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FreezeTB *M. tuberculosis* drug resistance screening assay

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We are still developing and optimizing this protocol

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Abstract

Alaska has the highest incidence of tuberculosis (TB) in the United States with disease disproportionately affecting Alaska Native/American Indian (AN/AI) populations and children under 14 years of age; who account for almost a third of cases. With a case fatality rate of 8% during TB treatment and a quarter of TB cases lacking sputum culture to enable drug resistance testing, the FreezeTB targeted next Generation Sequencing (tNGS) diagnostic initiative was launched to develop diagnostic genotypic drug resistance tools tailored to meet the challenges of care in Alaska, while being translatable to other settings.

We sought to develop a rapid and cost-effective method for identifying drug resistant mutations in Mycobacterium tuberculosis using targeted next generation sequencing (tNGS), FREEZE TB: Fast, Reliable, Economical Evaluation tool to Zap Endemic Tuberculosis. We designed a primer multiplex, amplification and barcoding protocol, and sequencing workflow for nanopore sequencers, including portable devices. We programmed an open-source (OS) software that detects mutations associated with drug resistance recognized by the World Health Organization. One hundred blinded M. tuberculosis sputum culture isolates from Alaska (59 resistant, 41 pan-susceptible) were selected and underwent analysis with our the FREEZETB lab and bioinformatic workflow, then compared to phenotypic drug susceptibility testing (pDST) using BACTEC MGIT 960.

Compared to WGS (n=79), FreezeTB tNGS and software provided the same mutation report in 96% (n=76/79) of samples. Compared to pDST, FreezeTB had almost perfectvery high (numerator / 79) agreement for RIF (0.904; 71/79) and EMB (1.0; 79/79), substantial agreement for ETO (0.784) and INH (0.795), moderate agreement for STR (0.556) and slight agreement for PZA (0.197) using Cohen’s kappa. FreezeTB is designed to detect resistance to fluoroquinolones, bedaquiline, pretomanid/delamanid, and linezolid;, however, no isolates had thiswere resistant to these drugsce pattern. Using a portable nanopore sequencer, each sample was sequenced for under $30.

FreezeTB provides a one-day laboratory and bioinformatic workflow for sequencing M. tuberculosis; offering an OS software with graphical user interface and a laboratory workflow costing under $30 per sample. Freeze TB can accurately determine resistance for rifampin, isoniazid, streptomycin, ethambutol and ethionamide and is designed to detect resistance in newer drugs, such as bedaquiline and pretomanid.

Decontamination

1 **Preparation of working solutions before beginning:**

1.1 **NALC-NaOH-Sodium citrate solution:** Combine 0.25 grams of NALC powder and dissolving fully in a 50 mL solution of 2% NaOH and 1.45% sodium citrate (a 1 liter stock solution of NaOH-sodium citrate can be made by combining 20 grams NaOH and 14.5 grams sodium citrate dihydrate and adding 1 Liter of distilled water). Ensure the solution is completely dissolved.

1.2 **Phosphate buffer** (0.067 M): Add 4.74 grams of disodium phosphate and 4.54 grams of monopotassium phosphate and add 1 Liter of distilled water. Ensure the solution is completely dissolved.

2 Decontamination Method

2.1 Take 700 microliters of direct sputum or sputum culture liquid isolate and add 700 microliters of NALC-NaOH0-sodium citrate solution in 1.5 or 2.0 mL microcentrifuge tubes. Process each sample individually and be careful not to cross-contaminate.

2.2 Vortex the tubes for and incubate at room temperature for ten minutes.

2.3 Centrifuge the tubes at 15,000 g for three minutes. Discard the supernatant by carefully pipetting off the supernatant without disturbing the pellet.

2.4 Add 1 mL of phosphate buffer (0.067 M) to resuspend the pellet.

2.5 Centrifuge at 15,000 g for three minutes. Carefully discard the supernatant again without disturbing the pellet.

DNA Extraction/Isolation

3 Working reagents:
PBS+ 0.1% Tween: Prepare 10% Tween/PBS by adding 1 mL Tween-80 to 9 mL PBS (mix well). Add 0.5 mL 10% Tween-80 in 50 mL PBS and mix well. Upscale accordingly if necessary.

4 Resuspend each (decontaminated) pellet in 200 µL PBS + 0.1 % Tween-80 + 20 µL Proteinase K and incubate at 56 °C for 10 minutes, including extraction PC and extraction NTC.

5 Heat kill step: incubate the tubes at 95 °C for 15 minutes.

6 Invert mix Reagent DX before use, then add 1 µL, mix by pipetting and transfer the solution by pipetting to the Qiagen Pathogen Lysis Tubes L bead tube.

7 Vortex the tubes for 1 minute.

8 To each tube, add 250 µL of Buffer ATL and 20 µL of Proteinase K and mix shortly by vortexing for 2 seconds.

9 Incubate at 56 °C for 10 minutes.

10 While the samples are being incubated, set up 350 µLof Buffer AL and 350 µL of 100% ethanol in a fresh 1.5 mL Eppendorf tube per sample.

11 After incubation (step 6) vortex the sample and transfer 350 µLfrom the bead tube (avoiding transfer of beads) to the tube containing Buffer AL and 100% ethanol (step 7). Vortex for 15 seconds.

12 Transfer 600 µL to a DNeasy spin column and centrifuge at 6,000 xg for 1 minute. Discard the flowthrough from the collection tube (by pouring) and return the insert into the same collection tube.

13 Transfer the remainder of the sample to the DNeasy spin column and centrifuge at 6,000 xg for 1 minute. Transfer the column to a new collection tube.

14 Add 500 µL Buffer AW1 to the column and centrifuge at 10,000 xg for 1 minute. Transfer the column to a new collection tube.

15 Add 500 µL Buffer AW2 to the column and centrifuge at 20,000 xg for 3 minutes.

16 Transfer the column to a fresh 1.5 mL Eppendorf tube and add 100 µL of Buffer EB/AE to the column membrane. Incubate at room temperature for 1 minute.

17 Centrifuge the sample at 10,000 xg for 1 minute.

18 Lift the column, collect the 100 µL eluate and add it back to the same column for a re-elution.

19 Centrifuge the sample at 10,000 xg for 1 minute. Discard the column and keep the eluate in the Eppendorf tube.

19.1 Samples can be stored in -20 or -80 C freezer at this step

Clean up and PCR

20 Collect 100 uL of eluted genomic DNA and clean each sample using a 1:1 ratio of AMPure XP beads

20.1 Gently pipette mix three times and place on a hula or spin mixer for 10 minutes

20.2 Prepare a solution of 80% ethanol

20.3 Collect samples and place on magnetic rack for at least two minutes, or until solution is clear

20.4 Remove supernatant without disturbing pellet

20.5 Wash beads with 500 uL of 80% ethanol without disturbing the pellet

20.6 Repeat the previous step and proceed

20.7 Allow beads to air dry on magnet rack for at least one minute, or until the beads lose glossy surface

20.8 Remove from magnet rack, elute beads into 20 µL of buffer EB by gently pipette mixing and allow to incubate at room temperature for 10 minutes

20.9 After incubation, place back on magnet rack for at least one minute or until the supernatant is clear

20.10 Move supernatant into a new DNA lobind tube, cleaned gDNA can be store at -20 or can move into next steps

21 Prepare thePCR master mix solution for the number of samples based on the chart

	A	B	C	D
	Components	Volume per 25 ul RXN	Final Concentration	Volume for 20 samples
	NEBNext Ultra II Q5 Master Mix	12.5 uL	1x	250 uL
	FreezeTB Primer Multiplex	5 uL	Variable per primer	100 uL
	Sample DNA	5 uL	Variable per sample	
	Nuclease Free Water	2.5 uL		50 uL
	Total Volume	25 uL		500 uL

22 Incubate samples in plate thermocycler with lid temperature 105 C following the protocol below

	A	B	C
	Stage	Temperature, C	Time
	Initial Denaturation	98	3 min
	35 Cycles	98	15 sec
		60	15 sec
		72	1:20 minutes
	Final Extension	72	4 minutes

	A	B	C
	Hold	10	

22.1 PCR samples can be stored at -20 °Cuntil ready to proceed

23 Move 25 uL of sample into a LoBind Tube, and add 15 uL of AMPure XP beads, or a 0.6:1 ratio.
Repeat for each sample.

23.1 Gently pipette mix three times and place on a hula or spin mixer for 10 minutes

23.2 Prepare a solution of 80% ethanol

23.3 Collect samples and place on magnetic rack for at least two minutes, or until solution is clear

23.4 Remove supernatant without disturbing pellet

23.5 Wash beads with 500 uL of 80% ethanol without disturbing the pellet

23.6 Repeat the previous step and proceed

23.7 Allow beads to air dry on magnet rack for at least one minute, or until the beads lose glossy surface

23.8 Remove from magnet rack, elute beads into 14 µL of buffer EB by gently pipette mixing and allow to incubate at room temperature for 10 minutes

23.9 After incubation, place back on magnet rack for at least one minute or until the supernatant is clear

23.10 Move supernatant into a new DNA lobind tube. Cleaned PCR product can be stored at -20 °C or can be moved into the next steps

Library Preparation (RBK-SQK-114.96)

24 Barcoding and Adaptor Ligation

24.1 Add 2.5 µL of a unique barcode to each well

24.2 Add 11 uL of unique PCR product to each well containing bar codes, pipette mix then spin down

24.3 Incubate samples in a thermocycler with the following protocol

	A	B
	Tempature, C	Time
	30	2:00
	80	2:00
	5	2:00

25 Pool all barcoded samples into a 1.5 mL DNA loBind tube

26 Perform a clean up using 0.6:1 ratio of AMPure XP beads

26.1 Gently pipette mix three times and place on a hula or spin mixer for 10 minutes

27 Prepare a solution of 80% ethanol

28 Collect samples and place on magnetic rack for at least two minutes, or until solution is clear

29 Remove supernatant without disturbing pellet

30 Wash beads with 500 uL of 80% ethanol without disturbing the pellet

31 Repeat the previous step and proceed

32 Allow beads to air dry on magnet rack for at least one minute, or until the beads lose glossy surface

33 Remove from magnet rack, elute beads into 14 µL of buffer EB by gently pipette mixing and allow to incubate at room temperature for 10 minutes

34 After incubation, place back on magnet rack for at least one minute or until the supernatant is clear

35 Move 12 uL of supernatant into a new DNA lobind tube.

36 In a fresh LoBind tube create a dilution of Rapid Adaptor (RA) using 1.5 uL of Rapid Adaptor (RA)
and 3.5 uL Adaptor Buffer (ADB)

36.1 Add 1 uL of diluted RA to 11 uL of barcoded DNA

36.2 Gently mix by flicking the tube, and spin down

36.3 Incubate at room temperature for 5 minutes, then place on ice

37 Priming and Loading minION flowcell

38 Prepare a mixture of priming mix with 1,170 uL of Flow Cell Flush (FCF), 5 uL of Bovine Serum Albumin (BSA) at 50 mg/ml, and 30 uL of Flow Cell Flush (FCT)

The addition of BSA is optional, but recommended per Oxford Nanopore. This assay has not been tested without its addition.

39 Set a pipette to 200 uL and insert the tip into the priming port of the flowcell

Turn the dial up slowly, drawing back until a small volume of buffer enters the pipette tip

40 Load 800 uL of priming mix into the flowcell via the priming port, ensuring no air bubbles are added to the priming port

41 Allow flowcell to incubate at room tempature for 5 minutes

Prepare library during this time

42 In a new DNA LoBind tube, add the following
Library beads will quickly settle and vortex before use

38 uL of Sequencing Buffer (SB)
26 uL of Library Beads (LIB)
12 uL DNA Library

43 Open both the priming port and the SpotON sample cover

Slowly load 200 uL of priming mix into the flowcell via the priming port just before loading the library; the library **must** be loaded within several minutes of this step

43.1 Gently mix library by pipetting, ensure beads are fully suspended

43.2 Add 76 uL of prepared library to the SpotOn port slowly, avoiding all addition of air bubbles

43.3 Close all ports and add light cover to flowcell sensor

Sequencing

44 Follow standard minKNOW sequencing set up.

This protocol was tested using the super high accuracy (SUP) basecalling model; however, if computational power is a limiting factor, such as on a laptop or small desktop, the high accuracy model (HAC) can be used as well.

POD5 output q5 minutes

Set the minimum passed read Q-score to 9

Set the run time to two hours. This time can be increased as desired; however, this assay was tested and proven to only need to be run for 2 hours of sequencing time. Longer times may yield better results for poorer samples, though this has not been thoroughly tested.