Instructions for Use (IFU)

Product Name: Pli-RCA TB RIF/INH LF AMP Kit
(To be used along with the Pli-RCA TB RIF/INH LF VIS Kit)
Molecular test kit for Detection of *Mycobacterium tuberculosis* and resistance to rifampicin and isoniazid.

Catalogue Number: Pli-RCA TB-AMP-25

Pack Size: 25 reactions

For Research use only (RUO)
NOT for diagnostic or clinical use

RUO

1. General Information

1.1.1. Brief Description of the Product

The Pli-RCA TB RIF/INH LF test is a qualitative *in vitro* test for the identification of *Mycobacterium tuberculosis* complex (MTC) and resistance to rifampicin and isoniazid from culture positive as well as 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 Pli-RCA TB RIF/INH LF test Kit consists of the amplification (Pli-RCA TB RIF/INH LF AMP Kit) and signal development kit (Pli-RCA TB RIF/INH LF VIS Kit).

The Pli-RCA TB RIF/INH LF 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>Pli-RCA TB RIF/INH LF 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>

1.1.2. Intended Use

The Pli-RCA TB RIF/INH LF AMP Kit is a qualitative molecular test used for amplification and detection of *Mycobacterium tuberculosis* and associated resistance genes to rifampicin and isoniazid. The assay is designed for research use only and is not intended for *in vitro* diagnosis. This kit must be used alongside the lateral flow detection kit **Pli-RCA TB RIF/INH LF VIS KIT**.

1.1.3. Principle of the Test

The Pli-RCA TB RIF/INH LF 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). Pli-RCA TB RIF/INH LF, can be used on culture positive samples or patient's sputum sample directly (without preceding culturing). The technique is easy to perform and has a turnaround time of 3 hours. Pli-RCA TB RIF/INH LF 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.

2. Product contents:

2.1.1. Kit Contents

The Kit contents supplied with the Pli-RCA TB RIF/INH LF 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

2.1.2. Storage and Handling:

- The Pli-RCA TB RIF/INH LF AMP Kit should be stored at -25 °C to -16 °C
- **The no. of freeze thaw cycles with the 25 test kit is 4. Beyond 4 times, the kit cannot be used and the results obtained beyond 4th cycle may not be considered for interpretation**

2.1.3. 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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2.1.4. 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)

2.1.5. 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.

3. Pre-amplification

3.1. 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 Pli-RCA TB RIF/INH LF cassettes to room temperature for equilibration.

3.2. **Prepare** the required number of PCR microcentrifuge tubes.

3.3. 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.

3.4. 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

3.5. Aliquot 15 µl of **Working solution 1** into all tubes.

3.6. Transfer 5 µl of DNA sample extracted from sputum to each PCR tube.

3.7. Close the PCR tubes, vortex gently and spin down in a microcentrifuge.

3.8. 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

4. Ligation

4.1 Prepare new microcentrifuge PCR tubes and label them accordingly.

4.2 Retrieve the tubes from the Thermocycler.

4.3 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.

4.4 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

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

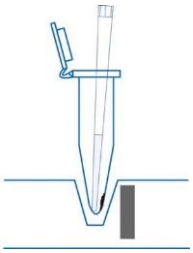
Important! *Although the assay contains reagents to eliminate accidental carry-over contamination from PCR product to future PCR reactions, one should take the utmost care to prevent contamination of PCR products to the laboratory environment. Change to a new pair of gloves after handling tubes containing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

4.8 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

5. Capture

5.1. Retrieve the samples from the Thermocycler.
5.2. 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.
5.3. 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.
5.4. Close the PCR microcentrifuge tubes and vortex gently until the color of the samples indicate homogenous **MAG** dispersion (dark brown).
5.5. Incubate samples with **MAG** for 5 minutes at room temperature.
5.6. Briefly spin down (~2 seconds) to remove any sample from the lids.
5.7. Move tubes to the Pli-RCA TB RIF/INH LF Magnetic Stand and let the magnetic beads collect at the magnet for approximately 10 seconds.
5.8. Using a pipette, carefully remove and discard all 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.*

5.9. 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*

5.10. 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 to remove any liquid from the tube lid and transfer to the Pli-RCA TB RIF/INH LF Magnetic Stand. Proceed to section 5.7 while allowing the beads to migrate.

6. RCA amplification

6.1 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.

6.2 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

6.3 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.*

6.4 Transfer 20.0 µl of **Working solution 3** to each tube.

6.5 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.

6.6 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**.

6.7 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

7. Digestion

7.1 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.

7.2 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

7.3 Retrieve samples from the thermocycler.
7.4 Transfer 5 µl of **Working solution 4** to each tube.
7.5 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.
7.6 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 Pli-RCA TB RIF/INH LF* VIS Kit *(Product code: TBRIFINH-VIS-25) referring to the IFU* (IFU/ EMPE TB RIF/INH -VIS)**

8. Performance Characteristics

8.1 Analytical Sensitivity

The analytical sensitivity of the Pli-RCA TB RIF/INH LF was evaluated using TB-negative sputum samples spiked with five serial dilutions of *Mycobacterium tuberculosis* H37Rv, ranging from 1000 CFU/mL to 200 CFU/mL.
The limit of detection (LOD) was determined to be **654.5 CFU/mL for ITS**, **516.7 CFU/mL for katG and inhA**, and **562.9 CFU/mL for rpoB**. When converted to CFU equivalents per PCR reaction, this corresponded to approximately **3.2 CFU for ITS**, **2.5 CFU for katG and inhA**, and **2.8 CFU for rpoB**.

8.2 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. The test did not show any cross reactivity with the tested NTMs:

9. 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.

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

Symbol	Description
REF	Product Catalogue Number
LOT	Product Lot/ Batch Number
	Use by date
	Storage conditions
	Manufacturer of the product
QTY	Quantity of the product filled in the vial
	Single Use Only
	Pack size
RUO	Research Use only
	Aspiration Hazard
	Important instruction
	Consult instructions for use
	Do not use if the package is damage
	Keep Dry

11. 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.

12. Purchaser notification

All materials and reagents provided with the Pli-RCA TB RIF/INH LF 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
Pli-RCA TB-AMP-25	25 reactions

13. Support

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

Corporate Headquarters
Stockholm, Sweden

Global Operations Center
Hyderabad, India

Instructions for Use (IFU)

Product Name: Pli-RCA TB RIF/INH LF VIS Kit
(To be used along with Pli-RCA TB RIF/INH LF AMP Kit)

Rapid test kit for detection of *Mycobacterium tuberculosis* and resistance genes to rifampicin and isoniazid

Catalogue Number: Pli-RCA TB-VIS-25

Pack Size: 25 reactions

For Research use only (RUO)

NOT for diagnostic or clinical use

RUO

1. General information

1.1. Brief Description of the Product

The Pli-RCA TB RIF/INH LF 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. Pli-RCA TB RIF/INH LF is designed to be performed with minimal and standard laboratory equipment and infrastructure using standard thermal cycler, heat block, microfuge, and vortex mixer.

The EMPE TB RIF/INH (LF) test Kit consists of the amplification (Pli-RCA TB RIF/INH LF AMP Kit) and signal development kit (Pli-RCA TB RIF/INH LF VIS Kit).

The Pli-RCA TB RIF/INH LF 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>
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<tr><th>Gene</th><th>Pli-RCA TB RIF/INH LF targets</th></tr>
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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>
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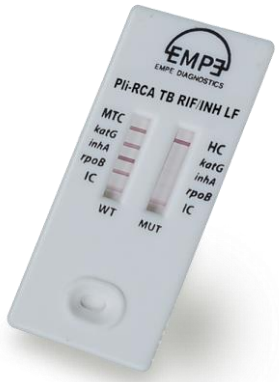


</td></tr>
<tr><td>Controls</td><td>There are two controls: (1) IC: Internal Control will not show up if there is any issue right from the sample preparation till the signal development. (2) HC: Hybridization Control in an internal quality control for the visualization reagent and the lateral flow cassette.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

1.2. Intended Use

The visualization test for EMPE TB RIF/INH (LF) kit is a qualitative molecular test for the detection of *Mycobacterium tuberculosis* and its associated resistance genes to rifampicin and isoniazid. The assay is designed for research use only and is not intended for *in vitro* diagnosis.

This kit must be used alongside the molecular test **Pli-RCA TB RIF/INH LF AMP KIT.**

1.3. Principle of the Test

The Pli-RCA TB RIF/INH LF 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 Pli-RCA TB RIF/INH LF,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. Pli-RCA TB RIF/INH LF VIS Kit consists of the visualization solutions and lateral flow cassette to see the results visually.

2. Product contents

2.1. Kit Contents:

The Kit contents supplied with the Pli-RCA TB RIF/INH LF VIS Kit as detailed below:

Product Code	Name of the Product	Quantity supplied with the kit
2-0017	VIS-1	2 x 937.5 µl
2-0031	VIS-2	1 x 1.0 ml
2-0036	Cassettes	1 x 25 Nos

2.2. Storage and Handling:

- The Pli-RCA TB RIF/INH LF VIS Kit should be stored at +2 °C to +8 °C

2.3. 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.

2.4. Materials required but not supplied:

- Disposable gloves
- Adjustable pipettes for200, and 1000 µl
- Disposable sterile pipette tips with filter: 10, 20, 200, and 1000 µl
- 1.5 ml microcentrifuge tubes
- Digested Product from Pli-RCA TB RIF/INH LF AMP kit
- Magnetic stand
- Timer

***Note: Carry out the assay steps after completion of Section 6.6 using the Pli-RCA TB RIF/INH LF* AMP Kit *(product code: Pli-RCA TB-AMP-25) referring to the IFU (*IFU/Pli-RCA TB-AMP/00)**

3. Signal development

3.1. Prepare the number of cassettes equivalent to the number of samples used in the experiment.
3.2. Remove **VIS-1** from the fridge, vortex **VIS-2**, and place the two solutions inside the fume hood.

*Important! VIS-1 contains **formamide**, a Health Hazard GHS08 chemical with the following hazard class/categories. Please refer to the associated Safety Data Sheet (SDS) for more information :*

- Respiratory sensitization, category 1
- Germ cell mutagenicity, categories 1A,1B,2
- Carcinogenicity, categories 1A,1B,2
- Reproductive toxicity, categories 1A,1B,2
- Specific Target Organ Toxicity – Single exposure, categories 1,2
- Specific Target Organ Toxicity – Repeated exposure, categories 1,2
- Aspiration Hazard, category 1

3.3. Transfer samples from the thermocycler to the fume hood
3.4. Prepare a development solution by mixing 75 µl of **VIS-1** and 40 µl of **VIS-2** per sample. Vortex and spin down. Calculate 10 % of additional assay volume to compensate for reagent loss. See example in Table 9.

Table-9 Development Solution

Component	Volume required for single assay
VIS-1	75 µl
VIS-2	40 µl
Solution volume	115 µl
Digested RCA product	25 µl
Final volume	140 µl

3.5. Aliquot 115 µl of development mix (**VIS-1**+**VIS-2**) into each sample.

*NOTE: The **Development solution** must be freshly prepared.*

3.6. Vortex (~3 seconds) and briefly spin down (~2 seconds)
3.7. Move the tubes to the Pli-RCA TB RIF/INH LF Magnetic Stand and let the magnetic beads collect at the magnet for approximately 10 seconds.
3.8. Aspirate 136 µl per sample and apply onto the cassette application window and start a 10 minutes timer.
3.9. After 10 minutes, inspect the cassette as described in section 8.

4. Signal interpretation

The Pli-RCA TB RIF/INH LF assay produces clear pink bands that are visible inside the sample reading windows of the cassette. As shown in Figure 1, bands represent detection of bacteria in the MTC, genotypes of resistance determining regions (in the genes *katG*, *inhA* and *rpoB*), Internal Control (IC) and Hybridization Control (HC).

The window on the left-hand side (marked as "WT") is dedicated for bands indicating MTC identity and detection of the wild-type allele of the resistance-coding gene. The right-hand side window (marked as MUT) is dedicated for bands indicating detection of mutations in the respective genes and the HC. The successful amplification of the Internal Control is indicated by the bands at the bottom of each window. A summary of the possible outcomes is given in Tables 10-11 at the end of this section.

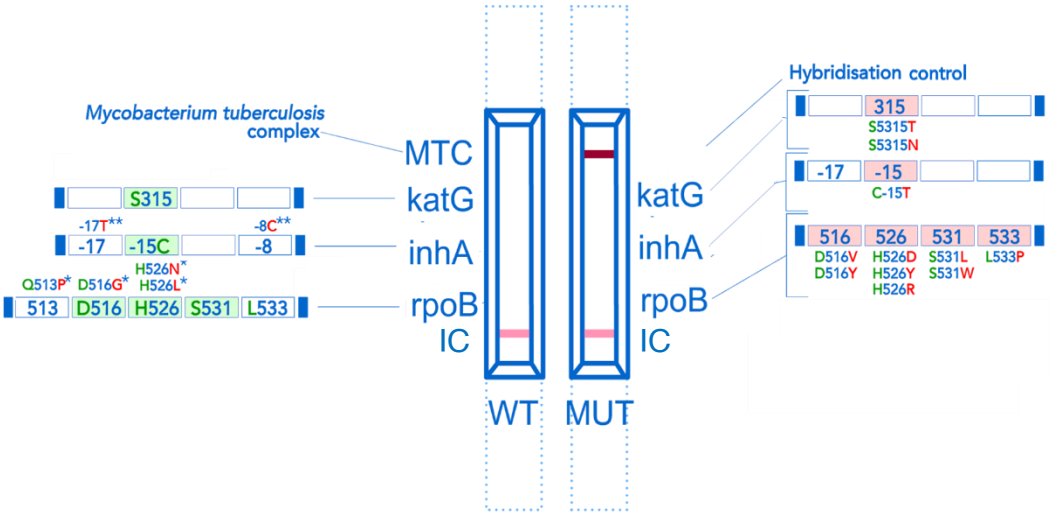
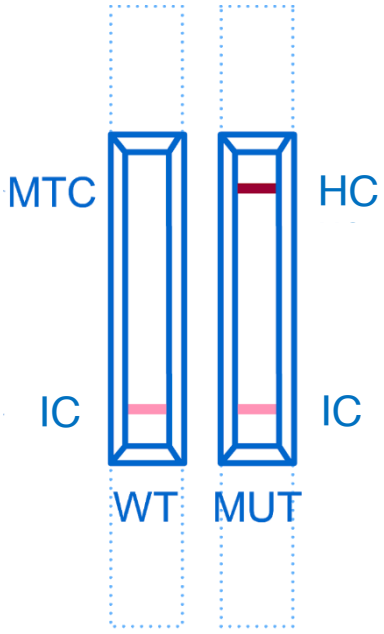


Figure 1. Overview of genomic loci represented by the respective bands of the Pli-RCA TB RIF/INH LF test.

Mycobacterium tuberculosis complex (MTC)

The Pli-RCA TB RIF/INH LF assay was designed to detect bacteria belonging to the MTC. The following species were confirmed to give signal in the test: *Mycobacterium tuberculosis, Mycobacterium africanum, Mycobacterium bovis, Mycobacterium microti, Mycobacterium canetti.* If the detected MTC bacteria are drug sensitive, wild type bands for all genes typically appear. For bacteria with drug resistance, one of several possible line patterns will appear, depending on the resistance profile.



If the MTC zone does not produce any visible signal, the tested sample does not contain bacteria belonging to the MTC. Following non-tuberculosis mycobacteria were shown not to produce any signal: *Mycobacterium chelonae, Mycobacterium gordonae, Mycobacterium kansasii, Mycobacterium avium, Mycobacterium fortuitum, Mycobacterium intracellulare, Mycobacterium xenopi, Mycobacterium interjectum, Mycobacterium malmoense, Mycobacterium abscessus, Mycobacterium marinum.*

4.1. Internal Control (IC)

The IC guards against false negatives due to an invalid test. When the test is performed correctly, the IC bands on both strips should develop. However, compromised test reagents or extreme levels of PCR inhibitors in the sample can cause the IC to fail. If the MTC is negative in these situations, the test should be interpreted as invalid.

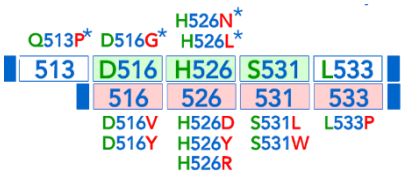
A positive MTC signal may also outcompete the IC. This has no impact on the test results since a positive result also validates the test.

4.2. Hybridization control (HC)

The HC band on the MUT strip should develop in every experiment during the signal development process. This indicates proper sample flow and function of the visualization chemicals. Depending on the development of the other bands, signal intensity of the HC band can vary between samples.

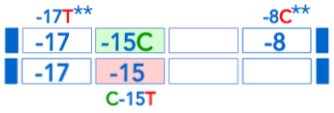
4.3. rpoB – wild type and mutation signals

A positive *rpoB* band on the WT side of the cassette indicates wild type variants of codon 516, 526, 531 (highlighted in green) in the rifampicin resistance determining region of *rpoB* gene. If a mutation is detected in any of the codons 516, 526, 531 or 533, a single band should appear in *rpoB* detection zone on the MUT side of the cassette. The test is designed to produce the signal if any of the mutations is present. Most common mutations in the *rpoB* gene, producing visible signal on the test, are listed in the figure in the margin.



4.4. inhA – wild type and mutation signals

A positive *inhA* band on the WT side of the cassette indicates wild type versions of -15 nucleotide of the *inhA* promoter (-15C, highlighted in green). If a -15C/T substitution (highlighted in red) is detected in this position, a band will appear in the *inhA* detection zone on the MUT side of the cassette.



4.5. katG – wild type and mutation signals

A positive *katG* band on the WT side of the cassette indicates wild type versions of 315 codon of the *katG* gene (highlighted in green). If a mutation is detected in codon 315, a band will appear in *katG* detection zone on the MUT side of the cassette. The test is designed to produce the signal if any of the mutations is present. The most common mutations in the *katG* 315 codon, producing visible signal on the test, are S315T and S315N.

Table 10. Expected Pli-RCA TB RIF/INH LF readout patterns and their respective interpretations of resistance indication.

MTC	WT			MUT					Interpretation
	rpoB	*katG*	*inhA*	*rpoB*	*katG*	*inhA*	HC	IC	
-	-	-	-	-	-	-	+	+	MTC not detected
+	+	+	+	-	-	-	+	+/-*	MTC detected /RIF & INH Sensitive
+	-	-	+	+	+	-	+	+/-*	MTC detected /RIF & INH Resistant
+	-	+	-	+	-	+	+	+/-*	MTC detected /RIF & INH Resistant
+	-	-	-	+	+	+	+	+/-*	MTC detected /RIF & INH Resistant
+	-	+	+	+	-	-	+	+/-*	MTC detected /RIF Resistant & INH Sensitive
+	+	-	+	-	+	-	+	+/-*	MTC detected /RIF Sensitive & INH Resistant
+	+	+	-	-	-	+	+	+/-*	MTC detected /RIF Sensitive & INH Resistant
+	+	-	-	-	+	+	+	+/-*	MTC detected /RIF Sensitive & INH Resistant

*When MTC is Pos, IC may be either Pos or Neg

Table 11. Examples of Indeterminate, Invalid or Hetero-resistant readout patterns of Pli-RCA TB RIF/INH LF

MTC	WT			MUT					Interpretation
	rpoB	*katG*	*inhA*	*rpoB*	*katG*	*inhA*	HC	IC	
+	-	-	+	-	+	-	+	+/-*	MTC detected /RIF Indeterminate & INH Resistant
+	-	-	+	+	-	-	+	+/-*	MTC detected /RIF Resistant & INH Indeterminate
+	+	+	-	-	-	-	+	+/-*	MTC detected /RIF Sensitive & INH Indeterminate
+	+	+	+	+	-	-	+	+/-*	MTC detected /RIF Hetero-resistant & INH Sensitive
+	+	+	+	-	-	+	+	+/-*	MTC detected /RIF Sensitive & INH Hetero-resistant
-	-	-	-	-	-	-	+	-	Invalid
-	-	-	-	-	-	-	-	-	Invalid

*When MTC is Pos, IC may be either Pos or Neg

The Pli-RCA TB RIF/INH LF technology relies on DNA amplification. Considering the multitude of MTB genetic variants, it is possible that for some strains the selected mutations will not be detected. This qualitative test can detect 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. All the bands on the strips do not have to be developed equally.

5. Performance Characteristics

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

5.1. Analytical Performance Characteristics:

The analytical performance characteristics of Pli-RCA TB RIF/INH LF test was conducted in-house on the stored sputum samples.

5.2. Analytical Sensitivity:

The analytical sensitivity of the Pli-RCA TB RIF/INH LF was evaluated using TB-negative sputum samples spiked with five serial dilutions of Mycobacterium tuberculosis H37Rv, ranging from 1000 CFU/mL to 200 CFU/mL.

The limit of detection (LOD) was determined to be 654.5 CFU/mL for ITS, 516.7 CFU/mL for katG and inhA, and 562.9 CFU/mL for rpoB. When converted to CFU equivalents per PCR reaction, this corresponded to approximately 3.2 CFU for ITS, 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 Pli-RCA TB RIF/INH LF 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

6. Discard of Kit components and Packaging materials

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

6.2. Discard the plastic cassettes into red color biohazard bag and follow local regulations for discarding biohazard waste.

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

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

Symbol	Description
REF	Product Catalogue Number
LOT	Product Lot/ Batch Number
	Use by date
	Storage conditions
	Manufacturer of the product
QTY	Quantity of the product filled in the vial
	Single Use Only
	Pack size
	Aspiration Hazard
	Important instruction
	Consult instructions for use
	Do not use if the package is damage
	Keep Dry
RUO	Research Use only

8. References

8.1. Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, USA 1985.

8.2. Isenberg HD. Clinical microbiology procedures handbook. American Society for Microbiology, Washington, D.C., USA 1992.

8.3. Global Laboratory Initiative. Mycobacteriology Laboratory Manual. Available from: https://www.who.int/tb/laboratory/mycobacteriology-laboratory-manual.pdf last accessed on November 26, 2021.

9. Purchaser notification

All materials and reagents provided with the Pli-RCA TB RIF/INH LF VIS 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	Packsize
Pli-RCA TB-VIS-25	25 reactions

10. Support

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

Corporate Headquarters
Stockholm, Sweden

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