EMPE DIAGNOSTICS

mfloDX® MDR Assay kit

Online specials

Catalog number: 9-001; 9-002; 9-003

Material	Label	Volume	Amount	Concentration	Storage	Stability
mfloDX® MDR-TB cassette		-	27 ×	-	8-25 °C Protect from light	When stored as stated, materials are stable for 6 months.
Bead Wash Buffer	BWB 4 ×	1 mL	1 x	4 ×	8-25 °C	
DNA Sample standard WT	DNA ssWT	30 µL	1 × of each	1×	-20 °C	
DNA Sample standard MDR	DNA ssMDR	30 µL				
Buffer 1	HLIG	300 µL		2 ×		
Buffer 2	AMP1	600 µL		1 ×		
Buffer 3	FRAG1	150 µL				
Buffer 4	AMP2	300 µL				
Buffer 5	FRAG2	150 µL				
Visualization solution1	COLOR1	450 µL	1 ×		4 °C Protect from light	
Enzyme1 – Ampligase	E1 AmpLig	16 µL				
Enzyme2+4 – Phi29	E2/4 Phi29	35 µL				
Enzyme3+5 – AluI	E3/5 AluI	35 µL				
Enzyme4 – T4 Ligase	E4 T4Lig	3 µL				
Visualization solution2	COLOR2	480 µL				Up to 8 freeze-that cycles without activity loss
Magnetic beads	MagBead	170 µL				
Magnetic stand			1 x			

Introduction

The *mfloDx*® MDR-TB is a qualitative test that can robustly distinguish single-nucleotide variants of the most common mutations in multidrug resistant *Mycobacterium tuberculosis* DNA [rpoB 531 (TCG/TTG) and katG 315 (AGC/ACC)], as well as verify the loss of the respective wild type alleles. This combinatorial molecular diagnostic test produces visual signals on lateral flow cassettes corresponding to the respective genotype markers. This kit includes 27 lateral flow cassettes and chemical reagents necessary to conduct the assay. Assay is optimized for TB-DNA obtained using heat lysis method after 4-6 weeks of bacteria culture in Löwenstein–Jensen medium.

Good to know before you start

Indented use

The mfloDX® MDR-TB Assay kit is intended for research use only applications (RUO). This product is not intended for the diagnosis, prevention, or treatment guidance in tuberculosis disease. We advise to read the following manual carefully, attention should be given to handling and exercising the procedures described.

Safety information

When working with chemicals provided with this kit, always wear a suitable lab coat, disposable gloves. Please make sure to operate the kit in a dedicated, DNAse free space. For further information, we refer to www.empediagnostics.com for appropriate safety data sheets (SDSs) available for each kit and kit component.

Visualization solution 1 contains formamide. Formamide (CAS 75-12-7) is classified as hazardous and volatile chemical. Formamide is suspected of causing cancer and may damage the unborn child. Do not inhale. The last steps of the protocol 6.5–6.10 should be done in the ventilated chemical cabinet.

Emergency information in English can be obtained between 9:00–17:00 from:
Swedish Poisons Information Center Tel: +46-010-456 6700

Storage of the *mfloDX*® MDR-TB Assay kit

The *mfloDX*® MDR-TB Assay kit is shipped frozen and should be stored as described above. Disposable cassettes and Bead Wash buffer can be stored at room temperature upon delivery. Formamide should be stored at 4°C in the fridge where light exposure is minimal.

Disposal of the *mfloDX*® MDR-TB Assay kit

Disposable cassettes are single-use-only and should be disposed as hazardous material, in accordance with federal, state and local regulations.

Materials and equipment required but not delivered with the kit

- Disposable gloves
- PCR microcentrifuge tubes (DNAse free)
- 1.5 mL microcentrifuge tubes (DNAse free)
- Pipette tips and pipettes
- Microcentrifuge (with rotor for PCR tubes)
- Vortexer
- Heated-lid PCR machine
- mqH2O
- Optional: PCR tube vertical rotator

Principles of the procedure

Susceptibility or resistance of *Mycobacterium tuberculosis* to rifampicin (RIF) or isoniazid (INH) is dictated by particular mutations in bacteria DNA. The *mfloDX*® principle combines proprietary technology (C2CA[1]) with simple and visual lateral flow (LF) readout to detect (genotype) mutations in TB. In the first step, the DNA isolated from bacteria (this kit was optimized on DNA obtained via heat lysis method[2] after 4-6 weeks of bacteria cultivation in Löwenstein–Jensen medium) is first fragmented with heat. Fragments of interest within rpoB and katG gene are then selectively targeted using amplifiable padlock probes[3] and finally purified with magnetic beads. After the first round of polymerization, amplified products are monomerized and subjected for the second round of amplification and monomerization followed by visualization on the disposable cassette. The developed visual bands will allow for accurate genotyping of rpoB 531 (TCG/TTG) and katG 315 (AGC/ACC). The protocol can be typically executed within 120 minutes.
A supplementary video presenting the procedure is available on www.empediagnostics.com.

The following symbol marks important notes are given for your reference. Please make sure to read the notes carefully.

The following symbol marks that it is possible to pause the experiment. If this is the case, transfer samples into the freezer and continue whenever it is convenient.

Introductory notice

Determine number of samples you desire to run. We recommend including three internal controls: two DNA sample standards provided with the kit (DNA ssWT and DNA ssMDR) and one negative sample control (water). Positive controls will provide a result for various TB genotypes assuring that the all assay steps were executed successfully. Negative control is expected to provide no visual signal (except the *Control line*). If 5 assays are intended to be tested, the final number of assays will be 8. The protocol is divided into 5 steps, and buffers and reagents are labelled according to their appearance in the workflow.

- Change pipette tips between samples
- All thawed buffers should be kept on ice if not frozen immediately after use
- Enzymes should only be removed from the freezer while preparing the working solutions

[1] Dahl F et al. 2004. Circle-to-circle amplification for precise and sensitive DNA analysis. *PNAS* **101**: 4548–4553.
[2] Coll P, et al. 2005. Molecular analysis of isoniazid and rifampin resistance in Mycobacterium tuberculosis isolates recovered from Barcelona. *Microb Drug Resist* **11**: 107–114.
[3] Nilsson M et al. 1994. Padlock probes: circularizing oligonucleotides for localized DNA detection. *Science* **265**: 2085–2088.

1. TB DNA Fragmentation and genotyping

DNA fragmentation step is necessary to facilitate interaction between amplifiable probes and TB DNA. EMPE Diagnostics AB provides two DNA ssWT and MDR standards which are fragmented already and do not need to be fragmented. To fragment the DNA, place samples in heated-lid thermocycler and incubate at 95°C for 30-70 minutes. Depending on the sampling procedures final time of the assay can be estimated experimentally.

A. TB DNA Genotyping

1.1 Prepare required number of PCR microcentrifuge tubes according to the number of assays to be run. *mfloDX*® MDR-TB assay kit recommends including three additional controls

1.2 Label the PCR microcentrifuge tubes

1.3 Thaw the Buffer1

1.4 Prepare the Step1 working solution in 1.5 mL microcentrifuge tube. Use a clean plastic tube each time you prepare this and every other working solution. Include 10% of additional assay volume to compensate for reagent loss

NOTE: Volume of a Step1 working solution is 20 µL. For a single assay, mix 10 µL of Buffer1, 1 µL of Enzyme1, 2 µL of sample (DNA, two DNA Sample standards or water for negative control) with 7 µL mqH2O to a total assay volume 20 µL (Table1). If multiple samples are tested, prepare the Step1 working solution by adding adjusted amount of water and Buffer1. 2 µL of sample should be separately added into individual tubes before adding a Step1 working solution.

Enzyme1 should be removed from the freezer and mixed with a working solution after all DNA samples have already been aliquoted.
Example: If 5 samples are being tested, prepare enough Step1 working solution for the 5 samples, 2 standards and 1 negative control plus additional 10% assay volume. For total of 8.8 samples, take 61.6 µL mqH2O, 88 µL Buffer1 and 8.8 µL Enzyme1 (Table1)

Step1			Volume added		
	Stock concentration	Final concentration	Single assay	8.8 assays (8+10%)	Your assay
Buffer1	2 ×	1 ×	10 µL	88 µL	
Enzyme1	-	-	1 µL	8.8 µL	
Sample	-	-	2 µL	(17.6 µL, not added)	
mqH2O	-	-	7 µL	61.6 µL	
		mix volume	20 µL		
		total volume	20 µL		

Table 1

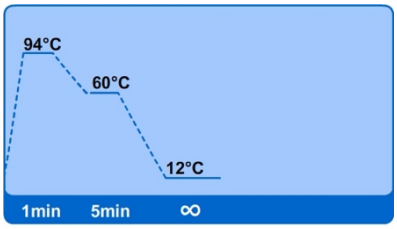
1.5 Aliquot 2 µL of samples (DNA, DNA Sample standards or water for negative control) into labeled PCR microcentrifuge tubes

1.6 Aliquot 18 µL of the Step1 working buffer into each tube

NOTE: To reduce risk of sample contamination, use automatic dispenser or change the pipette tips between samples

1.7 Close the PCR microcentrifuge tubes, spin down in the microcentrifuge (remove obvious bubbles)

1.8 Load samples into the PCR instrument and execute the following protocol:



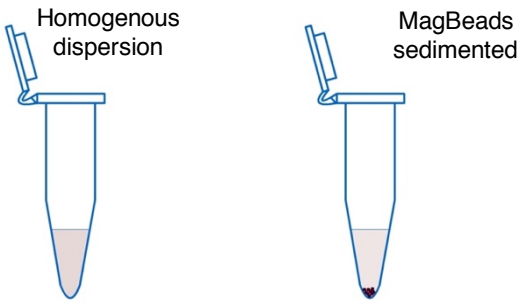
B. Sample purification

1.9 Remove samples from PCR instrument

1.10 Dilute 4×Bead Wash buffer 1:4 in mqH2O. 1 mL is provided with the kit and the whole buffer volume can be diluted at once

NOTE: Buffer can be stored at room temperature

1.11 Remove the MagBeads (magnetic beads) from the freezer and thaw the solution



1.12 Open all sample tubes and aliquot 5 µL of MagBeads into each sample tube

1.13 Close the PCR microcentrifuge tubes, vortex samples gently until the samples' color will indicate homogenous

MagBeads will sediment and should only be aliquoted from a homogenous solution. Vortex and spin MagBeads microcentrifuge tube gently to obtain homogenous aliquots in all assays. TB DNA genotyped during the Step1.A will be adsorbed on the MagBeads surface

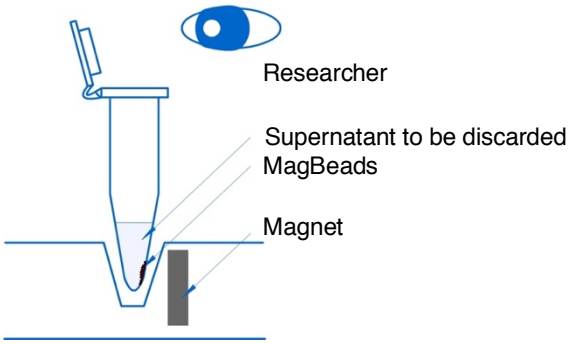
MagBeads dispersion (dark brown, no precipitate). Remove obvious bubbles

1.14 Incubate the MagBeads with samples for 5 minutes at room temperature on rotation (30 rpms are adequate)

NOTE: If a rotator is not available samples can be incubated on bench and vortexed gently every minute, for 5 minutes

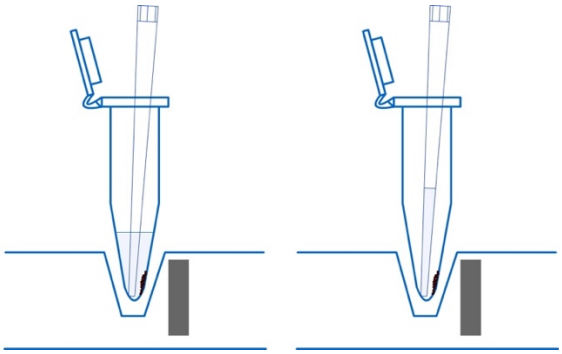
1.15 Transfer samples onto magnetic rack

NOTE: Magnets present inside the rack will concentrate the magnetic beads on the side of the PCR microcentrifuge tube. Wait one minute until all beads are concentrated on the side of the tube



1.16 Remove and discard the whole 25 µL of a supernatant volume with pipette.

NOTE: We recommend positioning the pipette tip at the bottom of the PCR tube. Work carefully not to disturb the MagBeads pellet present on the side of the tube

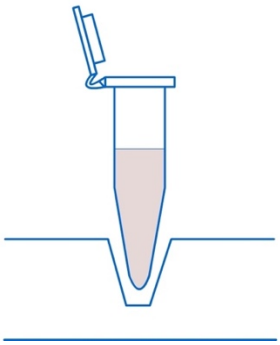


1.17 Transfer samples back onto regular, non-magnetic rack

1.18 Add 100 µL of the diluted 1×BeadWash buffer into each sample

NOTE: Electronic dispenser or multichannel pipette can be used to speed up work

1.19 Close the PCR microcentrifuge tubes, vortex gently until the samples color will indicate homogenous MagBeads dispersion (light brown, no precipitate)



2. Amplification

2.1 Thaw the Buffer2

2.2 Prepare the Step2 working solution in 1.5 mL microcentrifuge tube. Include 10% of additional assay volume to compensate for reagent loss

NOTE: Step2 working solution volume is 20 µL. For a single assay, mix 19.6 µL of Buffer2 with 0.4 µL of Enzyme2 (Table2). For the presented example, multiply the volumes required according to the Table 2

Step2			Volume added		
	Stock concentration	Final concentration	Single assay	8.8 assays (8+10%)	Your assay
Buffer2	1 x	1 x	19.6 µL	172.48 µL	
Enzyme2+4	-	-	0.4 µL	3.52 µL	
		mix volume	20 µL		
		total volume	20 µL		

Table 2

2.2 Transfer samples onto magnetic rack

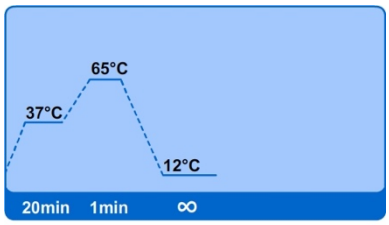
2.3 After the beads have aggregated on the magnet, remove supernatant from all samples

2.4 Transfer samples onto regular, non-magnetic rack

2.5 Aliquot 20 µL of Step2 working solution into all the tubes

2.6 Close the PCR microcentrifuge tubes, vortex gently until the samples' color will indicate homogenous MagBeads dispersion (dark brown, no precipitate)

2.7 Load samples into PCR instrument and execute the following protocol:



3. Digestion

3.1 Thaw the Buffer3

3.2 Prepare the Step3 working solution in 1.5 mL microcentrifuge tube. Include 10% of additional assay volume to compensate for reagent loss

NOTE: Step 3 working solution volume is 5 µL. For a single assay, mix 4.7 µL of Buffer3 with 0.3 µL of Enzyme3+5 (Table3). For the presented example, multiply the volumes required according to the Table 3

Step3			Volume added		
	Stock concentration	Final concentration	Single assay	8.8 assays (8+10%)	Your assay
Buffer3	1x	1x	4.7 µL	41.36 µL	
Enzyme3+5	-	-	0.3 µL	2.64 µL	
		mix volume	5 µL		
		total volume	25 µL		

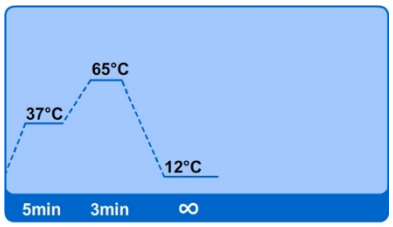
Table 3

3.3 Remove samples from the PCR instrument

3.4 Aliquot 5 µL of Step3 working solution into all the tubes

3.5 Close the PCR microcentrifuge tubes, vortex gently until the samples color will indicate homogenous MagBeads dispersion (dark brown, no precipitate)

3.6 Load samples into PCR instrument and execute the following protocol:



After this step, TB DNA adsorbed on the magnetic beads is released to the solution. Magnetic beads will be discarded, and supernatants transferred to clean PCR microcentrifuge tubes

3.7 Remove samples from PCR instrument
3.8 Transfer samples onto magnetic rack
3.9 Prepare required number of PCR microcentrifuge tubes reflecting number of assays on non-magnetic rack
3.10 Label the PCR microcentrifuge tubes
3.11 After the beads have aggregated, transfer supernatants into new PCR microcentrifuge tubes and discard the tubes from the magnetic rack with the MagBeads.

Samples can be stored in the freezer at this point

4. Second amplification

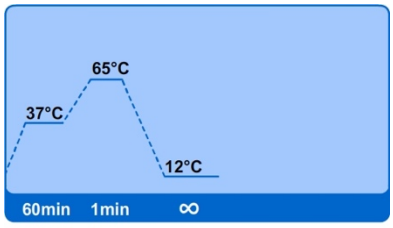
4.1 Thaw the Buffer4
4.2 Prepare the Step4 working solution in 1.5 mL microcentrifuge tube. Include 10% of additional assay volume to compensate for reagent loss

NOTE: Step4 working solution volume is 10 µL. For a single assay, mix 9.2 µL of Buffer 4 with 0.7 µL of Enzyme2+4 and 0.1 µL of Enzyme4 (Table3). For the presented example, multiply the volumes required according to the Table 3

Step4			Volume added		
	Stock concentration	Final concentration	Single assay	8.8 assays (8+10%)	Your assay
Buffer4	1x	1x	9.2 µL	80.96 µL	
Enzyme2+4	-	-	0.7 µL	6.16 µL	
Enzyme4	-	-	0.1 µL	0.88 µL	
		mix volume	10 µL		
		total volume	35 µL		

Table 4

4.3 Remove samples from PCR instrument
4.4 Aliquot 10 µL of Step4 working solution into all the tubes
4.5 Close the PCR microcentrifuge tubes, vortex gently
4.6 Load samples into PCR instrument and execute the following protocol:



5. Second digestion

5.1 Thaw the Buffer5

5.2 Prepare the Step5 working solution in 1.5 mL microcentrifuge tube. Include 10% of additional assay volume to compensate for reagent loss

NOTE: Step5 working solution volume is 5 µL. For a single assay, mix 4.2 µL of Buffer5 with 0.8 µL of Enzyme3+5 (Table5). For the presented example, multiply the volumes required according to the Table 5

Step5			Volume added		
	Stock concentration	Final concentration	Single assay	8.8 assays (8+10%)	Your assay
Buffer5	1x	1x	4.2 µL	36.96 µL	
Enzyme3+5	-	-	0.8 µL	7.74 µL	
		mix volume	5 µL		
		total volume	40 µL		

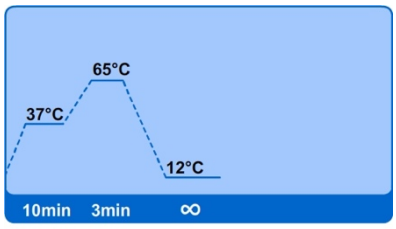
Table 5

5.3 Remove samples from the PCR instrument

5.4 Aliquot 5 µL of Step5 working solution into all the tubes

5.5 Close the PCR microcentrifuge tubes, vortex gently

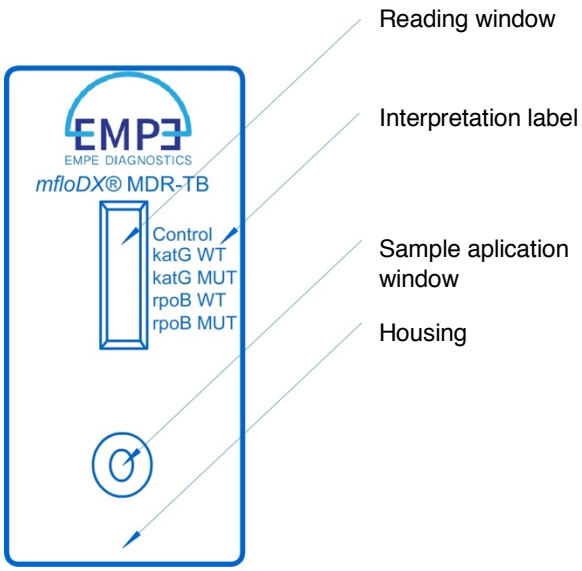
5.6 Load samples into PCR instrument and execute the following protocol:



Introductory notice to signal development

After the second digestion, reagents are ready to be applied on the disposable *mfloDX*® MDR-TB cassette. *mfloDX*® MDR-TB cassette consists of lateral flow nitrocellulose membrane inside the plastic housing. Sample application window is localized at the bottom of the cassette, below the reading window. Interpretation label is attached on the right side of the cassette next to the reading window to assist with the signal interpretation. Visual signal is obtained by mixing samples with two Visualization Solutions and after short incubation, dispensing whole sample volumes into the sample application

window. Signals typically develop within 1–2 minutes, however, up to 10 minutes are needed for the signal to develop fully.



Signal development reagents should be mixed and applied in a ventilated chemical cabinet while wearing a protective lab coat

6. Signal development

6.1 Prepare the number of *mfloDX*® MDR-TB cassettes equivalent to number of samples used in the experiment

6.2 Remove the cassettes from aluminum pouches and place cassettes in the chemical cabinet

NOTE: Pouch and a desiccant bag can be discarded to a regular trash

6.3 Label the cassettes in accordance with the sample naming

6.4 Remove Visualization solution1 from the fridge, thaw Visualization solution2 and place both solutions inside the chemical cabinet

NOTE: Gold particles present in the Visualization solution 2 can precipitate. Flick a tube and gently vortex prior use

6.5 Transfer samples from the PCR machine inside the cabinet

6.6 Aliquot 13.5 µL of the Visualization solution1 into each sample

6.7 Aliquot 15 µL of the Visualization solution2 into each sample

6.8 Incubate samples for 2 minutes

6.9 For every sample, aspirate total sample volume (68.5 µL) inside the sample application window on the corresponding cassette

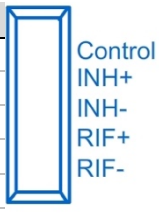
6.10 Interpret the results after 10 minutes

7. Signal interpretation

Successful asssay definition

The *mfloDX*® MDR-TB assay produces clear dark-red bands inside the cassettes that are visible inside the sample reading window. Configuration of the bands can be correlated with the interpretation label to conclude which mutations were present in the *Mycobacterium tuberculosis* DNA used in the experiment.

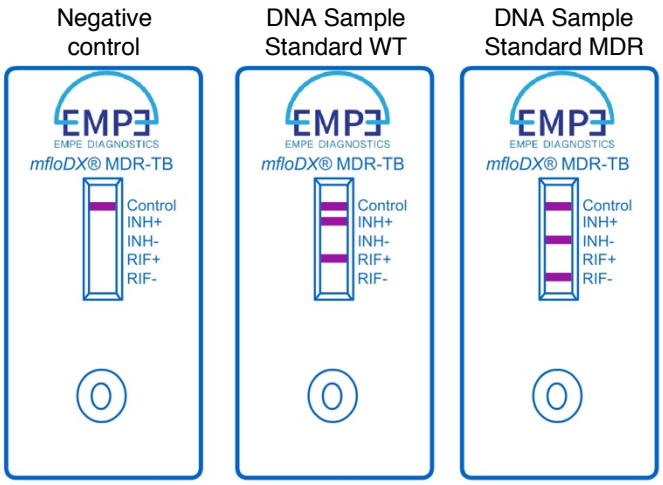
Line position	Label	Genotype	Interpretation
1	Control	-	Cassette and liquid flow control
2	INH+	katG315 WT	Bacteria susceptible to INH
3	INH-	katG315 ACC	Bacteria resistant to INH
4	RIF+	rpoB 531 WT	Bacteria susceptible to RIF
5	RIF-	rpoB 531 TTG	Bacteria resistant to RIF



Assay is considered to be executed properly if 3 bands are visible in the reading window. For negative control (where mqH2O was used instead of a DNA sample) only a *Control* band at position 1 should be present. For samples where a TB DNA was used, two additional bands are expected to develop.

Examples

DNA Sample Standard WT was prepared to develop the result expected for wild type TB bacteria. Conversely, DNA Sample Standard MDR should develop signal expected for multidrug resistant bacteria (resistant to INH and RIF).



8. Signal interpretation troubleshooting

Observation	Possible explanation	Action
No bands were developed	Visualization solution is not working	Make sure you are developing the signals in accordance with the presented protocol. Contact EMPE Diagnostics AB in observation is reproducible
No signal visible for DNA samples and sample standards but Control band is visible	Some steps of the protocol failed	Make sure the reagents were stored as recommended. Repeat the assay.
No signal visible for DNA samples but visible for sample standards	DNA samples used for the assay were not appropriate, DNA was degraded or inhibitors present in the reaction	Make sure that DNA used was obtained via heat lysis method form TB bacteria after 4-6 weeks of culture in Löwenstein–Jensen medium. DNA preparations should be conducted in DNAse free environment with certified DNAse free disposable tubes. Also, make sure the DNA was fragmented as described in the protocol
Both mutation and corresponding wild type signal are developed on the strip	It is possible that the TB DNA used was obtained from the heteroresistant strain. Also, it is possible that the original specimen contained more than one strain of TB. It is also possible that DNA was contaminated with a DNA from another specimen	

The *mfloDX*® technology relies on DNA amplification. Considering the myriad of MTB genetic variants is it possible that for some strains the selected mutations will not be detected. This qualitative test detects a DNA from both viable and non-viable bacteria. Considering this as well as multiple DNA amplification steps, the test cannot be used to quantitatively monitor progression of TB or be correlated to the original number of bacteria in the sample. Not all the bands on the strip have be developed equally. rpoB WT signal is often weeker compared to other lines of the strip.

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

9. Purchaser notification

Materials and reagents provided with the *mfloDX*® MDR-TB 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.

Obtaining Support

For help services please contact support@empediagnostics.com,
and for further information visit www.empediagnostics.com.

CONTACT INFORMATION

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