

Oct 31, 2025

FreezeTB *M. tuberculosis* drug resistance screening assay

 [Microbiology Spectrum](#)

DOI

<https://dx.doi.org/10.17504/protocols.io.261gek1ejg47/v1>

Bryce Inman¹, Jeremy Buttler¹, Tara Ness^{1,2}, Eric Bortz³

¹UAA; ²BCM; ³University of Alaska



Eric Bortz

University of Alaska

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.261gek1ejg47/v1>

External link: <https://doi.org/10.1128/spectrum.02335-25>

Protocol Citation: Bryce Inman, Jeremy Buttler, Tara Ness, Eric Bortz 2025. FreezeTB *M. tuberculosis* drug resistance screening assay. [protocols.io](#) <https://dx.doi.org/10.17504/protocols.io.261gek1ejg47/v1>

Manuscript citation:

Inman B, Butler J, George-Nichol S, Kovalenko G, Savidge T, Vergnetti Y, Pongratz C, Bee E, DiNardo AR, Kay A, Mandalakas A, Bortz E, Ness TE Application of FreezeTB, a targeted nanopore sequencing assay, for identification of drug resistance and lineages among pulmonary tuberculosis cases in Alaska. *Microbiology Spectrum* 14(1). doi: [10.1128/spectrum.02335-25](https://doi.org/10.1128/spectrum.02335-25)



License: This is an open access protocol distributed under the terms of the **[Creative Commons Attribution License](#)**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

Protocol in use; further development will be included from user feedback

Created: October 31, 2025

Last Modified: October 31, 2025

Protocol Integer ID: 231206

Keywords: lineage of mycobacterium tuberculosis, oxford nanopore technologies minion platform, pcr amplicon analysis of drug resistance mutation, mycobacterium tuberculosis, targeted next generation sequencing, next generation sequencing, freeze tb, drug resistance mutation, tuberculosis, mycobacterium, nanopore, bioinformatic software, low dependency bioinformatic software, pcr amplicon analysis, tb, drug resistance, primer ftb v11, mutation

Abstract

Freeze TB is a laboratory workflow and free, open-source (OS) low dependency bioinformatic software for targeted next-generation sequencing (tNGS) PCR amplicon analysis of drug resistance mutations and lineage of Mycobacterium tuberculosis. The protocol is optimized for nanopore sequencing on an Oxford Nanopore Technologies MinION platform.

This is primer fTB v11.

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-NaOH-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 μ L PBS + 0.1 % Tween-80 + 20 μ L 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 μ L, 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 μ L of Buffer ATL and 20 μ L 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 μ L of Buffer AL and 350 μ L of 100% ethanol in a fresh 1.5 mL Eppendorf tube per sample.
- 11 After incubation (step 6) vortex the sample and transfer 350 μ L from 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 μ L 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 μ L 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 μ L 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 μ L 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 °C until 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 μ L of a unique barcode to each well

24.2 Add 11 μ L 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 temperature 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.