

Document type: procedure	GENOTYPE MTBDR^{plus} FOR MDR-TB SCREENING
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1. PURPOSE

This SOP describes procedure for determination of *Mycobacterium tuberculosis* positivity and rifampicin/isoniazid resistance by utilizing the GenoType MTBDR*plus* test (Hain Lifesciences). Traditional microscopy takes weeks to establish a diagnosis of tuberculosis, with additional time required for conventional drug susceptibility testing. Testing may be performed on DNA isolated from cultures as well as smear positive direct patient material. The GenoType MTBDRplus assay is based on line probe assay (LPA) technology involving polymerase chain reaction (PCR) amplification and binding of amplicons to specific oligonucleotide probes immobilized on a membrane strip.

2. SCOPE

This SOP relates to molecular assays for mycobacterial speciation performed in the FIND Research Laboratory.

3. RESPONSIBILITIES

All staff members working in the FIND Uganda Research Laboratory are responsible for the implementation of this operating procedure.

All users of this procedure who do not understand it or are unable to carry it out as described are responsible for seeking advice from their supervisor.

4. CROSS-REFERENCES

Document Matrix_Uganda QP 01-03-03

Refer to SOPs listed under 07-01 (General Procedures), 07-02 (Specimen Handling), 07-06 (Equipment Use and Maintenance) and 07-05 (Molecular Methods).

See: *Document Matrix_Uganda QP01-03-03.doc*

Location: *Hard copy: FIND Uganda SOPs*

5. PROCEDURES

5.1. Equipment

Class II biological safety cabinet (BSC) that is inspected and certified at least bi-annually

Incubator $450\text{C} \pm 10\text{C}$

Sonicating Incubator

Centrifuge

Thermal cycler

5.2. Materials

PMN mix

10x polymerase incubation buffer

MgCl₂

Thermostable DNA polymerase

Distilled water

PCR tubes, DNase/RNase free

1% bleach

GenoType MTBDRplus kit (Hain Lifescience): DEN solution, HYB buffer, STR buffer, TwinCubator, DNA STRIP test membranes, Conjugate Concentrate (Con-C), Conjugate Buffer (CON-D), Substrate Concentrate (Sub-C), Substrate Buffer (Sub-D)

15 ml sterile graduated conical tubes for dilutions

Absorbent paper

Forceps

Timer

Sterile cotton-plugged tips FILTERED

Automatic pipettes: P10 in Pre-Amplification Room, P10, P200 in Processing lab, P1000, P200 in Post-Amplification room.

Disposable Pasteur pipettes

Discard containers

5.3. Master Mix Preparation

Always perform at the beginning of the day in Pre-Amplification Room wearing gloves. Wear lab coats remaining in pre-amplification room.

- Apply 1% bleach solution thoroughly to all surfaces of hood and worktop.
- For preparation of bleach solution:

See: *Laboratory Cleaning and Maintenance_Uganda QP 07-01-04.doc*

Location: *Hard copy: FIND Uganda SOPs*

- Remove thermostable DNA polymerase, PMN, buffer, H₂O, and MgCl₂ from refrigerator. Thaw final 3 reagents as needed.
- PMN mix is to be thawed and can be stored up to 2 weeks at 4°C. Mix can be refrozen up to 5-6 times.
- Carefully remove PCR tubes and close lids. Place in rack in hood and label.
- For volumes for master mix, apply volumes/tube x (specimen number +1 negative control) as follows:

35 ul PNM

5 ul 10x polymerase incubation buffer

2 ul MgCl₂

3 ul H₂O

0.2 ul Taq DNA polymerase

A table of volumes for common numbers of tubes is given below:

	Number of tubes								
	1	2	3	5	7	8	10	11	12
PNM	35µl	70 µl	105	175	245	280	350	385	420
Buffer	5 µl	10	15	25	35	40	50	55	60
MgCl ₂	2 µl	4	6	10	14	16	20	22	24
H ₂ O	3 µl	6	9	15	21	24	30	33	36
Taq polymerase	0.2 µl	0.4	0.6	1.0	1.4	1.6	2.0	2.2	2.4

- Aliquot 45 ul of Master Mix to each PCR tube.
- Add 5 ul H₂O to negative control tube.
- Disinfect all surfaces of PCR hood, and worktop, with 1% sodium hypochlorite solution.
- Remove lab coats and store in room.
- Bring PCR tubes out of room to Processing Lab.

Note: If poor banding intensity is obtained, an alternative Master Mix can be prepared which includes 8 µl of DNA and no water (see Product insert).

5.4. DNA Extraction

All steps to be performed in **Processing laboratory**.

- Thoroughly clean all surfaces of hood as well as external surfaces of all equipment in hood with 5% Lysol followed by 1% bleach.
- Spray gloves with 1% bleach before work. MAY GET INTO SPECIMENS FROM GLOVES!

- For preparation of cleaning solutions:

See: *Laboratory Cleaning and Maintenance_Uganda QP 07-01-04.doc*

Location: *Hard copy: FIND Uganda SOPs*

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- The hot plate should be switched on first when entering the laboratory to allow sufficient time for the temperature to equilibrate. *A microcentrifuge filled with distilled water should be placed in the hotplate and a thermometer inserted to measure the operating temperature of the hot plate (which does not correspond to the digital readout).*

5.4.1. DNA extraction from liquid culture

- Pipette 1 ml of liquid culture directly to conical vial.
- Centrifuge 15 min at 10000 x g.
- Discard supernatant and suspend pellet in 100ul H₂O.
- Incubate 20 min at 95°C in the hot plate.
- Incubate 15 min in ultrasonic bath.
- Centrifuge 5 min at maximum speed.
- Take 80 uL DNA supernatant to new 1.5 ml conical tube.
- Add 5 uL of DNA supernatant to corresponding PCR tubes.

5.4.2. DNA extraction directly from patient samples

- Sputum specimens should be decontaminated according to Specimen .
- Following suspension of the pellet in phosphate buffer, use a disposable Pasteur pipette to pipette 500µl of decontaminated sample to a 1.5ml microcentrifuge tube with screw cap.
- Centrifuge for 15 minutes at 10,000 x g.
- Discard supernatant and suspend pellet in 100ul molecular grade water.
- Incubate for 20 minutes at 95°C in the hot plate.
- Incubate for 15 minutes in the ultrasonic bath.

- Centrifuge for 5 minutes at 10,000 x g.
- Tubes should be loaded in the centrifuge and unloaded in the same tube alignment to make visualization of pellet easier for removing the supernatant.
- Carefully carry the tubes to the BSC. Uncap tubes one at a time, and carefully transfer 40-80ul of supernatant to a clean microcentrifuge tube using a P100 pipette.
- Careful attention must be paid to avoid touching the pipette tip to the pellet. If this is done, and pellet is drawn into the pipette tip, a small amount of liquid can be expelled back into the tube until only clear supernatant remains.
- Add 5µl of DNA supernatant to corresponding PCR tubes.

5.5 Amplification

All steps take place in **Post-Amplification Room**.

- If PCR tubes have bubbles at base, remove by swinging arm with tubes in hand in arc.
- Transfer PCR tubes to middle section of thermocycler.
- Select Program User=FINN and choose program of 30 cycles [10 + 20 cycles] for cultures, using Hot Star polymerase (MTBDR Hot 30), or 40 cycles [10+ 30 cycles] for specimens (MTBDR Hot 40).
- Check program parameters and follow menu options to start appropriate program.

Amplification Profile:

	Culture samples	direct pt. material
5 min 95°C	1 cycle	1 cycle
30 sec 95°C	10 cycles	10 cycles
2 min 58°C		
25 sec 95°C		
40 sec 53°C	20 cycles	30 cycles
40 sec 70°C		

8 min 70°C 1 cycle 1 cycle

5.6 Hybridization

All steps to be performed in **Post-Amplification Room** wearing gloves.

- Pre-warm HYB and STR solutions (green and red) to 45°C in water bath (15 minutes total).
- Pre-warm TwinCubator to 45°C.
- Pipette 20 ul DEN (denaturing solution) to each well of tray to be used.
- For each repetitive addition step, may use same pipette tip if wells/samples not touched. **If the wells are touched a fresh pipette tip must be used.**
- Add 20 ul of corresponding amplified DNA sample to each well, and mix well by pipetting up and down several times.
- Incubate for 5 minutes at room temperature.
- Remove DNA strips from tube (shake strips down to end of tube then remove carefully holding the end of the strip with forceps) and mark them with provided red pen or pencil.
- Add 1 ml HYB (hybridization solution) to each well and gently shake to homogenize solution.
- Add 1 strip to each well with colored marker facing up. If strips turn over, re-position them with a fresh pipette tip.
- Place tray on TwinCubator and press "START" to incubate for 30 minutes at 45°C. From this point, press right arrow on TwinCubator once to advance steps in protocol.
- When alarm goes off, press right arrow to stop.
- Pour off HYB into the sink, holding it low and close to the drain to avoid amplicon transmission. **Remove remaining solution by forcefully tapping tray against paper towels on benchtop.** Membranes will not fall out!
- Wipe off condensation that forms on lid before every incubation step.
- Add 1 ml STR (stringent buffer) per well and incubate for 15 minutes in TwinCubator at 45°C. Press right arrow to start.
- Prepare diluted Conjugate and Substrate in 15 ml conical vials by diluting 1:100 with corresponding Con-D and Sub-D. Colours of small tubes (concentrates) correspond to colors of dilution buffer tubes. Wrap Substrate dilution in aluminum foil. Prepare fresh Conjugate and

Substrate dilutions every day, **but may re-use old conical tubes after washing thoroughly in distilled water.**

- When alarm goes off, press right arrow. Completely remove STR as previously described for HYB removal.
- Add 1 ml RIN (rinse solution) per well. Press right arrow to incubate for 1 minute on TwinCubator..
- Hit right arrow after alarm. Remove RIN and add 1 ml of diluted Conjugate per well.
- Press right arrow to incubate for 30 minutes on TwinCubator.
- Rinse sink with 1% sodium hypochlorite solution.
- Press right arrow upon alarm. Remove solution and wash for 1 minute with 1 ml RIN per well on TwinCubator. Pour out solution and repeat rinse with 1 ml RIN per well for 1 min.
- Remove RIN and wash with 1 ml distilled water per well on TwinCubator.
- Remove water and add 1 ml of diluted substrate per well.
- Place on TwinCubator under aluminum foil for a maximum of 10 minutes. Look for colour reaction to indicate reaction completion after 4-5 minutes. If colour reaction is too weak, replace the foil and re-incubate for several more minutes, up to a maximum of 10 minutes.
- Wash twice for 1 minute each with distilled water. Do not remove the volume of water as this will assist transfer of strips to result sheet.
- **Trays can be reused a few times. Wash in water and repeat rinse in distilled water. Occasionally wash in 1% sodium dodecyl sulphate solution.**
- Record cleaning of laboratories and equipment in *Laboratory Cleaning and Maintenance Logbook*.

Use: *Laboratory Cleaning and Maintenance Logbook_form.doc*

Location: *Hard copy: FIND Uganda SOPs*

5.7. Interpretation of Results

- Use forceps to transfer strips to the *GenoType MTBDRRplus Results Sheet* provided with the kit, or downloadable from Hain Lifescience website.

Use: *Genotype MTBDRplus Results Sheet.pdf*

Location: *Hard copy: FIND Uganda SOPs*

- Read results by lining strips up to code provided with kit.
- In order for results to be valid, CC (conjugate control) and AC (amplification control) bands must appear for every sample. The presence of TUB band indicates that *M.tuberculosis* complex is present in the sample.
- A mutation in the relevant gene (and resistance to the relevant drug) is signified by either an absent wild type band and/or the presence of a mutant band for each gene cluster.
- The rpoB, katG and inhA each have a control band which must be present in order to interpret the results.
- rpoB predicts RIF resistance, katG predicts high level INH resistance, inhA predicts low level INH resistance.
- For results to be valid the bands must be of intensity equal to or greater than the intensity of the AC band.
- In order for a batch of results to be valid, the negative control strip must have a CC and AC band present, but no other bands must be visible.
- If a positive result is obtained with the negative control, the results of the whole batch must be repeated and measures taken to remove amplicon contamination from all rooms and equipment.
- Refer to the product insert for interpretation of banding patterns and troubleshooting.

6. REFERENCES

GenoType MTBDRplus product insert. Hain Lifescience. Version 1.0; 2/2007.

Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME. Rapid molecular screening for drug-resistant tuberculosis in a high-volume public health laboratory in South Africa. *Am J Respir Crit Care Med*. 2008 Apr 1;177(7):787-92. Epub 2008 Jan 17.

7. CHANGE HISTORY

New version # / date	Old version # / date	No. of changes	Description of changes	Source of change request