Instruction for Use

mfloDx™ MDR-TB AMP Kit

(To be used along with *mfloDx*™ MDR-TB VIS Kit)

Brief Description of the Product

The *mfloDx*™ MDR-TB test is a qualitative *in vitro* test for the identification of *Mycobacterium tuberculosis* complex (MTC) and resistance to rifampicin and isoniazid from smear positive sputum samples. Our multiplex molecular test indicates presence of *Mycobacterium tuberculosis* and its genotypic resistance profile by developing a visual signal within 3 h. The *mfloDx*™ is designed to be performed with minimal and standard laboratory equipment and infrastructure using standard thermal cycler, heat block, microfuge, and vortex mixer.

The *mfloDx*™ MDR-TB test Kit consists of the amplification (*mfloDx*™ MDR-TB AMP Kit) and signal development kit (*mfloDx*™ MDR-TB VIS Kit).

The *mfloDx*™ MDR-TB test can detect 15 targets coding for the wild type and their clinically significant mutations causing resistance to:

- rifampicin (*rpoB* codons 516, 526, 531, 533), and to
- isoniazid; *katG* (codon 315) and *inhA* (promoter region -15).

<table>
<tr><th>Targets</th><th>Input requirement</th></tr>
<tr><td>Diagnostic targets</td><td>15 DNA targets for the detection of Mycobacterium tuberculosis complex, wild type and mutations coding for the sensitivity and resistance to Rifampicin and Isoniazid.</td></tr>
<tr><td>Genetic targets</td><td>
<table>
<tr><th>Gene</th><th>mfloDx™ MDR-TB targets</th></tr>
<tr><td>rpoB</td><td>516 G/T
516 A/T
526 C/G
526 C/T
526 A/G
531 C/T
531 C/G
533 G/C</td></tr>
<tr><td>katG</td><td>315 G/C
315 G/A</td></tr>
<tr><td>inhA</td><td>-15 C/T</td></tr>
</table>


</td></tr>
<tr><td>Controls</td><td>There are two controls: (1) IC: Internal Control will not show up if there is any issue right from the sample preparation till the signal development. (2) HC: Hybridization Control in an internal quality control for the visualization reagent and the lateral flow cassette.</td></tr>
</table>

Intended Use

The ***mfloDx***™ MDR-TB AMP Kit is a qualitative *in-vitro* diagnostic test used for amplification and detection of *Mycobacterium tuberculosis* and its resistance to Rifampicin and Isoniazid genes. The assay is designed for **"Professional Use Only"** in diagnostic laboratory under **technical supervision** as an aid to detect patients suspected of being infected with TB and resistance to Rifampicin and Isoniazid. This kit has to be used along with a *Rapid test kit for detection of* ***Mycobacterium tuberculosis*** *and resistance to Rifampicin and Isoniazid (****mfloDx***™ ***MDR-TB VIS Kit)***

Principle of the Test

The *mfloDx*™ diagnostic platform is based on two well-established technologies: padlock probe-dependent rolling circle amplification (RCA, an isothermal nucleic acid amplification method) and sensitive lateral flow nucleic acid biosensor chemistry (signal development readout). The *mfloDx*™ MDR-TB, can be used on patient's sputum sample directly (without preceding culturing). The technique is easy to perform and has a turnaround time of 3 hours. The *mfloDx*™ MDR-TB AMP Kit consists of primers and padlock probes used to amplify DNA segments (sequences) by a technology called, rolling circular amplification (RCA). These padlock probes are specifically designed to identify the presence of MTC and its antibiotic resistance to rifampicin and isoniazid in fast and accurate manner. Padlock probes are highly selective DNA probes that forms circles upon hybridizing to their complementary region of DNA enabling specific and multiplex detection of single nucleotide variations. The digested amplified products are applied on the lateral flow cassette to see the visual results.

1. Product contents:

1.1 Kit Contents

The Kit contents supplied with the *mfloDx*™ MDR-TB AMP Kit as detailed below:

Product Code	Name of the Product	Quantity supplied with the kit
2-0001	PCR mix	1 x 366.0 µl
2-0002	LIG mix	1 x 450.0 µl
2-0003	RCA mix	1 x 480.0 µl
2-0004	DIG mix	1 x 112.5 µl
2-0039	Enzyme E-1	1 x 9.0 µl
2-0040	Enzyme E-2	1 x 25.0 µl
2-0041	Enzyme E-3	1 x 20.0 µl
2-0042	Enzyme E-4	1 x 12.5 µl
2-0025	MAG	1 x 75.0 µl
2-0035	BWB	2 x 1.25 ml

1.2 Storage and Handling:

- The *mfloDx*™ MDR-TB AMP Kit should be stored at -25 °C to -16 °C
- ***The No. of freeze thaw cycles with the 25 test kit is 4 No.s. Beyond 4 times, the kit cannot be used and the results obtained beyond 4th cycle may not be considered for interpretation***

1.3 Precautions:

- Always wear suitable PPE while handling the clinical specimens.
- Treat all biological specimens as potentially infectious, and strictly adhere to the GLI guidelines while handling the biological specimens.
- All techniques involving open tubes/containers must be carried out in a class II biosafety cabinet
- Wash hands thoroughly after handling specimens and test reagents.
- Use only for the detection of the *M. tuberculosis* complex using sediments prepared following the NALC-NaOH decontamination protocol.
- This test may only be used with raw sputum samples or concentrated sediments prepared from induced or expectorated sputa.

- When processing more than one sample at a time, follow necessary precautions to prevent cross contamination between samples.

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1.4 Materials required but not supplied

- Thermocycler
- Drybath / Heating block
- Microcentrifuge
- Magnetic stand
- Vortex
- Timer
- Disposable gloves
- Sample decontamination reagents as well as necessary equipment
- Adjustable pipettes for 10, 20, 200, and 1000 µl
- Disposable sterile pipette tips with filter: 10, 20, 200, and 1000 µl
- 1.5 ml microcentrifuge tubes
- 0.2 ml PCR tubes
- Nuclease free Water (for negative controls)

1.5 Specimen collection, transport, and storage

All specimens should be collected and transported as recommended by CDC and GLI [1,2,3], or according to the institutional laboratory sample collection procedure manual. It must be ensured that until decontamination, specimens are kept in sterile plastic containers at a temperature of 2 °C to 8 °C. Specimens used for decontamination must not be older than 7 days. After decontamination and resuspension of the bacteria pellet with phosphate buffer, samples can be stored at 2 °C or 8 °C for a maximum of 7 days until performing DNA extraction. Clinical specimens must be processed using the NALC-NaOH method according to the GLI guidelines [3]. After decontamination, the cell pellet should be resuspended in a maximum of 1.0 to 1.5 ml of phosphate buffer. Due to the potential inhomogeneity of the specimen, the decontaminated sample must be mixed before removing the aliquot to be analyzed; otherwise, the sensitivity of the test might be influenced. Handling of potentially infectious specimens must be carried out in a class II safety cabinet.

2. Pre-amplification

2.1 Thaw the **PCR mix** at room temperature and thaw all the other mixes (**LIG mix**, **RCA mix**, **DIG mix**, **VIS-2**) and the magnetic beads (**MAG**) at 2-8 ºC. Move the required number of *mfloDx*™ MDR-TB cassettes to room temperature for equilibration.

2.2 Prepare the required number of PCR microcentrifuge tubes.

2.3 Label the PCR microcentrifuge tubes. Vortex the **PCR mix** at least 3 seconds and spin down. Prepare **Working solution 1** in a 1.5 ml microcentrifuge tube by mixing 14.64 µl **PCR mix** and 0.36 µl Enzyme **E-1** per test. Vortex ~3 seconds and spin down. Calculate 10 % of additional assay volume to compensate for reagent loss. See example in Table 1.

2.4 Keep the Enzyme **E-1** in a benchtop cooler or on ice. Return it to the freezer immediately after use.

Table-1 Working Solution-1

Component	Volume required for single assay
PCR mix	14.64 µl
Enzyme **E-1**	0.36 µl
Solution volume	15.0 µl
DNA sample	5.0 µl
Final volume	20.0 µl

2.5 Aliquot 15 µl of **Working solution 1** into all tubes.
2.6 Transfer 5 µl of DNA sample extracted from sputum to each PCR tube.
2.7 Close the PCR tubes, vortex gently and spin down in a microcentrifuge.
2.8 Load samples into a Thermocycler and execute the protocol described in Table 2.

Table-2 PCR Program		
Temperature	**Time**	**Phase**
37 ˚C	5 min	Holding stage
95 ˚C	5 min	Holding stage
95 ˚C	10 sec	x 25 cycles
64 ˚C	30 sec	
72 ˚C	10 sec	
95 ˚C	10 sec	x 15 cycles
64 ˚C	30 sec	
72 ˚C	30 sec	
8 ˚C	∞	Holding stage

3. Ligation

3.1 Prepare new microcentrifuge PCR tubes and label them accordingly.
3.2 Retrieve the tubes from the Thermocycler.
3.3 Vortex and spin down the thawed **LIG mix**. Prepare **Working solution 2** in a 1.5 mL microcentrifuge tube by mixing 18 µl **LIG mix** and 1 µl Enzyme **E-2** per test. Vortex 3 seconds and spin down. Calculate 10 % of additional assay volume to compensate for reagent loss. See example in Table 3.
3.4 Keep the Enzyme **E-2** in a benchtop cooler or on ice. Return it to the freezer immediately after use.

Table-3 Working Solution-2	
Component	**Volume required for single assay**
LIG mix	18 µl
Enzyme **E-2**	1 µl
Solution volume	19 µl
PCR product	1 µl
Final volume	20 µl

3.5 Aliquot 19 µl of **Working solution 2** into all labeled new PCR tubes.
3.6 Transfer 1 µl of the amplified PCR product to the corresponding PCR tube.
3.7 Close the PCR tubes, vortex gently and spin down in a microcentrifuge.

Important! Although the assay contains reagents to eliminate accidental carry-over contamination from PCR product to future PCR reactions, one should take the utmost care to prevent contamination of PCR products to the laboratory environment. Change to a new pair of gloves after handling tubes containing PCR products.

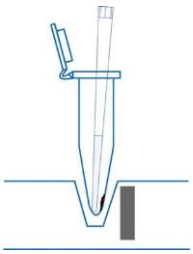
3.8 Load samples into a Thermocycler and execute the protocol described in Table 4.

Table-4 Ligation Program		
Temperature	**Time**	**Phase**
94 ˚C	1 min	Holding stage
56 ˚C	5 min	Holding stage
8 ˚C	∞	Holding stage

4. Capture

4.1 Retrieve the samples from the Thermocycler.
4.2 Vortex the thawed **MAG** suspension**.** Take extra care to make sure that magnetic beads settled at the bottom are redispersed. It is important that the **MAG** is always homogenous. If necessary, flick the beads down rather than centrifuging.
4.3 Open all sample tubes and aliquot 3 µl of **MAG** into each sample tube. If this takes more than a minute, vortex the **MAG** again to keep the solution homogenous.
4.4 Close the PCR microcentrifuge tubes and vortex gently until the color of the samples indicate homogenous **MAG** dispersion (dark brown).
4.5 Incubate samples with **MAG** for 5 minutes at room temperature.
4.6 Briefly spin down (~2 seconds) to remove any sample from the lids.
4.7 Move tubes to the *mfloDx*™ Magnetic Stand and let the magnetic beads collect at the magnet for approximately 10 seconds.
4.8 Using a pipette, carefully remove and discard all of the liquid. Do not attempt to decant supernatant

NOTE: Pay attention to which side of the tubes the magnetic beads have collected. We recommend positioning the pipette tip at the bottom of the PCR tube in a direction opposite to the magnetic beads. Work carefully not to disturb or aspirate the magnetic bead pellet present on the side of the tube. If beads are accidentally aspirated, pipette them back and allow them to collect at the magnet.



4.9 Add 100 µl of the **BWB** into each sample

NOTE: A multichannel pipette and a reagent reservoir for the BWB can be used to speed up work

4.10 Close the PCR microcentrifuge tubes and vortex gently until the color of the tubes indicates homogenous **MAG** dispersion (light brown, no precipitate). Briefly spin the tubes in order to remove any liquid from the tube lid and transfer to the *mfloDx*™ Magnetic Stand. Proceed to section 4.7 while allowing the beads to migrate.

5. RCA amplification

5.1 Vortex and spin down the thawed **RCA mix**. Prepare **Working solution 3** in a 1.5 ml microcentrifuge tube by mixing 19.2 µl **RCA mix** and 0.8 µl Enzyme **E-3** per test. Vortex ~3 seconds and spin down. Calculate 10 % of additional assay volume to compensate for reagent loss. See example in Table 5 below.
5.2 Keep the Enzyme **E-3** in a benchtop cooler or on ice. Return it to the freezer immediately after use.

Table-5 Working Solution-3	
Component	**Volume required for single assay**
RCA mix	19.2 µl
Enzyme **E-3**	0.8 µl
Solution volume	20.0 µl

5.3 Carefully remove all the liquid (the **BWB** from the previous step) from all samples.

Important! Continue immediately with the next steps to avoid the MAG of drying out. If a high number of samples are processed, steps 6.3 – 6.5 can be performed batchwise, 8 tubes at a time.

5.4 Transfer 20.0 µl of **Working solution 3** to each tube.
5.5 Close the PCR microcentrifuge tubes and vortex gently until the color of the samples indicates homogenous **MAG** dispersion (dark brown, no precipitate). Remove bubbles or liquid in the lids by gently flicking the tubes. Do not spin the samples.
5.6 If sample is still present in the lid of a tube, give the tubes a quick spin (~1-2 seconds) and a gentle vortex to redisperse the **MAG**.
5.7 Load samples into the Thermocycler and execute the protocol described in Table 6.

Table-6 RCA Program		
Temperature	**Time**	**Phase**
37 ˚C	20 min	Holding stage
65 ˚C	1 min	Holding stage
8 ˚C	∞	Holding stage

6. Digestion

6.1 Vortex and spin down the thawed **DIG mix**. Prepare **Working solution 4** in a 1.5 ml microcentrifuge tube by mixing 4.5 µl **DIG mix** and 0.5 µl Enzyme **E-4** per test. Vortex ~3 seconds and spin down. Calculate 10% of additional assay volume to compensate for reagent loss. See example in Table 7.
6.2 Keep the Enzyme **E-4** in a benchtop cooler or on ice. Return it to the freezer immediately after use.

Table-7 Working Solution-4	
Component	**Volume required for single assay**
DIG mix	4.5 µl
Enzyme **E-4**	0.5 µl
Solution volume	5 µl
RCA product	20 µl
Final volume	25 µl

6.3 Retrieve samples from the thermocycler.
6.4 Transfer 5 µl of **Working solution 4** to each tube.
6.5 Close the PCR microcentrifuge tubes and vortex gently. It is common that magnetic beads aggregate and sediment at this stage. After the RCA amplification, it is not always possible to redisperse the beads. Remove liquid in the lids by gently flicking the tubes. Do not spin the samples.
6.6 Load samples into the Thermocycler and execute the following incubation protocol:

Table-8 Digestion Program		
Temperature	**Time**	**Phase**
37 ˚C	3 min	Holding stage
8 ˚C	∞	Holding stage

***Note: Carryout the rest of the assay steps using mfloDx™* MDR-TB VIS Kit *(Product code: MDRTB-VIS-25) referring to the IFU (*IFU/MDRTB-VIS)**

7. Performance Characteristics

The performance evaluation of *mfloDx*™ MDR-TB test was conducted at ICMR-NIRT, Chennai. The test had diagnostic accuracy, specificity, sensitivity, PPV and NPV of 98%, 100%, 97%, 100% and 91%, respectively, from pooled sputum (smear positive and smear negative) samples. The Clinical sensitivity from smear positive samples for TB detection, RIF and INH resistance detection was 100% with a PPV and diagnostic accuracy of 100%.

Analytical Performance Characteristics:

The analytical performance characteristics of *mfloDx*™ MDR-TB test was conducted in-house on the stored sputum samples.

Analytical Sensitivity:

The analytical sensitivity of *mfloDx*™ MDR-TB AMP Kit and *mfloDx*™ MDR-TB VIS Kit was evaluated by spiking the TB negative sputum samples with five level of dilutions of *M. tuberculosis* H37Rv from 1000 CFU/ml to 200 CFU/ml.

The result of LOD is **654.5 CFU/ml, *katG* and *inhA* 516.7 CFU/ml and *rpoB* 562.9 CFUml**. When extrapolating the CFU equivalent to **per PCR reaction**, it **showed 3.2 CFU for ITS and 2.5 CFU for *katG* and *inhA* and 2.8 CFU for *rpoB*.**

Cross Reactivity: Cross Reactivity with closely related *Mycobacterium* species was evaluated by testing *M. kansasii, M. intracellularae, M. simiae and M. fortuitum* in duplicates at 100pg/µl concentration using *mfloDx*™ MDR-TB test. The test did not show any cross reactivity with the tested NTMs as detailed in the Figure below:

NTM/ MTC	Result
M. kansasii Replicate 1	Negative
M. kansasii Replicate 2	Negative
M. intracellularae Replicate 1	Negative
M. intracellularae Replicate 2	Negative
M. simiae Replicate 1	Negative
M. simiae Replicate 2	Negative
M. fortuitum Replicate 1	Negative
M. fortuitum Replicate 2	Negative
M. tuberculosis H37Rv Replicate 1	Positive
M. tuberculosis H37Rv Replicate 2	Positive
Negative	Negative

8. Discard of Kit components and Packaging materials

- Discard the kit components empty vials, packaging material into the black color plastic bag and follow regulations for discarding.

Strictly adhere to the protocol presented to ensure correct test results and avoid sample-to-sample contamination.

9. Symbol Keys (referred from ISO 15223-1 standard guidelines)

Symbol	Description
	Product Catalogue Number
	Product Lot/ Batch Number
	Use by date
	Storage conditions
	Manufacturer of the product
	Quantity of the product filled in the vial
	Single Use Only
	Pack size
	*Invitro* Diagnostic medical device
	Aspiration Hazard
	Important instruction
	Consult instructions for use

10. References

1. Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services, Centres for Disease Control and Prevention, Atlanta, USA 1985.
2. Isenberg HD. Clinical microbiology procedures handbook. American Society for Microbiology, Washington, D.C., USA 1992.
3. Global Laboratory Initiative. Mycobacteriology Laboratory Manual. Available from: https://www.who.int/tb/laboratory/mycobacteriology-laboratory-manual.pdf last accessed on November 26, 2021.

11. Purchaser notification

All materials and reagents provided with the *mfloDx*™ MDR-TB AMP kit must be used by, or directly under the supervision of, a technically qualified individual. Read the Safety Data Sheet provided for each product available at www.empediagnostics.com.

Product Code	Pack size
MDRTB-AMP-25	25 Tests

Obtaining Support
For help services please contact info@empediagnostics.com
and for further information visit www.empediagnostics.com.

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