shots which should come after 2.6.4. There are 3 shots in total in this step. I have noted it as 2.6.4 - 2 on the clapboard & 2.6.4 - 3 on the clapboard

2.7. Estimate the protein content using a commercially available bicinchoninic acid assay kit **[1]**. Aliquot the proteins **[2]**, add sodium azide to a final concentration of 0.2% **[3]**, and store at minus 80 degrees Celsius **[4]**.

2.7.1. Talent adding the protein to a bicinchoninic acid assay kit.
Added shot: 2.7.1-2
Videographer's Note: The authors have added a shot which should come after 2.7.1. I have noted it as 2.7.1 - 2 on the clapboard

2.7.2. Talent aliquoting the concentrated protein.
Videographer's Note: Shot 2.7.2 & 2.7.3 are shot together. Slated as 2.7.2 for both. The final shot which features both steps together is the Ok take

2.7.3. Talent adding sodium azide.

2.7.4. Talent placing the aliquots into a minus 80 degrees Celsius freezer.

3. Preparation and Fractionation of CFPs by Liquid Phase Isoelectric Focusing (IEF)

Demonstrator: Anbarasu Deenadayalan and Madhavan Dhanapal

3.1. Solubilize *Mycobacterium tuberculosis* culture filtrate protein in 60 milliliters of isoelectric focusing or IEF *(I-E-F)* separation buffer containing 8 molar urea, 1 millimolar dithiothreitol, 5% glycerol, 2% digitonin, and 2 percent ampholytes **[1]**.

3.1.1. Talent dissolving the proteins in the specified IEF buffer inside a beaker.

3.2. Fill the anode chamber with 0.1 molar phosphoric acid **[1]** and the cathode chamber with 0.1 molar sodium hydroxide **[2]**. Assemble the preparative isoelectric focusing cell

according to the manufacturer's instructions **[3]**.

3.2.1. Talent pouring 0.1 molar phosphoric acid into the anode chamber.

3.2.2. Talent pouring 0.1 molar sodium hydroxide into the cathode chamber.

3.2.3. Assembled preparative isoelectric focusing cell.

3.3. Load up to 350 milligrams of protein onto the IEF system **[1]**. Focus the proteins at 4 degrees Celsius using a cooling circulatory water system **[2]**. Apply a constant power of 12 watts **[3]** until the voltage stabilizes at approximately 1,400 volts, before continuing for an additional 30 minutes **[4]**.

3.3.1. Talent pipetting protein sample into the IEF system loading tray.

3.3.2. Talent connecting the system to a cooling circulatory water system.

3.3.3. SCREEN: A constant power of 12 watts being applied. *Videographer: If this shot is performed on a computer screen, please make sure the screen is clearly visible in the frame.*
Videographer: Shot on camera

3.3.4. SCREEN: Monitor showing increasing voltage reaching ~1400 volts and stabilizing. *Videographer: If this shot is performed on a computer screen, please make sure the screen is clearly visible in the frame.*
Videographer: Shot on camera

3.4. Harvest the IEF fractions **[1]** and determine their pH **[2]**. Analyze each aliquot by SDS-PAGE *(S-D-S-Page)* **[3-TXT].** ~~and visualize proteins using silver staining **[4].**~~

3.4.1. Talent adding the IEF fractions into labeled tubes.

3.4.2. Talent measuring pH of the IEF fractions.

3.4.3. Talent loading aliquots on SDS-PAGE gels. **TXT: Visualize proteins using silver staining**

~~3.4.4. The proteins visualized using silver staining.~~

4. SDS-PAGE Separation and Whole-Gel Elution of Culture Filtrate Proteins

Demonstrator: Anbarasu Deenadayalan and Madhavan Dhanapal

4.1. Prepare samples for SDS-PAGE by mixing the IEF fractions with a commercially available 6x *(six-ex)* SDS-PAGE sample buffer **[1]**. Heat the mixture at 95 degrees Celsius for 5 minutes **[2]**.

4.1.1. Talent pipetting fractions into tubes and adding 6× SDS-PAGE buffer.

4.1.2. Talent placing the tubes in a heat block at 95 degrees Celsius.

4.2. Load up to 10 milligrams of protein into a 16 by 20-centimeter polyacrylamide gel with a 4 percent stacking gel and a 12.5 percent resolving gel **[1]**. Run the electrophoresis at a constant current of 50 milliamperes until the dye front is 2 centimeters from the bottom **[2]**.

4.2.1. Talent loading protein into a 16 cm × 20 cm polyacrylamide gel with a 4% stacking gel and a 12.5% resolving gel.

4.2.2. The electrophoresis being run at a constant current of 50 milliamperes. *Videographer: If this shot is performed on a computer screen, please make sure the screen is clearly visible in the frame.*
Added shot: 4.2.2-2
Videographer: Shot on camera, Added shot: 4.2.2-2 on clapboard

~~4.2.3. The dye front is 2 centimeters from the bottom.~~

4.3. Now, equilibrate the gel in an elution buffer for 10 minutes **[1-TXT]**. Transfer the gel to the whole-gel eluter instrument **[2]**. Elute proteins by applying a constant current of 250 milliamperes for 1 hour **[3]**.

4.3.1. Talent submerging the gel in equilibration buffer. **TXT: Elution buffer: 60 mM of Tris, 40 mM of CAPS, pH 9.4**

4.3.2. Talent placing the gel into the whole-gel elution device.

4.3.3. A constant current of 250 milliamperes being applied to the gel. *Videographer: If this shot is performed on a computer screen, please make sure the screen is clearly visible in the frame.*
Videographer: Shot on camera

4.4. Next, collect approximately 30 fractions of 2.5 milliliters each **[1]**. Determine protein concentration using the bicinchoninic acid method following the manufacturer's instructions **[2]**. Analyze 10 micrograms of protein from each fraction by SDS-PAGE **[3-TXT]**.

4.4.1. Talent collecting eluted fractions into labeled tubes.
Videographer: Use 2.7.1

4.4.2. Talent measuring protein concentration using BCA assay kit.

4.4.3. Talent loading 10 micrograms from each sample on SDS-PAGE. **TXT: Visualize using silver staining**
Videographer: Use 3.4.3

4.4.4. ~~The protein visualized using silver staining~~.

Results

5. Results

5.1. This image displays a two-dimensional SDS PAGE analysis, where 100 micrograms of culture filtrate proteins produced **[1]** nearly 100 distinct protein spots **[2]**, indicating a highly complex protein composition **[3].**

5.1.1. LAB MEDIA: Figure 1.

5.1.2. LAB MEDIA: Figure 1. *Video Editor: Emphasize the brown spots.*

5.1.3. LAB MEDIA: Figure 1.

5.2. SDS PAGE analysis of three representative fractions from different separation runs showed distinct banding patterns, confirming variability in protein composition across the samples **[1].**

5.2.1. LAB MEDIA: Figure 2.

5.3. The pH values of the separated fractions gradually increased from 2.54 to 12.9 **[1]**, with the majority of proteins concentrated in fractions with pH between 4 and 6 **[2]**.

5.3.1. LAB MEDIA: Figure 3. *Video Editor: Highlight the blue plot.*

5.3.2. LAB MEDIA: Figure 3. *Video Editor: Highlight the red lines at 4 and 6.*

5.4. SDS PAGE analysis of whole-gel eluted isoelectric focusing fraction 12 revealed that each fraction contained 1 to 3 protein bands, confirming the successful separation and resolution of proteins into distinct bands **[1]**.

5.4.1. LAB MEDIA: Figure 4.

Pronunciation Guide:

1. Mycobacterium

Pronunciation link: https://www.merriam-webster.com/dictionary/mycobacterium Merriam-Webster+1Merriam-Webster+7Merriam-Webster+7Merriam-Webster+7

IPA: /ˌmaɪkoʊbækˈtɪəriəm/

Phonetic Spelling: my-koh-bæk-TEER-ee-əm

2. Proteomics

Pronunciation link: https://www.merriam-webster.com/dictionary/proteomics Merriam-Webster+3Merriam-Webster+3Merriam-Webster+3

IPA: /ˌproʊtiˈoʊmɪks/

Phonetic Spelling: proh-tee-OH-miks

3. Proteome

Pronunciation link: https://www.merriam-webster.com/dictionary/proteome Merriam-Webster+4Merriam-Webster+4Merriam-Webster+4Merriam-Webster+15Merriam-Webster+15Merriam-Webster+15

IPA: /proʊˈtiːoʊm/

Phonetic Spelling: proh-TEE-ohm

4. Isoelectric

Pronunciation link: https://www.merriam-webster.com/dictionary/isoelectric Merriam-Webster+7Merriam-Webster+7Merriam-Webster+7

IPA: /ˌaɪsoʊɪˈlɛktrɪk/

Phonetic Spelling: eye-soh-i-LEK-trik

5. Isoelectric focusing

Pronunciation link: https://www.merriam-webster.com/dictionary/isoelectric%20focusing Merriam-Webster+3Merriam-Webster+3Merriam-Webster+3Merriam-Webster+15Merriam-Webster+15Merriam-Webster+15

IPA: /ˌaɪsoʊɪˈlɛktrɪk ˈfoʊkəsɪŋ/

Phonetic Spelling: eye-soh-i-LEK-trik FOH-kuh-sing

6. Pellicle

Pronunciation link: https://www.merriam-webster.com/dictionary/pellicle Merriam-Webster+5Merriam-Webster+5Merriam-Webster+5

IPA: /ˈpɛlɪkəl/

Phonetic Spelling: PEL-i-kul

7. Digitonin

Pronunciation link: https://www.merriam-webster.com/dictionary/digitonin Merriam-Webster+2Merriam-Webster+2Merriam-Webster+3Merriam-Webster+3Merriam-Webster+3

IPA: /ˌdɪdʒəˈtoʊnɪn/

Phonetic Spelling: dij-uh-TOH-nin

8. Glycerol

Pronunciation link: https://www.merriam-webster.com/dictionary/glycerol Merriam-Webster+6Merriam-Webster+6Merriam-Webster+6

IPA: /ˈglɪsərɒl/ or /ˈglɪsəˌrɑl/ (variations exist)

Phonetic Spelling: GLIS-uh-rol or GLIS-uh-rawl

9. Tuberculosis
Pronunciation link: https://www.merriam-webster.com/dictionary/tuberculosis Merriam-WebsterMerriam-Webster+11Merriam-Webster+11Merriam-Webster+11Merriam-Webster+9Merriam-Webster+9Merriam-Webster+9
IPA: /ˌtuːbərkjəˈloʊsɪs/
Phonetic Spelling: too-bur-kyuh-LOH-sis
10. Cyclic
Pronunciation link: https://www.merriam-webster.com/dictionary/cyclic Merriam-Webster+8Merriam-Webster+8Merriam-Webster+8
IPA: /ˈsaɪklɪk/
Phonetic Spelling: SY-klik