INNO-LiPA Rif.TB

RUO

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BIOTECHNOLOGY FOR HEALTHCARE

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Symbols used

	Manufactured by
RUO	For research use only Not for use in diagnostic procedures
LOT	Lot number
REF	Catalog number
	Use by

*INNOGENETICS® is a Registered Trademark of Innogenetics N.V.

	Consult instructions for use
	Temperature limits
	Contains sufficient for < X > tests
STRIPS	Strips
CONJ 100x	Conjugate 100x
CONJ DIL	Conjugate Diluent
DENAT SOLN	Denaturation Solution
HYBRIDIZ SOLN	Hybridization Solution
RINSE SOLN 5x	Rinse Solution 5x
SUBS BCIP/NBT 100x	Substrate BCIP/NBT 100x
SUBS BUF	Substrate Buffer
STRIN WASH SOLN	Stringent Wash Solution

English

Intended use

The INNO-LiPA Rif.TB is a line probe assay (LiPA) for *in vitro* use, which allows for the detection of *Mycobacterium tuberculosis* complex and the detection of wild types and mutations in the *rpo*B gene region related to rifampicin resistance, after amplification of DNA extracted from grown bacteria on solid media.

Resistance to rifampicin is caused by any nucleotide mutation giving rise to an amino-acid change in the gene encoding for the ß-subunit of the RNA polymerase (*rpo*B). These mutations are mainly located in a relevant part (codon 509 to 534) of the *rpo*B gene.[1]

Test principle

The INNO-LiPA Rif.TB is based on the reverse hybridization principle. Amplified biotinylated DNA material is hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips. After hybridization, streptavidin labeled with alkaline phosphatase is added and bound to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen results in a purple/brown precipitate, and results can be visually interpreted.

To perform the INNO-LiPA Rif.TB assay, amplification of the rifampicin resistance region of the *rpo*B gene should be performed (see INNO-LiPA Rif.TB Amplification instructions for use).

Using the amplicon generated by the INNO-LiPA Rif.TB amplification as target, the INNO-LiPA Rif.TB assay detects the presence of a pathogen belonging to the *M. tuberculosis* complex by the presence of a specific *M. tuberculosis* complex probe (MTB) on strip. Simultaneously, the presence of point mutations, insertions and deletions in the relevant part of the *rpo*B gene (codon 509 to 534) is detected by a set of five overlapping S-probes that span the relevant region of the gene. These S-probes exclusively hybridize to the wild-type sequence. If a mutation is present in one of the target regions, the mismatch created will prevent the amplicon from hybridizing with the corresponding probe under the stringent conditions applied. Consequently, the absence of a hybridization probe is indicative of the presence of a mutation and hence implies a resistant genotype.
In addition, the presence of four particular mutations is confirmed by four corresponding R-probes (R2 = D516V, R4a = H526Y, R4b = H526D, R5 = S531L).

INNO-LiPA Rif.TB: steps involved

step 1	Amplification of the rifampicin resistance region of the rpoB gene (INNO-LiPA Rif.TB amplification).
step 2	Hybridization of the biotinylated amplicon to the strip, followed by stringent wash.
step 3	Addition of conjugate and substrate, resulting in color development.
step 4	Visual interpretation of the signal pattern.

Reagents

Description, preparation for use and recommended storage conditions

- When stored at 2 - 8°C (opened as well as unopened, and stored in the original vials) all reagents, including the coated test strips, are stable until the expiry date of the kit. **Do not freeze any of the reagents.** Do not use the reagents beyond the expiry date of the kit.
- The kit should be stored isolated from any source of contaminating DNA, especially amplified DNA products.
- All reagents and the plastic tubes containing the test strips should be brought to room temperature (20 - 25°C) approximately 30 minutes before use and should be returned to the refrigerator immediately after use.
- Alterations in physical appearance of the kit components may indicate instability or deterioration.
- To minimize the possibility that strips curl before use, it is recommended to store the tube horizontally.

Reagents supplied:

Component	Quantity	Ref.	Description
Strips	1 x 20	51376	Containing 20 INNO-LiPA Rif.TB strips marked with a red Marker Line.
Denaturation Solution	1 x 1 ml	56828	Alkaline solution containing EDTA. This vial should be closed immediately after use; prolonged exposure of this solution to air leads to a rapid deterioration of the denaturing strength.
Hybridization Solution	1 x 80 ml	56645	SSC buffer containing 0.1% SDS, to be pre-warmed to a temperature of at least 37°C and not exceed 62°C.
Stringent Wash Solution	1 x 200 ml	56646	SSC buffer containing 0.1% SDS, to be pre-warmed to a temperature of at least 37°C and not exceed 62°C.
Rinse Solution 5x	1 x 80 ml	56831	Phosphate buffer containing NaCl, Triton® and 0.05% MIT/0.5% CAA as preservative, to be diluted 1:5 in distilled water before use. Prepare 4 ml diluted Rinse Solution for each test trough + 5 ml in excess for manual testing. For *Auto*-LiPA, prepare 12 ml diluted Rinse Solution for each test trough + 20 ml in excess.

			The Rinse working solution is stable for 2 weeks at 2 - 8°C.
Conjugate Diluent	1 x 80 ml	56833	Phosphate buffer containing NaCl, Triton, protein stabilizers and 0.01 % MIT/ 0.1% CAA as preservative.
Conjugate 100x	1 x 0.8 ml	56832	Streptavidin labeled with alkaline phosphatase in Tris buffer containing protein stabilizers and 0.01% MIT/ 0.098% CAA as preservative, to be diluted 1:100 in Conjugate Diluent. Prepare 1 ml diluted Conjugate for each test trough + 1 ml in excess for manual testing. For *Auto*-LiPA, prepare 2 ml diluted Conjugate for each test trough + 10 ml in excess. The Conjugate working solution is stable for 24 hours at room temperature (20 - 25°C) when stored in the dark.
Substrate Buffer	1 x 180 ml	56835	Tris buffer containing NaCl, $MgCl_2$, and 0.01 % MIT/0.1% CAA as preservative.
Substrate BCIP/NBT 100x	1 x 0.8 ml	56834	BCIP and NBT in DMF, to be diluted 1:100 in Substrate Buffer. Prepare 1 ml diluted Substrate for each test trough + 1 ml in excess for manual testing. For *Auto*-LiPA, prepare 2 ml of diluted Substrate for each test trough + 10 ml in excess. The Substrate working solution is stable 24 hours at room temperature (20 - 25°C) when stored in the dark.
Incubation tray	2	-	Containing 12 troughs each.
Reading card	1	-	For identification of positive lines.
Data reporting sheet	2	-	For storage of developed strips.

Materials required but not provided

- INNO-LiPA Rif.TB amplification kit.
- Water bath with shaking platform (80 rpm; with inclined lid; temperature adjustable to minimum 62°C ± 0.5°C).
- Aspiration apparatus.
- Calibrated thermometer.
- Distilled or deionized water.
- Disposable gloves.
- Disposable sterile pipette tips (preferably cotton-plugged).
- Tweezers for strip handling.
- Graduated cylinders (10, 25, 50, and 100 ml).

- Orbital, reciprocal or rocking platform shaker.
  Recommendations
  For an orbital shaker:
  - the diameter of the circular motion should be equal or superior to 13 mm
  - recommended speed for a 13 mm circular motion is 160 rpm.

  For a reciprocal shaker:
  - recommended speed for the to and from motion is 80 movements per minute.

  For a rocking platform shaker:
  - the shaking angle should not exceed 13° to avoid spilling of liquid
  - recommended speed is 50 rpm.
- Adjustable pipettes to deliver 1 - 20 µl, 20 - 200 µl, and 200 - 1000 µl.
- Dispensing Multipipette (Eppendorf, optional).
- Timer, 2 hours (± 1 minute).
- Vortex mixer or equivalent.

Safety and environment

- **Please refer to the Material Safety Data Sheet (MSDS) and product labelling for information on potentially hazardous components. The most recent MSDS version is available on the website www.innogenetics.com.**

 R20/21, R36, R61, S36/37, S45, S53

Toxic! (T) Harmful by inhalation and in contact with the skin. Irritating to eyes. May cause harm to the unborn child.
Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). Avoid exposure - obtain special instructions before use. Restricted to professional users.
Contains Dimethylformamide, 5-Bromo-4-chloro-3-indolyl phosphate p-Toluidine salt: Substrate BCIP/NBT 100x.


R43, S24-37

Irritant! (Xi) Avoid contact with skin. May cause sensitization by skin contact. Wear suitable gloves. **Contains 2-Chloroacetamide:** Rinse Solution, Substrate Buffer and Conjugate Diluent.


R34, S28-36/37/39-45

Corrosive! (C) Causes burns. After contact with skin, wash immediately with plenty of soap and water. Wear suitable protective clothing, gloves, and eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

- Specimen should be handled as potentially infectious. Therefore, all biological materials should be considered as being potentially infectious and should be handled as such. Only adequately trained personnel should be permitted to perform the test procedure. All biological materials should be disposed of in accordance with established safety procedures.
  - Autoclave for at least 15 minutes at 121°C.
  - Incinerate disposable material.
  - Mix liquid waste with sodium hypochlorite so that the final concentration is ± 1% sodium hypochlorite. Allow to stand overnight before disposal. CAUTION: Neutralize liquid waste that contains acid before adding sodium hypochlorite.
- Use of personal protective equipment is necessary: gloves and safety spectacles when manipulating dangerous or infectious agents.
- Waste should be handled according to the institution's waste disposal guidelines. All federal, state, and local environmental regulations should also be observed.

Specimens

Specific sample requirements for DNA amplification are provided with the package insert for INNO-LiPA Rif.TB Amplification. Since biotinylated amplified DNA material has to be used as target for INNO-LiPA Rif.TB, an amplification kit, utilizing biotinylated

oligonucleotide primers, is available as an accompanying tool for the amplification of the rifampicin resistance region of the *rpo*B gene of *M. tuberculosis* complex.

Remarks and precautions

- Do not mix reagents with different lot numbers.
- Avoid microbial contamination of reagents.
- Use tweezers to handle strips. Do not touch strips with your hands as the oils from your hands could interfere with hybridization and color development.
  Use only pencil to write on the strips. The assay reagents may remove ink from the strips.
- Make sure that the test strips are placed in the troughs with the side bearing the coated membrane facing upwards (this side is marked).
- The strips are designed to be used only once!
- All vessels used to prepare Conjugate and Substrate working solutions should be cleaned thoroughly and rinsed with distilled water.
- Use a new sterile pipette tip (cotton-plugged) for each specimen.
- To prevent PCR contamination, maximize the physical separation of the pre- and post-amplification steps.
  Do not return samples, equipment, or reagents to the area where you performed the previous step. If you need to return to a previous work area, first perform the appropriate anti-contamination safeguards.
- For *in vitro* use only.
- For professional use only.
- The use of autoclaved disposable lab material is recommended.
- Do not reuse disposable lab material.
- Do not reuse troughs.

Test procedure

Please read 'Remarks and precautions' before performing the test.

NOTE:
- Throughout the different incubation steps, the test strips should always remain in the same trough.

- **Before incubation, check the temperature of the water bath using a calibrated thermometer, and adjust the temperature if necessary before placing the tray in the water bath.**
  Always close the lid.

Procedural notes

- Incubation at **exactly 62.0°C ± 0.5°C** during hybridization and stringent wash are the most critical steps in avoiding false positive (temperature too low) or false negative/very weak signals (temperature too high). A shaking water bath with a **closed** lid (80 rpm) allows a good control of temperature variations. Strict temperature control (within 0.5°C from the set point of 62°C) with a calibrated thermometer is necessary.
  The shaking of the solutions over the strips is critical in achieving maximum sensitivity and homogeneous staining. During shaking, the strip surface should be completely submerged. Do not use a hot air shaker.
- The amplitude of the motion generated by both the shaking water bath (hybridization procedure) and the orbital, reciprocal shaker or rocking platform (color development procedure) is critical in achieving maximum sensitivity and homogeneous staining.
  The amplitude should be as high as possible. However, spilling of liquid over the edges of the troughs must be avoided.
  The hybridization and stringent wash incubation steps should be performed in a shaking water bath (80 rpm, with closed lid).
  All 20 - 25°C incubation steps should be performed using an orbital shaker (160 rpm) or a rocker shaker (50 rpm).
- For hybridization and stringent wash, the troughs should be placed on the shaking platform of the water bath. Adjust the water level between 1/3 and 1/2 of the height of the trough. Make sure that the troughs do not float on the water.
  The water should be in direct contact with the troughs.
- Shaking during incubation of the strips should be performed in such a way that both the liquid and the test strips move back and forth in the trough, without liquid being spilled over the edge of the troughs.
- Always **close** the lid of the water bath during incubations in order to avoid false positive signals.

- Do not cover the tray. During the hybridization and stringent wash incubations, the troughs can be left uncovered in the water bath. Covering the troughs with microplate sealers may cause cross-contamination.
- The liquid is aspirated from the trough with a pipette, preferably attached to a vacuum aspirator. The tray is held at an angle to allow all liquid to flow to one end of the trough. Add 1 ml of the appropriate solution to each trough and follow the protocol. Repeat this step as many times as are indicated in the test procedure (a dispensing Multipipette (Eppendorf) is useful for this purpose).
  NOTE:
  - Do not allow the strips to dry between the washing steps.
  - Make sure the surfaces of the strips are not damaged when aspirating. Preferably aspirate the liquid at the top of the strip above the marker line.
  - Make sure the whole strip is thoroughly washed by complete submersion in the solution.
  - Alter the speed of the orbital shaker when necessary.
- Incubation steps for the color development should be between 20 and 25°C. If the temperature is below 20°C, weaker results may be obtained. If the temperature is above 25°C, high background and/or false positive signals may be obtained.
- The specific incubation times should be strictly respected to guarantee correct performance of the assay.

Samples

1. Mycobacterial *rpo*B gene amplified product (use 10 µl).
2. Blank amplified control sample (Negative Control) (use 10 µl).

Hybridization

1. Heat a shaking water bath to 62°C ± 0.5°C. Check the temperature using a calibrated thermometer, and adjust the temperature if necessary. Pre-warm the Hybridization Solution and Stringent Wash Solution to at least 37°C and do not exceed 62°C. Mix before use. All crystals should be dissolved.
2. Using tweezers, remove the required number of INNO-LiPA Rif.TB strips from the tube (1 strip per sample) and pencil an identification

number above the red marker line on the strip. Always include a strip for the Negative Control sample (no DNA added).

3. Take the required number of test troughs (1 trough per strip) and place them in the tray.
4. Pipette 10 µl of Denaturation Solution into the upper corner of each trough.
   NOTE:
   - Close the vial immediately after use.
5. Add either 10 µl amplified biotinylated product, or 10 µl Negative Control sample to the Denaturation Solution and carefully mix by pipetting up and down. Always use cotton-plugged sterile pipette tips. Allow denaturation to proceed for 5 minutes at room temperature (20 - 25°C).
6. Shake the pre-warmed ready-for-use Hybridization Solution and gently add 1 ml to the denatured amplified product into each trough. Mix by gentle shaking. Take care not to contaminate neighbouring troughs during pipetting.
7. Immediately place the strip into the trough with the marked side (red Marker Line) of the membrane facing upwards. The strips should be completely submerged in the solution.
   NOTE:
   - Wear disposable gloves and use tweezers.
8. Place the tray into the 62°C ± 0.5°C shaking water bath (approximately 80 rpm; see 'Procedural notes'), close the lid, and incubate for 30 minutes.

Stringent wash

1. After hybridization, remove the tray from the water bath.
2. Hold the tray at a low angle and aspirate the liquid from the trough with a pipette, preferably attached to a vacuum aspirator. Add 1 ml pre-warmed Stringent Wash Solution into each trough and rinse by rocking the tray for 60 seconds at room temperature. Aspirate the solution from each trough.
3. Repeat this washing step once (see also Test Procedure - Procedural notes).
4. Finally, aspirate the solution and incubate each strip in 1 ml pre-warmed Stringent Wash Solution in the shaking water bath at 62°C ± 0.5°C for 10 ± 2 minutes. Close the lid of the water bath. Before incubation, check the temperature of the water

bath using a calibrated thermometer, and adjust the temperature if necessary. Always close the lid.
NOTE:
- Prepare Rinse Solution and Conjugate Solution during the stringent wash incubation (See Reagents).

Color development

All subsequent incubations are carried out at **20 - 25°C on a shaker**. If the temperature is below 20°C, weaker results may be obtained. If the temperature is above 25°C, high background and/or false positive signals may be obtained.
During the incubations, the liquid and test strips should move back and forth in the trough for homogeneous staining.

1. Wash each strip twice for 60 - 90 seconds using 1 ml of the diluted Rinse Solution (see Reagents and Test Procedure - Procedural notes). Aspirate.
2. Add 1 ml of the diluted Conjugate Solution (see Reagents) to each trough and incubate for 30 ± 3 minutes while shaking. Aspirate.
   NOTE:
   - Dilute Substrate about 10 minutes prior to the end of the conjugate incubation (see Reagents).
3. Wash each strip twice for 60 - 90 seconds using 1 ml of the diluted Rinse Solution and wash once more using 1 ml Substrate Buffer. Aspirate.
4. Add 1 ml of the prepared Substrate Solution (See Reagents) to each trough and incubate for 30 ± 3 minutes while shaking. Aspirate.
5. Stop the color developments by washing the strips in 1 ml distilled water while shaking for at least 3 minutes.
6. Using tweezers, remove the strips from the troughs and place them on absorbent paper. Let the strips dry completely before reading the results. Allow the developed strips to dry completely before covering and storing. Developed dry strips should be stored preferably in the dark at room temperature (20 - 25°C); unused or developed strips should be kept away from intense light and heat.

Automated test procedure: *Auto*-LiPA and *Auto*-LiPA 48

The LiPA test procedure is extremely well suited for automation. Therefore, the *Auto*-LiPA and *Auto*-LiPA 48 is designed to fully handle hybridization, stringent wash, and color development steps. The *Auto*-LiPA and *Auto*-LiPA 48 is featured as a walk-away system, with automated heating and cooling and with automated aspiration and pipetting.
Please select the MYCOBV5 program to run the INNO-LiPA Rif.TB test on the *Auto*-LiPA, and the MYCOBV6 program to run INNO-LiPA Rif.TB test on the *Auto*-LiPA 48.
Any changes to the volumes of the working solutions to be prepared when following the automated test procedure can be found in the section 'Reagents - Reagents supplied'.
For more information and specific protocols on the *Auto*-LiPA/*Auto*-LiPA 48, please contact Innogenetics® or your local distributor.

Results

Reading

Figure 1 illustrates the position of the different oligonucleotide probes on the INNO-LiPA Rif.TB strip. A line is considered positive when a clear purple/brown band appears at the end of the test procedure.

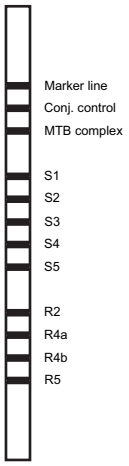


Figure 1: Location of the different probes on the INNO-LiPA Rif.TB strip. A red Marker Line is drawn on the top the strip for orientation. The Conjugate Control line (conj. control) provides an internal control for the color development reaction. The MTB line is a specific probe for M. tuberculosis complex.

Validation

- It is required to include one negative control each time a test is performed. As with any new laboratory procedure, the inclusion of additional positive and negative controls should be considered until a high degree of confidence is reached in the ability to correctly perform the test procedure.
- The assay result of each negative control should be no apparent signal for any of the lines on the strip, except for the conjugate control line.
- Color intensities between lines on a strip may differ from one line to the other.

Interpretation of the results

- The uppermost red line is the Marker Line.
- The first positive line is the Conjugate Control line. This line should be lined up with the conjugate control line on the plastic reading card. This line controls for the addition of reactive Conjugate and Substrate Solution during the detection procedure. It should always be positive and should have approximately the same intensity on each strip in the same test run.
- The second positive line ("MTB complex" on reading card) contains a probe specific for the *M. tuberculosis* complex, and controls for the addition of amplified material for hybridization. This line is positive if DNA amplicons from *M. tuberculosis* complex strains are present. Strains from other microbial taxa react negatively on this probe.
- When all the S-probes (S1, S2, S3, S4 and S5) give a positive signal while all the R-probes are negative, the *M. tuberculosis* strain is sensitive to rifampicin (Fig. 2A; for exceptions, see Limitations).
- When at least one negative signal with the S-probes is obtained, the *M. tuberculosis* strain is resistant to rifampicin (Fig. 2B, C, D, E, F, G). If the resistance to rifampicin is caused by one of the four most frequently observed mutations (D516V, H526Y, H526D, S531L), a positive reaction should also be obtained with one of the four R-probes (R2, R4a, R4b, R5, respectively). A positive reaction of the R-probe should be accompanied with a negative reaction on the corresponding S-probe (S2, S4, S4, or S5, respectively) (Fig. 2C, D, E, F; for exceptions, see Limitations).
- There are however some exceptions. The pattern obtained may deviate from the regular patterns (Table 1) when:
  - the sample contains more than one strain (mixture or contamination) (Figure 3);
  - more than one mutation is present in the sample examined (Figure 4).

  By way of example some aberrant patterns are schematically shown in figures 3 and 4. It should be noted that when an aberrant pattern is observed, at least one of the strains present in the sample deviates from the wild type and is most probably resistant.

- In conclusion: a sample should be considered as harboring a resistant strain if any INNO-LiPA pattern is observed that deviates from the wild-type pattern.

Table 1: INNO-LiPA Rif.TB regular probe patterns

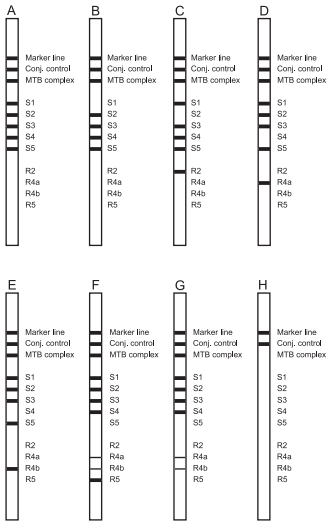
PROBES										INTERPRETATION	Pattern
Rif.TB	S1	S2	S3	S4	S5	R2	R4a	R4b	R5		
-	-	- (1)	-	-	-	-	-	-	-	No *M. tuberculosis*	-
+	+	+	+	+	+	-	-	-	-	Wild type (2)	WT
+	-	+	+	+	+	-	-	-	-	Mutation in probe region 1	ΔS1
+	+	-	+	+	+	-	-	-	-	Mutation in probe region 2	ΔS2
+	+	-	+	+	+	+	-	-	-	D516V	R2
+	+	+	-	+	+	-	-	-	-	Mutation in probe region 3	ΔS3
+	+	+	+	-	+	-	-	-	-	Mutation in probe region 4	ΔS4
+	+	+	+	-	+	-	+	-	-	H526Y	R4a
+	+	+	+	-	+	-	-	+	-	H526D	R4b
+	+	+	+	+	- (3)	-	-*	-*	-	Mutation in probe region 5	ΔS5
+	+	+	+	+	-	-	-*	-*	+	S531L	R5

* These probes react weakly positive when S5 is negative.

(1) May occasionally be positive with non-*M. tuberculosis* strains.

(2) Theoretically, there is a possibility that a sample contains a mixture of a WT strain and a mutant strain (not being recognized by one of the R-probes). In this case all S-probes will be positive and all the R-probes will be negative. If there is no noticeable difference in color intensity of the different S-lines, this mixture will be interpreted as sensitive.

(3) When dealing with a mutation at codon 533, it is possible that the S5 probe does not disappear completely.



Strips	Probes										Pattern	Result
	TB	S1	S2	S3	S4	S5	R2	R4a	R4b	R5		
A	+	+	+	+	+	+	-	-	-	-	Wild type	Sensitive
B	+	-	+	+	+	+	-	-	-	-	ΔS1	Resistant
C	+	+	-	+	+	+	+	-	-	-	R2	Resistant
D	+	+	+	+	-	+	-	+	-	-	R4a	Resistant
E	+	+	+	+	-	+	-	-	+	-	R4b	Resistant
F	+	+	+	+	+	-	-	-	-	+	R5	Resistant
G	+	+	+	+	+	-	-	-	-	-	ΔS5	Resistant
H	-	-	-	-	-	-	-	-	-	-	-	No *M. tuberculosis*

Figure 2: Examples of results obtained with INNO-LiPA Rif.TB strips

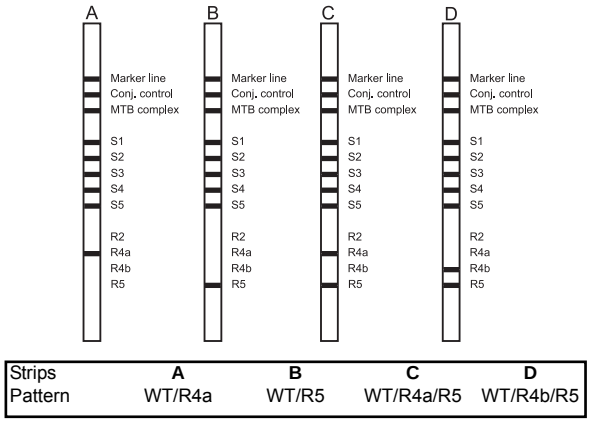


Figure 3: Examples of samples containing more than one strain

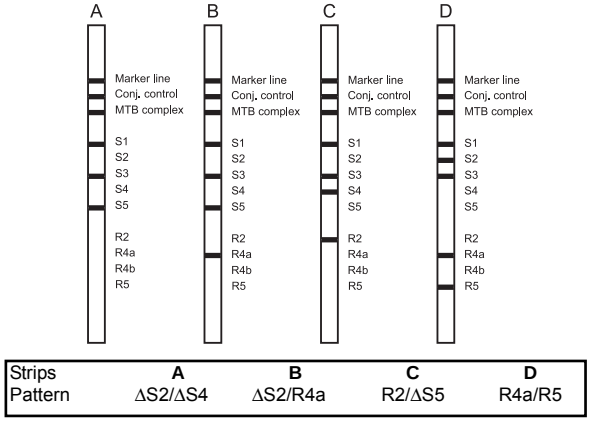


Figure 4: Examples of samples with double mutations

Limitations of the procedure

- Use of this product should be limited only to personnel well trained in the techniques of amplification and hybridization.
- Polymerase inhibition might be the reason for complete failure of the assay.
- Although low quantity and quality DNA can be used, and the main handlings are pipetting and aspirating, only good laboratory practice and careful performance of the procedures specified allow specific hybridization and correct genotyping.
- This kit detects wild types of *M. tuberculosis* complex and mutations in the relevant region (codon 509 to 534) of its *rpo*B gene related to rifampicin resistance. Only for two described mutations (514insF and 514insFM) inside this region, INNO-LiPA Rif.TB gave a wild type pattern while the strain was rifampicin resistant *in vitro*. Mutations outside this region, as well as unknown mechanisms of resistance to rifampicin will not be detected by this assay.
- False positive reactions of probes R4a, R4b, and/or S5 may occur when a mutation is present in the S5 probe. For instance, when dealing with mutation S531L, weak false positive reactions of probes R4a and R4b will be visible in addition to a regular R5-pattern (meaning MTB, S1, S2, S3, S4, and R5 are positive). When dealing with mutation L533P, a weak false positive reaction of the S5 will occur, together with false positive reactions of probe R4a and R4b.
- During evolution towards resistance, a mixture of a WT strain and a mutant strain (not being recognized by one of the R-probes) may be interpreted as sensitive.

References

1. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet 1993;341:647-650.