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Title: Amplicon Sequencing using the Long-Read Sequencing Technologies

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **09/08/2025**

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Current Protocol Length

Number of Steps: 23
Number of Shots: 54

Introduction

1.1. **Morwasehla Modjadji:** I investigate whether portable long-read sequencing can provide TB drug susceptibility results in resource-limited settings with accuracy comparable to gold-standard methods, advancing accessible, high-quality tuberculosis diagnostics.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 3*

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

1.2. **Morwasehla Modjadji:** Utilizing targeted next generation sequencing as a diagnostic tool for drug-resistant tuberculosis by offering a comprehensive resistant profile directly from clinical samples without bioinformatic expertise.

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

~~What technologies are currently used to advance research in your field?~~

~~1.3. **Brendon Mann:** Next-generation sequencing, including both whole genome and targeted approaches, is transforming how we detect drug resistance and understand transmission dynamics in tuberculosis.~~

~~1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.~~ NOTE: Remove it. Video quality is not good

What advantage does your protocol offer compared to other techniques?

1.4. **Morwasehla Modjadji:** Our protocol utilizing long-read sequencing has the potential to provide quicker turnaround times, simpler workflows, and real-time resistance detection, facilitating comprehensive TB diagnostics and enhancing outbreak tracking in resource-limited environments with minimal infrastructure.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 1*

Ethics Title Card

This research has been approved by the Human Research Ethics Committee (HREC) at Stellenbosch University

Protocol

2. ONT Library Preparation for Deep Sequencing

Demonstrator: Morwasehla Modjadji

2.1. To begin, calculate 100 nanograms of each amplicon sample based on the concentration **[1]** and transfer the required volume into a clean PCR tube **[2]**. To determine the total mass of amplicons in the sample, use the given formula **[3]**.

2.1.1. WIDE: Talent calculating volume needed to obtain 100 nanograms based on the sample concentration.

2.1.2. Talent transferring the calculated volume into a clean polymerase chain reaction tube using a pipette.

2.1.3. TEXT ON A PLAIN BACKGROUND

Total mass (ng) = concentration (ng/µL) × volume (µL).

2.2. Adjust the total volume of each sample to 12.5 microliters using nuclease-free water **[1]**. Mix the samples gently by pipetting up and down 10 times **[2]** and briefly spin them in a microcentrifuge **[3]**.

2.2.1. Talent pipetting nuclease-free water into the polymerase chain reaction tubes.

2.2.2. Talent pipetting the contents of the tube up and down gently.

2.2.3. Talent placing the tubes in a microcentrifuge and initiating a brief spin.

2.3. Next, add 1.75 microliters of end-repair and adenylation reaction buffer to each sample, followed by 0.75 microliter of end-repair and adenylation enzyme mix **[2]**.

2.3.1. Talent pipetting 1.75 microliters of buffer into the polymerase chain reaction tube. Authors: Please keep all the mentioned reagents in the frame.

2.4. After mixing and spinning the samples, place the tubes in a thermocycler **[1]** at 25 degrees Celsius for 30 minutes, followed by 65 degrees Celsius for 30 minutes **[2]**.

2.4.1. Talent placing the tubes into a thermocycler.

2.4.2. Show the thermocycler display set 25 degrees Celsius for 30 minutes and 65 degrees Celsius for 30 minutes.

2.5. For native barcode ligation, add the components shown on the screen in new PCR tubes

[1]. Mix the reactions gently by pipetting **[2]** and spin briefly using a microcentrifuge **[3-TXT]**.

2.5.1. TEXT ON A PLAIN BACKGROUND

Nuclease-free water: 2.75 µL

End-prepped DNA: 1 µL

Native barcodes: 1.25 µL

DNA ligation enzyme mix: 5 µL

2.5.2. Talent pipetting the reaction mixture gently to mix.

2.5.3. Talent placing the tubes into a microcentrifuge for a brief spin. **TXT: Incubation: RT, 20 min**

2.6. Next, add 1 microliter of EDTA to each tube, then mix thoroughly and spin briefly as shown earlier **[3]**.

2.6.1. Talent pipetting 1 microliter of ethylenediaminetetraacetic acid into each tube.

2.7. Pool the barcoded samples into a 1.5-milliliter microcentrifuge tube **[1]**. Vortex the DNA cleanup beads to resuspend them completely **[2]** and add a volume equal to 0.7 times the pooled sample volume **[3]**.

2.7.1. Talent pipetting all barcoded reactions into a single 1.5 milliliter microcentrifuge tube.

2.7.2. Talent vortexing the DNA cleanup bead suspension.

2.7.3. Talent pipetting calculated volume of DNA beads into the pooled sample tube.

2.8. Mix the bead-sample solution and incubate it on a Hula Mixer at room temperature for 10 minutes **[1]**.

2.8.1. Talent mixing the pooled sample and beads by pipetting gently and keeping it aside.

2.9. Next, to prepare 2 milliliters of 80 percent ethanol, mix 1,600 microliters of pure ethanol with 400 microliters of nuclease-free water **[1]**.

2.9.1. Talent pipetting ethanol and nuclease-free water into a tube to prepare 80 percent ethanol.

2.10. Place the tube on a magnetic rack for 5 minutes until the eluate appears clear and colourless **[1]**. Then, remove and discard the supernatant without disturbing the pellet

[2]. Wash the beads with 700 microliters of freshly prepared 80 percent ethanol **[3]** and centrifuge the tube briefly **[4]**. After placing the tube on the magnetic rack, remove the residual ethanol **[5-TXT]**.

2.10.1. Talent placing the bead-sample tube on a magnetic rack.

2.10.2. Talent carefully aspirating and discarding the supernatant from the tube.

2.10.3. Talent adding 700 microliters of ethanol wash to the pellet.

2.10.4. Talent spinning the tube in a microcentrifuge briefly.

2.10.5. Talent removing the remaining ethanol from the tube placed on the magnetic rack using a pipette. **TXT: Air dry the beads for 30 s**

2.11. Now, remove the tube from the magnetic rack **[1]**, resuspend the beads in 35 microliters of nuclease-free water by gentle flicking **[2]**, and incubate at 37 degrees Celsius for 10 minutes **[3]**.

2.11.1. Talent taking the tube off the magnetic rack.

2.11.2. Talent adding nuclease-free water to the tube and flicking it.

2.11.3. Talent placing the tube into a heating block or incubator set at 37 degrees Celsius.

2.12. Return the tube to the magnetic rack until the eluate becomes clear and colourless **[1]**. Then, transfer 35 microliters of the eluate into a clean 1.5-milliliter microcentrifuge tube for further use **[2]**.

2.12.1. Talent placing the tube back onto the magnetic rack.

2.12.2. Talent transferring the clear eluate into a new 1.5 milliliter tube using a pipette.

2.13. For adaptor ligation, mix the given components in a 1.5-milliliter low-binding microcentrifuge tube **[1]**. Centrifuge the tube briefly and incubate at room temperature for 20 minutes **[2]**.

2.13.1. TEXT ON A PLAIN BACKGROUND
Pooled barcoded sample: 30 µL
Native Adapter (NA): 5 µL
Rapid ligation reaction buffer: 10 µL
Rapid-acting T4 DNA ligase: 5 µL

2.13.2. Talent placing the tube into a microcentrifuge and initiating a short spin.

2.14. Vortex the DNA cleanup beads to resuspend them completely **[1]**. Add 20 microliters of beads to the tube and mix gently **[2]**. Incubate on a Hula Mixer for 10 minutes at room

temperature **[3]**.

2.14.1. Talent vortexing the DNA bead suspension tube.

2.14.2. Talent pipetting 20 microliters of beads into the ligation reaction tube and mixing.

2.14.3. Talent placing the tube on a Hula Mixer at room temperature.

2.15. Then, place the tube on a magnetic rack to pellet the beads and aspirate the supernatant without disturbing the pellet **[1]**. Add 125 microliters of Short Fragment Buffer and gently flick the tube to resuspend the beads **[2]**, centrifuge briefly **[3]**, and return the tube to the magnetic rack **[4]**.

2.15.1. Talent placing the reaction tube on a magnetic rack and carefully removing the supernatant.

2.15.2. Talent adding 125 microliters of Short Fragment Buffer to the tube and flicking the tube.

2.15.3. Talent briefly centrifuging the tube.

2.15.4. Talent placing the tube back on the magnetic rack.

2.16. Aspirate the supernatant again after the beads have pelleted **[1-TXT]**.

2.16.1. Talent removing the supernatant from the magnetic rack**. TXT: Repeat wash 1x**

2.17. Now, remove the tube from the magnetic rack **[1]** and resuspend the beads in 15 microliters of elution buffer by gentle pipetting or flicking **[2]**.

2.17.1. Talent taking the tube off the magnetic rack.

2.17.2. Talent resuspending the beads in Elution Buffer with gentle motion.

2.18. Briefly centrifuge the tube to collect the contents **[1]**, then incubate it at 37 degrees Celsius for 10 minutes to elute the DNA **[2]**. Place the tube on a magnetic rack until the eluate is clear and colorless **[3]**, then aspirate 15 microliters of eluate and transfer it into a clean 1.5-milliliter microcentrifuge tube **[4-TXT]**.

2.18.1. Talent placing the tube in a microcentrifuge for a brief spin.

2.18.2. Talent placing the tube into a heating block or incubator set at 37 degrees Celsius.

2.18.3. Talent returning the tube to a magnetic rack and waiting for bead separation.

2.18.4. Talent pipetting 15 microliters of clear eluate into a new microcentrifuge tube. **TXT: Use 1µL eluate for quantification**

2.19. To prepare the flow cell priming solution, mix the given components thoroughly **[1]**.

2.19.1. TEXT ON A PLAIN BACKGROUND.

BSA (50 mg/mL): 5 µL

Flow Cell Tether: 30 µL

Flow Cell Flush: 1170 µL

2.20. Open the priming port of the flow cell **[1]**, aspirate approximately 20 microliters of buffer to remove air bubbles **[2]**, and load 800 microliters of the priming mix into the port without introducing air bubbles **[3-TXT]**.

2.20.1. Talent opening the priming port of the nanopore sequencing flow cell.

2.20.2. Talent aspirating buffer from the priming port using a pipette to remove air bubbles.

2.20.3. Talent slowly loading 800 microliters of priming mix into the flow cell without bubbles. **TXT: Allow the flow cell to sit for 5 min**

2.21. To prepare the DNA library, thoroughly mix the components shown on the screen **[1]**.

2.21.1. TEXT ON A PLAIN BACKGROUND
Sequencing Buffer: 37.5 µL
Library Beads or Library Solution: 25.5 µL
20 fmol DNA library: 12 µL

2.22. Gently lift the sample port cap of the flow cell **[1]**, load 200 microliters of priming mix into the priming port without introducing air bubbles **[2]**.

2.22.1. Talent carefully opening the sample port on the nanopore flow cell.

2.22.2. Talent slowly pipetting 200 microliters of priming mix into the port to avoid bubbles.

2.23. After mixing the prepared DNA library, load 75 microliters of the library dropwise into the sequencing flow cell sample port **[1-TXT]**. Close the flow cell sample port cap, ensuring the bung securely enters the sample port **[2]**, and then close the priming port tightly **[3-TXT]**.

2.23.1. Talent loading the library into the flow cell port one drop at a time, waiting between drops. **TXT: Wait for each drop to be fully absorbed before adding the next**

2.23.2. Talent pressing the flow cell cap down gently, ensuring the bung fits into the port.

2.23.3. Talent snapping the priming port shut. **TXT: Connect the sequencing device to a computer and start run via the software**

Results

3. Results

3.1. Gel electrophoresis confirmed amplicon integrity across nearly all samples, but lanes corresponding to samples 89 and 136 displayed faint or absent bands **[1].** Samples 89 and 136 had insufficient amplicon concentrations, leading to their exclusion from long-read sequencing **[1].**

3.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the lane labeled "89" and "136"where the band is faint.*

3.1.2. LAB MEDIA: Table 2. *Video editor: Highlight the "Amplicon concentration" values for "89" and "136."*

3.2. Across all targeted genes, the long-read and short-read platforms achieved high coverage depths, with the eis *(E-I-S)* gene showing the highest average values **[1]** and rrs *(R-R-S)* gene exhibiting the lowest coverage depths **[2].**

3.2.1. LAB MEDIA: Figure 3. *Video editor: Highlight the violin plot for the gene "eis" showing both the orange (Illumina) and blue (ONT) shapes at the top of the value scale*.

3.2.2. LAB MEDIA: Figure 3. *Video editor: Highlight the violin plot for "rrs" showing the lowest positioned shapes on the graph.*

3.3. Despite the range in coverage depth, both platforms maintained a high coverage breadth of 99.9% across all resistance-associated genes **[1].**

3.3.1. LAB MEDIA: Figure 3.

3.4. Drug resistance variants for Ethambutol, Fluoroquinolones, Kanamycin, Amikacin, and Capreomycin were detected with 100% concordance across short- and long-read sequencing platforms **[1].**

3.4.1. LAB MEDIA: Table 4. *Video editor: Highlight the rows labeled "EMB", "FQs", "KAN", "AMK", and "CAP" showing equal R values for both platforms*.

3.5. No resistance-associated mutations were found for Linezolid, Bedaquiline, and Clofazimine using either sequencing approach **[1].** Streptomycin resistance detection showed lower concordance between platforms, with only 71.43% agreement **[1].**

3.5.1. LAB MEDIA: Table 4. *Video editor: Highlight the rows "LIN", "BDQ", and "CFZ".*

3.5.2. LAB MEDIA: Table 4. *Video editor: Highlight the "SM" row showing discrepancy in "R" values between short- and long-read platforms.*

3.6. Detection sensitivity for key genes—rpoB *(R-P-O-B)*, katG/fabG1/ahpC/inhA, fabG1/inhA *(Kat-G-Fab-G-One-A-H-P-C-I-N-H-A-Fab-G-I-N-H-A)*, and pncA *(P-N-C-A)*—ranged from 89.47% to 96.77% between platforms **[1].**

3.6.1. LAB MEDIA: Table 4 *Video editor: Highlight the resistance detection concordance column and rows*

1. **Amplicon**
   - Pronunciation link: https://www.merriam-webster.com/dictionary/amplicon
   - IPA: /ˈæm.plɪˌkɑn/
   - Phonetic: AM-pli-kon
2. **Nanograms**
   - Pronunciation link: https://www.merriam-webster.com/dictionary/nanogram
   - IPA: /ˈnæn.oʊˌgræm/
   - Phonetic: NAN-oh-gram
3. **Thermocycler**
   - (Not always in standard dictionaries; derived from “thermo-” + “cycler”)
   - IPA (approx): /ˌθɜr.moʊˈsaɪ.lər/
   - Phonetic: THER-moh-sigh-lər
4. **Ethylenediaminetetraacetic (EDTA)**
   - Pronunciation link: https://www.merriam-webster.com/dictionary/ethylenediaminetetraacetic (if available)
   - IPA (approx): /ˌiːθəˌliːn-daɪˈæmɪn-tɛtrəəˈsɛtɪk/
   - Phonetic: ee-thy-leen-dye-AM-in-eh-tetra-ASE-tik
5. **Barcode**
   - Pronunciation link: https://www.merriam-webster.com/dictionary/barcode
   - IPA: /ˈbærˌkoʊd/
   - Phonetic: BAR-kohd
6. **Vortex / Vortexing**
   - Pronunciation link: https://www.merriam-webster.com/dictionary/vortex
   - IPA: /ˈvɔr.tɛks/
   - Phonetic: VOR-teks
   - For “vortexing”: /ˈvɔr.tɛksɪŋ/ → VOR-teks-ing
7. **Ligase / Ligation**
   - Pronunciation link (ligase): https://www.merriam-webster.com/dictionary/ligase
   - IPA: /ˈlaɪˌgeɪs/
   - Phonetic: LYE-gays
   - “Ligation”: /laɪˈgeɪʃən/ → lye-GAY-shun
8. **Eluate**
   - Pronunciation link: https://www.merriam-webster.com/dictionary/eluate
   - IPA: /ˈɛl.juˌeɪt/
   - Phonetic: EL-yu-ate

9. **Flow cell / Priming port**
    - "Flow" is straightforward; "cell" /sɛl/; "priming" /ˈpraɪmɪŋ/ (PRY-ming); "port" /pɔrt/
10. **Stellenbosch** (as in Stellenbosch University)
    - Pronunciation link: No standard English dictionary, but often approximated as /ˈstɛlənˌbɒʃ/
    - Phonetic: STEL-len-bosh
11. **Tuberculosis**
    - Pronunciation link: https://www.merriam-webster.com/dictionary/tuberculosis
    - IPA: /ˌtuːbərkəˈloʊsɪs/
    - Phonetic: too-ber-kuh-LOH-sis
12. **Eis (gene name, "eis")**
    - As a gene, often just spelled out "E-I-S" (letters) → /i aɪ ɛs/
    - Phonetic: EYE-ESS
13. **rpoB, katG, fabG1, ahpC, inhA, pncA**
    - Gene names are typically read letter by letter:
        - rpoB → "R-P-O-B" /ɑr pi oʊ bi/
        - katG → "K-A-T-G" /keɪ eɪ ti dʒi/
        - fabG1 → "F-A-B-G-one" /ɛf eɪ bi dʒi wʌn/
        - ahpC → "A-H-P-C" /eɪ eɪtʃ pi si/
        - inhA → "I-N-H-A" /aɪ ɛn eɪtʃ eɪ/
        - pncA → "P-N-C-A" /pi ɛn si eɪ/