FINAL SCRIPT: APPROVED FOR FILMING

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Title: Mycobacterial DNA Extraction Using Bead Beating in Custom Buffer Followed by NGS Workflow

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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: 25
Number of Shots: 52

Introduction

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

1.1. **John Metcalfe:** Our diagnostics research focuses on improving drug resistance testing for tuberculosis in limited resource settings, including speed and pragmatism.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.2*

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

1.2. **John Metcalfe:** In TB diagnostics, there are more comprehensive genotypic and a number of newer rapid phenotypic assays; there are consistent pushes to get such tests closer to point of care but a number of pragmatic barriers to address.

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. **John Metcalfe:** Resource challenges – from reagents to infrastructure to logistics – continue to be significant barriers. With anything beyond the technical complexity of the Cepheid Xpert assay, protocol standardization is difficult due to resource availability in different settings. M.tb DNA extraction prior to tNGS assays as detailed in this work is a prime example.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:5.1*

What research gap are you addressing with your protocol?

1.4. **Alina Nalyvayko:** There is a critical need to expand sequencing-based TB diagnostics, but many labs still lack a reliable way to rapidly extract high-quality DNA. Our goal is to share a method that's simple, practical, and suitable for low-resource or point-of-care settings.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.8*

What advantage does your protocol offer compared to other techniques?

1.5. **Alina Nalyvayko:** Here we present a simple, low-cost protocol designed for use in resource-limited settings. It has been validated for NGS applications and is compatible with automated liquid handling systems.

1.5.1. INTERVIEW: Named Talent says

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

Testimonial Questions (OPTIONAL):

Videographer: Please ensure that all testimonial shots are captured in a wide-angle format, while also maintaining sufficient headspace, given that the final videos will be rendered in a 1:1 aspect ratio.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Alina Nalyvayko** : We believe that publishing with JoVE can help us reach more clinical labs, especially new diagnostics sites. A video demonstration can improve training, ensure consistency across operators, and increase access to this mycobacterial DNA extraction method - making it easier to implement regardless of prior experience.

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

Ethics Title Card

This research has been approved by the Biosafety Committee at the University of California San Francisco (UCSF)

Protocol

2. Preparation of Samples for Mycobacterial DNA Extraction

Demonstrator: Alina Nalyvayko

2.1. To begin, combine sodium chloride solution, Tris-hydrochloride buffer, Triton X-100 *(tri-ton-Ex-One-hundred)* , and EDTA to make the Triton buffer **[1-TXT]**. Mix by stirring and add ultrapure water to reach a final volume of 100 milliliters **[2]**. Filter sterilize the buffer before use **[3]**.

2.1.1. WIDE: Talent measuring and combining reagents into a beaker. **TXT: 2 mL of 5 M NaCl, 1 mL of 1 M Tris-HCl (pH 8), 1 mL of Triton X-100, and 200 µL of 0.5 M EDTA**

2.1.2. Talent mixing constituents using a stir plate, then filling the final volume with ultrapure water to reach the 100 milliliters mark.

2.1.3. Talent filter sterilizing the buffer.

2.2. Next, prepare 100 milliliters of Low EDTA Tris-EDTA buffer by combining 1 milliliter of 1 molar Tris-HCl *(Tris -eighch-see-el)* and 20 microliters of 0.5 molar EDTA **[1]**. Mix and fill with ultrapure water to reach a final volume of 100 milliliters **[2]**. Then filter sterilize the buffer **[3].**

2.2.1. Talent pipetting Tris-HCl and EDTA into a beaker and mixing using a stir bar and a stir plate.

2.2.2. Talent adding ultrapure water to complete the volume.

Added shot: Talent filter sterilizing the buffer.

2.3. To prepare the lysis tubes, use a scalpel blade to carefully cut the bottom off a 1.5-milliliter screw cap tube just below the inflection point **[1]**.

2.3.1. Talent holding a tube and slicing the bottom with a scalpel.

2.4. Cut the tip off a P1000 *(P-One-Thousand)* pipette tip and make a V-shaped wedge near the end **[1]**. Wedge the cut bottom of the screw cap tube into the V-shaped pipette tip to form a scoop **[2]**.

2.4.1. Talent trimming a pipette tip and making a V-wedge with scissors.

2.4.2. Talent showing the assembled “spoon”.

2.5. Fill a sterile container with 0.1-millimeter Zirconia-Silicate Beads **[1]**. Using the prepared scoop, transfer approximately 200 milligrams of beads into 1.5-milliliter screw cap tubes **[2]**.

2.5.1. Talent pouring beads into a sterile container.

2.5.2. Talent scooping and dispensing beads into screw cap tubes.

2.6. For the preparation of the bacterial cell culture, transfer 5 milliliters of *Mycobacterium tuberculosis* culture into a 15-milliliter conical centrifuge tube **[1].** Centrifuge it at maximum speed for 10 minutes **[2]**.

2.6.1. Shot of *Mycobacterium tuberculosis* culture being pipetted into a 15-milliliter conical centrifuge tube

2.6.2. Talent placing the culture tube into the rotor inside a biosafety cabinet, then into the centrifuge.

2.7. Now use a 10-milliliter serological pipette to carefully remove all but approximately 500 microliters of the supernatant without disturbing the pellet **[1]**. Use a P1000 pipette to remove the remaining supernatant **[2]**.

2.7.1. Talent removing the upper layer of liquid with a serological pipette.

2.7.2. Talent removing remaining liquid carefully with a micropipette.

2.8. Resuspend the pellet in 350 microliters of custom Triton buffer **[1]** then mix well by pipetting up and down **[2-TXT]**. Heat the sample in a dry heat bath for 30 minutes at 95 degrees Celsius **[3].**

2.8.1. Talent adding 350 µL of custom Triton buffer to the tube.

2.8.2. Talent mixing the buffer with the pellet using a pipette. **TXT: If required, inactivate the sample**

Added shot: Talent placing sample into dry heat bath for 30 minutes at 95 °C.

2.9. To prepare the sputum, transfer 1 to 5 milliliters of sputum sample into a sterile 50-milliliter centrifuge tube **[1]**.

~~2.9.1. Talent transferring sputum into labeled sterile tube.~~ Shot of Sputum already in 1 mL aliquot in 50 mL tube.

3. Liquefaction of Sample for Extraction of Mycobacterial DNA

3.1. To perform dithiothreitol liquefaction, add four volumes of 10 millimolar dithiothreitol

to the sputum sample **[1]**. Then vortex thoroughly for 30 seconds **[2-TXT]**. ~~After incubating the sample at room temperature for 7 minutes, vortex again for 30 seconds **[3 TXT]**.~~

3.1.1. Talent pipetting dithiothreitol into sputum sample.

3.1.2. Talent vortexing the tube vigorously. **TXT: Repeat DTT liquefaction 1 more time; For viscous samples perform up to 5 incubation - vortex cycles**

3.1.3. ~~Talent vortexing again after incubation.~~
NOTE: Shot deleted by authors

3.2. Centrifuge the sample at maximum speed for 10 minutes **[1]**. Then use a 10-milliliter serological pipette to discard all but 500 microliters of the supernatant **[2]**. Remove the rest of the supernatant with a P1000 pipette without disturbing the pellet **[3]**.

3.2.1. Talent placing sample tube into the rotor, then the centrifuge.

3.2.2. Talent discarding upper liquid layer using a serological pipette.

3.2.3. Talent using micropipette to remove remaining liquid.

3.3. Resuspend the pellet in 350 microliters of custom Triton buffer **[1]**.

3.3.1. Talent mixing pellet with buffer using pipette and transferring to a labeled tube pre-filled with beads.

3.4. To perform the NALC-sodium hydroxide liquefaction, add 4 volumes of the NALC-sodium hydroxide solution ~~according to manufacturer's instructions **[1]**. Add four volumes of NALC- sodium hydroxide~~ to the sputum sample **[1]**.

3.4.1. Talent preparing and pipetting NALC-NaOH solution into sample.

~~3.4.2. Talent mixing NALC-NaOH with sputum.~~
NOTE: Shot deleted by authors

3.5. Vortex the mixture for 30 seconds **[1].** Then incubate the tube for 7 minutes at room temperature **[2-TXT]**.

3.5.1. Talent vortexing the tube. AUTHOR'S NOTE: 3.5.1 and 3.5.2 shot as one take.

3.5.2. Shot of tube being placed on a bench. **TXT: Repeat the cycle once or up to five times for viscous samples**

3.6. Now add PBS up to the 50-milliliter mark **[1]**. Vortex the contents of the tube to mix well **[2].** Then centrifuge the tube at maximum speed for 10 minutes **[3]**.

3.6.1. Talent filling tube with buffer up to 50 mL mark.

3.6.2. Shot of the tube being vortexed.

3.6.3. Talent placing sample in centrifuge and setting the parameters.

3.7. After centrifugation, with a 50-milliliter serological pipette, discard the supernatant as demonstrated earlier **[1].** Then resuspend pellet in 350 microliters of custom Triton buffer **[2-TXT]**.

3.7.1. Talent using micropipette for final removal.

3.7.2. Talent pipetting buffer to resuspend pellet. **TXT: Inactivate sample if needed AUTHOR'S NOTE: Can use the shot 2.8.3 here again, "talent placing sample into a dry heat bath"**

4. Mycobacterial DNA Extraction and qPCR Enumeration

4.1. First, transfer 350 microliters of inactivated sample into a labeled 1.5-milliliter screw cap tube containing 250 microliters of 0.1-millimeter Zirconia-Silicate Beads **[1]**. Bead beat the lysate at 6.5 meters per second for 45 seconds with 2 minutes rest between cycles **[2-TXT].**

4.1.1. Talent pipetting sample into bead-containing tube.

4.1.2. Talent operating bead beater for sample disruption. **TXT: Repeat for a total of three cycles**

4.2. Centrifuge the sample at maximum speed for 2 minutes **[1].** Then transfer 150 microliters of the supernatant to a new labeled tube **[2-TXT]**.

4.2.1. Talent placing the tube in a centrifuge and setting the parameters.

4.2.2. Shot of 150 µL of supernatant being pipetted into a new tube. **TXT: Avoid transferring any beads or debris**

4.3. Next, vortex cleanup magnetic beads that have been equilibrated at room temperature for 30 minutes, to resuspend them **[1]**. Transfer 180 microliters of magnetic beads to the DNA sample **[2].** Pipette up and down 10 times to mix **[3]**.

4.3.1. Talent vortexing magnetic beads after equilibration.

4.3.2. Talent pipetting beads into sample.

4.3.3. Shot of the suspension being pipetted up and down.

4.4. After a 2-minute incubation at room temperature, place the tube on a magnetic rack and wait 2 minutes for the solution to clear **[1]**. Then use a 200-microliter pipette to discard the supernatant **[2]**.

4.4.1. Talent placing sample tube onto magnetic rack.

4.4.2. Talent carefully discarding clear supernatant.

4.5. With the tube still on the magnetic rack, add 500 microliters of freshly-prepared 70% ethanol along the opposite wall and wait for 30 seconds **[1-TXT]**. At the end of the last wash, remove residual ethanol with a 10-microliter pipette and air dry for 2 minutes **[2]**.

4.5.1. Talent adding ethanol to the sample tube gently. **TXT: Repeat ethanol washes for a total of 2 washes**

4.5.2. Talent performing the second ethanol wash.

4.5.3. Talent removing ethanol and letting beads dry.

Added shot: Shot of "glossy beads"

4.6. As soon as beads turn opaque, remove the tube from the magnetic rack **[1].** Resuspend in 20 microliters of Low EDTA Tris buffer **[2-TXT]**. Mix by pipetting or vortexing to ensure all the beads are in solution before a 5-minute incubation at room temperature **[3].**

4.6.1. Shot of tube being removed from the magnetic rack.
Added shot: Close-up of "dry beads".

4.6.2. Talent adding 20 µL buffer to resuspend magnetic beads. **TXT: Do not allow beads to over dry or crack**

4.6.3. Talent mixing and setting aside tube for incubation.

4.7. Next, place the tube on a magnetic rack for 2 minutes until clear **[1]**. Then transfer less than 20 microliters of the eluted DNA to a new labeled tube to avoid bead carryover **[2]**.

4.7.1. Shot of the tube on the magnetic rack with clearing solution.

4.7.2. Talent transferring 15 µL of clear DNA solution to a fresh tube.

4.8. To quantify mycobacterial DNA using a quantitative PCR targeting 99 nucleotides of the mycobacterial *atpE* *(A-T-P-E)*, assemble a qPCR master mix on ice. Adjust master mix based on the amount of samples and standards, including a 10% overage to account for pipetting loss. **[1]**.

4.8.1. Talent pipetting reagents into a 1.5 mL tube on ice.
AND
TEXT ON PLAIN BACKGROUND:

Reaction Mixture per Sample
Universal Probe Master Mix (2x): 5 µL

Forward Primer (5'-AATTCCTGGTGTAGCGGTGG-3', 10 µM): 0.4 µL
Reverse Primer (5'-GTTTACGGCGTGGACTACCA-3', 10 µM) 0.4 µL
TaqMan probe (5'-VIC-AGGAGGAACACCGGTGGCGA-MGB-3', 10 µM): 0.2 µL
DNA Template: 2 µL
Nuclease-Free Water : 2 µL
Video Editor: Please play both shots side by side

4.9. Run the thermal cycler with 95 degrees Celsius for 60 seconds, followed by 35 cycles of 95 degrees Celsius for 10 seconds and 60 degrees Celsius for 30 seconds with ramp rate of 2.11 degrees Celsius per second **[1]**. Run all samples, standards, and controls in triplicate **[2-TXT]**.

4.9.1. Talent setting the cycle conditions on the thermal cycler.
Videographer: Please capture the instrument screen for this step

4.9.2. Talent placing triplicate samples in the thermal cycler. **TXT: Generate standard curves using serial DNA dilutions and analyze using software**

Results

5. Results

5.1. Pure *Mycobacterium tuberculosis* cultures yielded the highest DNA concentrations among all tested conditions **[1].**

5.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the orange box plot labeled "Culture"*

5.2. Spiked sputum samples showed progressively lower DNA yields and increased variation with decreasing bacterial input **[1],** especially in the 10,000 bacteria group **[2].**

5.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the green box plot labeled "Sputum spiked with 50,000 bacteria" and the blue box plot labeled "Sputum spiked with 10,000 bacteria"*

5.2.2. LAB MEDIA: Figure 2. *Video editor: Please highlight the blue box plot labeled "Sputum spiked with 10,000 bacteria"*

Pronunciation Guide:

1. Mycobacterial

- **Pronunciation link:** No confirmed link found
- **IPA (American):** /ˌmaɪkoʊbækˈtɪriəl/
- **Phonetic Spelling:** my-koh-bak-TEER-ee-uhl

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2. Bedaquiline

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/bedaquiline
- **IPA (American):** /bəˈdækwɪˌliːn/
- **Phonetic Spelling:** buh-DAK-wih-leen

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3. Tuberculosis

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/tuberculosis
- **IPA (American):** /tjuˌbɝkjəˈloʊsɪs/ or /təˌbɝkjəˈloʊsɪs/
- **Phonetic Spelling:** too-ber-kyuh-LOH-sis

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4. Genotypic

- **Pronunciation link:** No confirmed link found
- **IPA (American):** /ˌdʒiːnəˈtɪpɪk/
- **Phonetic Spelling:** JEE-nuh-tip-ik

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5. Phenotypic

- **Pronunciation link:** No confirmed link found
- **IPA (American):** /ˌfiːnəˈtɪpɪk/
- **Phonetic Spelling:** FEE-nuh-tip-ik

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6. Pragmatism

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/pragmatism
- **IPA (American):** /ˈprægməˌtɪzəm/
- **Phonetic Spelling:** PRAG-muh-tih-zum

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7. Tris-hydrochloride

- **Pronunciation link:** No confirmed link found
- **IPA (American):** /trɪs haɪdrəˈklɔːraɪd/
- **Phonetic Spelling:** tris hy-droh-KLOR-ide

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8. Zirconia-silicate

- **Pronunciation link:** No confirmed link found
- **IPA (American):** /zɝˈkoʊniə ˈsɪlɪkeɪt/
- **Phonetic Spelling:** zur-KOH-nee-uh SIL-ih-kate

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9. Dithiothreitol

- **Pronunciation link:** No confirmed link found

- **IPA (American):** /daɪˌθaɪoʊˈθriːɪˌtɔːl/
- **Phonetic Spelling:** dye-THY-oh-three-ih-tawl

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10. Bead beating

- **Pronunciation link:** No confirmed link found
- **IPA (American):** /biːd ˈbiːtɪŋ/
- **Phonetic Spelling:** beed BEE-ting