



(86) Date de dépôt PCT/PCT Filing Date: 1995/06/09
(87) Date publication PCT/PCT Publication Date: 1995/12/14
(45) Date de délivrance/Issue Date: 2009/01/20
(85) Entrée phase nationale/National Entry: 1996/12/06
(86) N° demande PCT/PCT Application No.: EP 1995/002230
(87) N° publication PCT/PCT Publication No.: 1995/033851
(30) Priorité/Priority: 1994/06/09 (EP94870093.5)

(51) Cl.Int./Int.Cl. *C40B 40/06* (2006.01),
C07H 21/04 (2006.01), *C12N 15/31* (2006.01),
C12N 15/54 (2006.01), *C12Q 1/68* (2006.01),
C12Q 1/18 (2006.01)

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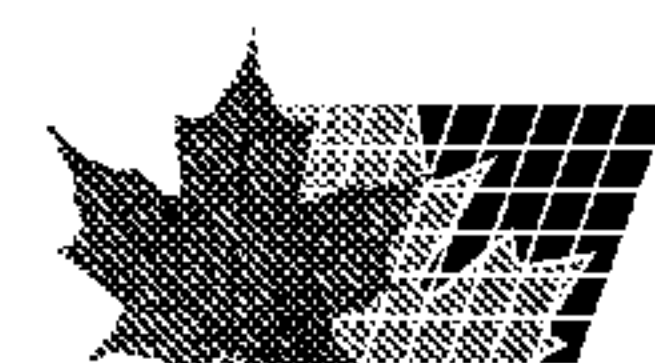
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(54) Titre : PROCEDE DE DETECTION DU SPECTRE DE RESISTANCE ANTIBIOTIQUE DE L'ESPECE
MYCOBACTERIUM

(54) Title: METHOD FOR THE DETECTION OF THE ANTIBIOTIC RESISTANCE SPECTRUM OF MYCOBACTERIUM
SPECIES

(57) **Abrégé/Abstract:**

Method for the detection of the antibiotic resistance spectrum of Mycobacterium species present in a sample, possibly coupled to the identification of the Micobacterium species involved, comprising the steps of: (i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample; (ii) if need be amplifying the relevant part of the antibiotic resistance genes present in said sample with at least one suitable primer pair, (iii) hybridizing the polynucleic acids of step (i) or (ii) with at least one of the *rpoB* gene probes, as specified in table 2, under the appropriate hybridization and wash conditions; (iv) detecting the hybrids formed in step (iii); (v) inferring the Mycobacterium antibiotic resistance spectrum, and possibly the Mycobacterium species involved from the differential hybridization signal(s) obtained in step (iv).





WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68		A3	(11) International Publication Number: WO 95/33851
			(43) International Publication Date: 14 December 1995 (14.12.95)
(21) International Application Number: PCT/EP95/02230 (22) International Filing Date: 9 June 1995 (09.06.95) (30) Priority Data: 94870093.5 9 June 1994 (09.06.94) EP (34) Countries for which the regional or international application was filed: AT et al.		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). 2192364 Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
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(54) Title: METHOD FOR THE DETECTION OF THE ANTIBIOTIC RESISTANCE SPECTRUM OF MYCOBACTERIUM SPECIES			
(57) Abstract Method for the detection of the antibiotic resistance spectrum of <i>Mycobacterium</i> species present in a sample, possibly coupled to the identification of the <i>Micobacterium</i> species involved, comprising the steps of: (i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample; (ii) if need be amplifying the relevant part of the antibiotic resistance genes present in said sample with at least one suitable primer pair; (iii) hybridizing the polynucleic acids of step (i) or (ii) with at least one of the <u>rpoB</u> gene probes, as specified in table 2, under the appropriate hybridization and wash conditions; (iv) detecting the hybrids formed in step (iii); (v) inferring the <i>Mycobacterium</i> antibiotic resistance spectrum, and possibly the <i>Mycobacterium</i> species involved from the differential hybridization signal(s) obtained in step (iv).			

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METHOD FOR THE DETECTION OF THE ANTIBIOTIC RESISTANCE SPECTRUM
OF MYCOBACTERIUM SPECIES

The present invention relates to the field of drug-resistant mycobacteria.

5 The present invention relates to probes, primers, methods and kits comprising the same for the detection of mycobacterial nucleic acids in biological samples.

Identification of most clinically relevant Mycobacterium species, in particular of Mycobacterium tuberculosis is tedious and time consuming due to culture-procedures which can take up to 6 weeks. Rapid diagnosis of Mycobacterium infection is very important since
10 the disease might be life-threatening and highly contagious. Only recently some methods - all making use of one or another amplification process - have been developed to detect and identify Mycobacterium species without the need for culture (Claridge et al., 1993). Most of these methods are still in evaluation and their benefit in routine applications remains questionable. Moreover, these methods do not solve the problem of Mycobacterium drug-
15 resistance detection which still relies on culture.

Since the frequency of multidrug resistance in tuberculosis is steadily increasing (Culliton, 1992), it is clear now that early diagnosis of M. tuberculosis and the rapid recognition of resistance to the major tuberculostatics are essential for therapy and an optimal control of the resurgent epidemic.

20 The antibiotics used for treatment of M. tuberculosis infections are mainly isoniazid and rifampicin either administered separately or as a combination of both. Occasionally, pyrazinamide, ethambutol and streptomycin are used; other classes of antibiotics like (fluoro)quinolones may become the preferred tuberculostatics in the future.

Since most multidrug resistant mycobacteria also lost susceptibility to rifampicin, rifampicin-resistance is considered to be a potential marker for multidrug resistant tuberculosis. For this reason, the detection of resistance to rifampicin might be of particular
25 relevance.

For the majority of the M. tuberculosis strains examined so far, the mechanism responsible for resistance to rifampicin (and analogues like rifabutin) has been elucidated.
30 Rifampicin (and analogues) block the RNA polymerase by interacting with the β -subunit of this enzyme. Telenti et al. (1993a) found that mutations in a limited region of the β -subunit of the RNA polymerase of M. tuberculosis give rise to insensitivity of the RNA polymerase

for rifampicin action. This region is limited to a stretch of 23 codons in the rpoB gene. The authors describe 17 amino acid changes provoking resistance to rifampicin (Telenti et al., 1993b). These amino acid changes are caused by point mutations or deletions at 15 nucleotides or 8 amino acid codons respectively scattered over a stretch of 67 nucleotides or 23 amino acid codons.

Telenti et al. (1993a and b) described a PCR-SSCP method to screen for the relevant mutations responsible for rifampicin resistance (SSCP refers to single-strand conformation polymorphism). SSCP analysis can be performed either by using radio-activity or by using fluorescent markers. In the latter case sophisticated and expensive equipment (an automated DNA-sequencing apparatus) is needed. The SSCP approach described has also other limitations with respect to specificity and sensitivity which might impede its routine use. Specimen can only be adequately analysed directly if a significant load of bacteria (microscopy score : > 90 organisms/field) is observed microscopically and on crude DNA samples strand-separation artefacts may be observed which complicate the interpretation of the results.

Kapur et al. (1994) describe 23 distinct rpoB alleles associated with rifampicin resistance. In addition to the mutations described by Telenti et al. (1993a), some new mutant rpoB alleles are described. However, the most frequently occurring alleles remain the same as those described before.

In M. leprae, the molecular basis for rifampicin resistance was described by Honoré and Cole (1993). Here too, resistance stemmed from mutations in the rpoB gene, which encodes the beta subunit of RNA polymerase of M. leprae. Only a limited number of resistant M. leprae strains (9) were analysed, and in most of them (8/9) resistance was due to a mutation affecting the Ser-425 residue.

Clinically important mycobacteria other than M. tuberculosis and M. leprae often show an innate, be it variable, resistance to rifampicin. This is the case for M. avium and M. intracellulare, human pathogens for which only limited treatment options are available. Guerrero et al. (1994) compared the rpoB-gene sequences of different M. avium and M. intracellulare isolates with that of M. tuberculosis. Differences are present at the nucleotide level but a full amino acid identity was found with rifampicin-sensitive M. tuberculosis. These findings suggest that another mechanism of resistance, possibly a permeability barrier, applies for M. avium and M. intracellulare.

The specific detection of point mutations or small deletions can elegantly be

approached using hybridization procedures such as the reverse hybridization assay. However, the complexity observed in the relevant part of the rpoB gene does not allow a straightforward probe development. As will be exemplified further, it was one of the objects of the present invention to design a specific approach allowing the detection of most if not all mutations found so far in a fast and convenient way without the need for sophisticated equipment.

The mechanism of resistance to isoniazid (INH) is considerably more complex than that for rifampicin. At least two gene products are involved in INH-resistance. First, there is catalase-peroxidase which is believed to convert INH to an activated molecule. Hence, strains which do not produce catalase-peroxidase by virtue of a defective or deleted katG gene are not anymore susceptible to INH (Zhang et al., 1992; Stoeckle et al., 1993). In this context it should be mentioned that the association between INH-resistance and the loss of catalase activity was already noted in the fifties (Middlebrook, 1954 a and b; Youatt, 1969).

The second molecule involved is the inhA gene product, which is believed to play a role in the mycolic acid biosynthesis. It is postulated that the activated INH molecule interacts either directly or indirectly with this product and probably prevents proper mycolic acid biosynthesis. This hypothesis is based on the recent observation that overexpression of the wild type inhA gene or a particular amino acid change (S94A) in the inhA gene product confers resistance to INH (Banerjee et al., 1994).

In short, and somewhat simplified we can state that in certain M. tuberculosis strains resistance to INH might be mediated by :

- the loss of catalase-peroxidase activity
- the presence of certain amino acid changes in the inhA protein
- the expression level of the wild type inhA protein

Also, other mechanisms might be involved in conferring resistance to INH and related drugs. The importance of these factors in the total spectrum of INH-resistance mechanisms has yet to be assessed. This issue can be addressed by means of DNA probe techniques if reliable DNA probes can be developed from the available DNA-sequences of the katG gene (EMBL n° X68081) and inhA gene (EMBL n° U02492) of M. tuberculosis. These probe-tests could then also be applied for detection of drug resistance in biological samples.

For the detection of resistance to streptomycine and (fluoro)quinolones the same approach as for rifampicin can be followed. Resistance to these antibiotics is also induced

by point mutations in a limited region of one or more genes. Point mutations in the gyrase gene confer resistance to (fluoro)quinolones (EMBL n° L27512). Streptomycin resistance is correlated with mutations in either the 16S rRNA gene or the gene of a ribosomal protein S12 (rpsL) (Finken et al., 1993; Douglas and Steyn, 1993; Nair et al., 1993).

5 Resistance due to nucleotide changes in the katG, rpoB and rpsL genes have been described in international application WO 93/22454. For each of the different genes in M. tuberculosis only one of the many possible mutations was specified in detail, nl. R461L for katG, S425L (equivalent to S531L described by Telenti et al. and the present invention) for rpoB and K42R for rpsL.

10 It is an object of the present invention to provide a rapid and reliable detection approach for determination of antibiotic resistance of mycobacterial species present in a biological sample.

 More particularly, it is an aim of the present invention to provide a rapid and reliable method for determination of resistance to rifampicin (and/or rifabutin) of M. tuberculosis present in a biological sample.

15 It is also an object of the present invention to provide methods enabling the detection and identification of Mycobacterium species in a biological sample, directly coupled to the monitoring of the antibiotic resistance spectrum.

 It is more particularly an aim of the present invention to provide a method to detect the presence of Mycobacterium tuberculosis in a biological sample directly coupled to the detection of rifampicin (and/or rifabutin) resistance.

 It is more particularly an aim of the present invention to provide a method to detect the presence of Mycobacterium leprae in a biological sample directly coupled to the detection of rifampicin (and/or rifabutin) resistance.

25 It is also an aim of the present invention to select particular probes able to discriminate wild-type sequences from mutated sequences conferring resistance to one or more drugs.

 It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type sequences from mutated sequences conferring resistance to rifampicin (and/or rifabutin).

30 It is more particularly an aim of the present invention to select a particular set of probes, able to discriminate wild-type sequences from mutated sequences conferring

resistance to rifampicin (an/or rifabutin) with this particular set of probes being used under the same hybridisation and wash-conditions.

It is moreover an aim of the present invention to combine a set of selected probes able to discriminate wild-type sequences from mutated sequences conferring resistance to rifampicin (and/or rifabutin) with another set of selected probes able to identify the mycobacteria species present in the biological sample, whereby all probes can be used under the same hybridisation and wash-conditions.

It is also an aim of the present invention to select primers enabling the amplification of the gene fragment(s) determining the antibiotic resistance trait of interest.

It is more particularly an aim of the present invention to select primers enabling the amplification of the rpoB-gene fragment determining resistance to rifampicin (and analogues).

Another aim of the invention is to provide kits for the detection of antibiotic resistance in mycobacteria, possibly coupled to the identification of the mycobacterial species involved.

All the aims of the present invention have been met by the following specific embodiments.

The selection of the preferred probes of the present invention is based on the Line Probe Assay (LiPA) principle which is a reverse hybridization assay using oligonucleotide probes immobilized as parallel lines on a solid support strip (Stuyver et al. 1993; international application WO 94/12670). This approach is particularly advantageous since it is fast and simple to perform. The reverse hybridization format and more particularly the LiPA approach has many practical advantages as compared to other DNA techniques or hybridization formats, especially when the use of a combination of probes is preferable or unavoidable to obtain the relevant information sought.

It is to be understood, however, that any other type of hybridization assay or format using any of the selected probes as described further in the invention, is also covered by the present invention.

The reverse hybridization approach implies that the probes are immobilized to a solid support and that the target DNA is labelled in order to enable the detection of the hybrids formed.

The following definitions serve to illustrate the terms and expressions used in the present invention.

The target material in these samples may either be DNA or RNA e.g. genomic DNA

or messenger RNA or amplified versions thereof. These molecules are also termed polynucleic acids.

The term "probe" refers to single stranded sequence-specific oligonucleotides which have a sequence which is complementary to the target sequence to be detected.

5 The term complementary as used herein means that the sequence of the single stranded probe is exactly complementary to the sequence of the single-stranded target, with the target being defined as the sequence where the mutation to be detected is located. Since the current application requires the detection of single basepair mismatches, very stringent conditions for hybridization are required, allowing in principle only hybridization of exactly
10 complementary sequences. However, variations are possible in the length of the probes (see below), and it should be noted that, since the central part of the probe is essential for its hybridization characteristics, possible deviations of the probe sequence versus the target sequence may be allowable towards head and tail of the probe, when longer probe sequences are used. These variations, which may be conceived from the common knowledge in the art,
15 should however always be evaluated experimentally, in order to check if they result in equivalent hybridization characteristics than the exactly complementary probes.

Preferably, the probes are about 5 to 50 nucleotides long, more preferably from about 10 to 25 nucleotides. The nucleotides as used in the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides
20 containing modified groups which do not essentially alter their hybridisation characteristics. Probe sequences are represented throughout the specification as single stranded DNA oligonucleotides from the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein T is replaced by U).

25 The probes according to the invention can be prepared by cloning of recombinant plasmids containing inserts including the corresponding nucleotide sequences, if need be by cleaving the latter out from the cloned plasmids upon using the adequate nucleases and recovering them, e.g. by fractionation according to molecular weight. The probes according to the present invention can also be synthesized chemically, for instance by the conventional
30 phospho-triester method.

The term "solid support" can refer to any substrate to which an oligonucleotide probe can be coupled, provided that it retains its hybridization characteristics and provided that the

background level of hybridization remains low. Usually the solid substrate will be a microtiter plate, a membrane (e.g. nylon or nitrocellulose) or a microsphere (bead). Prior to application to the membrane or fixation it may be convenient to modify the nucleic acid probe in order to facilitate fixation or improve the hybridization efficiency. Such
5 modifications may encompass homopolymer tailing, coupling with different reactive groups such as aliphatic groups, NH_2 groups, SH groups, carboxylic groups, or coupling with biotin, haptens or proteins.

The term "labelled" refers to the use of labelled nucleic acids. Labelling may be carried out by the use of labelled nucleotides incorporated during the polymerase step of the
10 amplification such as illustrated by Saiki et al. (1988) or Bej et al. (1990) or labelled primers, or by any other method known to the person skilled in the art. The nature of the label may be isotopic (^{32}P , ^{35}S , etc.) or non-isotopic (biotin, digoxigenin, etc.).

The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product which is
15 complementary to the nucleic acid strand to be copied. The length and the sequence of the primer must be such that they allow to prime the synthesis of the extension products. Preferably the primer is about 5-50 nucleotides long. Specific length and sequence will depend on the complexity of the required DNA or RNA targets, as well as on the conditions of primer use such as temperature and ionic strenght.

20 The fact that amplification primers do not have to match exactly with the corresponding template sequence to warrant proper amplification is amply documented in the literature (Kwok et al., 1990).

The amplification method used can be either polymerase chain reaction (PCR; Saiki et al., 1988), ligase chain reaction (LCR; Landgren et al., 1988; Wu & Wallace, 1989;
25 Barany, 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al., 1990; Compton, 1991), transcription-based amplification system (TAS; Kwoh et al., 1989), strand displacement amplification (SDA; Duck, 1990; Walker et al., 1992) or amplification by means of Q β replicase (Lizardi et al., 1988; Lomeli et al., 1989) or any other suitable method to amplify nucleic acid molecules known in the art.

30 The oligonucleotides used as primers or probes may also comprise nucleotide analogues such as phosphorothiates (Matsukura et al., 1987), alkylphosphorothiates (Miller et al., 1979) or peptide nucleic acids (Nielsen et al., 1991; Nielsen et al., 1993) or may

contain intercalating agents (Asseline et al., 1984).

As most other variations or modifications introduced into the original DNA sequences of the invention these variations will necessitate adaptations with respect to the conditions under which the oligonucleotide should be used to obtain the required specificity and sensitivity. However the eventual results of hybridisation will be essentially the same as those obtained with the unmodified oligonucleotides.

The introduction of these modifications may be advantageous in order to positively influence characteristics such as hybridization kinetics, reversibility of the hybrid-formation, biological stability of the oligonucleotide molecules, etc.

The "sample" may be any biological material taken either directly from the infected human being (or animal), or after culturing (enrichment). Biological material may be e.g. expectorations of any kind, broncheolavages, blood, skin tissue, biopsies, lymphocyte blood culture material, colonies, liquid cultures, soil, faecal samples, urine etc.

The probes of the invention are designed for attaining optimal performance under the same hybridization conditions so that they can be used in sets for simultaneous hybridization; this highly increases the usefulness of these probes and results in a significant gain in time and labour. Evidently, when other hybridization conditions would be preferred, all probes should be adapted accordingly by adding or deleting a number of nucleotides at their extremities. It should be understood that these concomitant adaptations should give rise to essentially the same result, namely that the respective probes still hybridize specifically with the defined target. Such adaptations might also be necessary if the amplified material should be RNA in nature and not DNA as in the case for the NASBA system.

For designing probes with desired characteristics, the following useful guidelines known to the person skilled in the art can be applied.

Because the extent and specificity of hybridization reactions such as those described herein are affected by a number of factors, manipulation of one or more of those factors will determine the exact sensitivity and specificity of a particular probe, whether perfectly complementary to its target or not. The importance and effect of various assay conditions, explained further herein, are known to those skilled in the art.

First, the stability of the [probe : target] nucleic acid hybrid should be chosen to be compatible with the assay conditions. This may be accomplished by avoiding long AT-rich sequences, by terminating the hybrids with G:C base pairs, and by designing the probe with

an appropriate T_m . The beginning and end points of the probe should be chosen so that the length and %GC result in a T_m about 2-10°C higher than the temperature at which the final assay will be performed. The base composition of the probe is significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due to additional hydrogen bonding. Thus, hybridization involving complementary nucleic acids of higher G-C content will be stable at higher temperatures.

Conditions such as ionic strength and incubation temperature under which a probe will be used should also be taken into account when designing a probe. It is known that hybridization will increase as the ionic strength of the reaction mixture increases, and that the thermal stability of the hybrids will increase with increasing ionic strength. On the other hand, chemical reagents, such as formamide, urea, DMSO and alcohols, which disrupt hydrogen bonds, will increase the stringency of hybridization. Destabilization of the hydrogen bonds by such reagents can greatly reduce the T_m . In general, optimal hybridization for synthetic oligonucleotide probes of about 10-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

It is desirable to have probes which hybridize only under conditions of high stringency. Under high stringency conditions only highly complementary nucleic acid hybrids will form; hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two nucleic acid strands forming a hybrid. The degree of stringency is chosen such as to maximize the difference in stability between the hybrid formed with the target and the nontarget nucleic acid. In the present case, single base pair changes need to be detected, which requires conditions of very high stringency.

Second, probes should be positioned so as to minimize the stability of the [probe : nontarget] nucleic acid hybrid. This may be accomplished by minimizing the length of perfect complementarity to non-target organisms, by avoiding GC-rich regions of homology to non-target sequences, and by positioning the probe to span as many destabilizing mismatches as possible. Whether a probe sequence is useful to detect only a specific type of organism depends largely on the thermal stability difference between [probe:target] hybrids and [probe:nontarget] hybrids. In designing probes, the differences in these T_m values should be

as large as possible (e.g. at least 2°C and preferably 5°C).

The length of the target nucleic acid sequence and, accordingly, the length of the probe sequence can also be important. In some cases, there may be several sequences from a particular region, varying in location and length, which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another which differs merely by a single base. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly complementary base sequence will normally primarily determine hybrid stability. While oligonucleotide probes of different lengths and base composition may be used, preferred oligonucleotide probes of this invention are between about 5 to 50 (more particularly 10-25) bases in length and have a sufficient stretch in the sequence which is perfectly complementary to the target nucleic acid sequence.

Third, regions in the target DNA or RNA which are known to form strong internal structures inhibitory to hybridization are less preferred. Likewise, probes with extensive self-complementarity should be avoided. As explained above, hybridization is the association of two single strands of complementary nucleic acids to form a hydrogen bonded double strand. It is implicit that if one of the two strands is wholly or partially involved in a hybrid that it will be less able to participate in formation of a new hybrid. There can be intramolecular and intermolecular hybrids formed within the molecules of one type of probe if there is sufficient self complementarity. Such structures can be avoided through careful probe design. By designing a probe so that a substantial portion of the sequence of interest is single stranded, the rate and extent of hybridization may be greatly increased. Computer programs are available to search for this type of interaction. However, in certain instances, it may not be possible to avoid this type of interaction.

The present invention provides in its most general form for a method to detect the antibiotic resistance spectrum of Mycobacterium species present in a sample, possibly coupled to the identification of the Mycobacterium species involved, comprising the steps of:

- (i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample;
- (ii) if need be amplifying the relevant part of the antibiotic resistance genes present in said sample with at least one suitable primer pair;
- (iii) hybridizing the polynucleic acids of step (i) or (ii) with at least one of the rpoB gene

29233-1

11

probes as mentioned in table 2, under appropriate hybridization and wash conditions:

- (iv) detecting the hybrids formed in step (iii):
- (v) inferring the Mycobacterium antibiotic resistance spectrum, and possibly the Mycobacterium species involved from the differential hybridization signals(s) obtained in step (iv).

The relevant part of the antibiotic resistance genes refers to the regions in the rpoB, katG, inhA, 16S rRNA, rpsL and gyrase genes harboring mutations causing resistance to rifampicin, isoniazid, streptomycin and (fluoro)quinolones as described above.

According to a preferred embodiment of the present invention, step (iii) is performed using a set of probes meticulously designed as such that they show the desired hybridization results, when used under the same hybridization and wash conditions.

According to another aspect of the invention, there is provided method for the detection of at least one of rifampicin resistance and rifabutin resistance of Mycobacterium species present in a sample, comprising the steps of: (i) hybridizing polynucleic acids in said sample with a set of wild-type probes, each probe of said set spanning more than one polymorphic site of the polynucleic acid, and said set overlapping a complete mutation region of a rpoB gene of the polynucleic acid, under appropriate hybridization and wash conditions; (ii) detecting the

29233-1

11a

hybrids formed in step (i); and (iii) determining the rifampicin susceptibility (sensitivity versus resistance) of the Mycobacterium species present in the sample from the differential hybridization signal(s) obtained in step (iii).

5 Optionally, the method for the detection of at least one of rifampicin resistance and rifabutin resistance of Mycobacterium species present in a sample further comprises one or both of the following steps prior to the hybridization step (i): releasing, isolating or
10 concentrating the polynucleic acids present in the sample; and amplifying the relevant part of the rpoB gene with at least one suitable primer pair.

 More specifically, the present invention provides a method for detection of rifampicin (and/or rifabutin)
15 resistance of M. tuberculosis present in a biological sample, comprising the steps of:

- (i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample;
- (ii) if need be amplifying the relevant part of the
20 rpoB gene present in said polynucleic acids with at least one suitable primer pair;
- (iii) hybridizing the polynucleic acids of step (i) or (ii) with a selected set of rpoB wild-type probes under appropriate hybridization and wash
25 conditions, with said set comprising at least one of the following probes (see Table 2):

S1 (SEQ ID NO 1)

S11 (SEQ ID NO 2)

29233-1

11b

S2 (SEQ ID NO 3)

S3 (SEQ ID NO 4)

S33 (SEQ ID NO 5)

S4 (SEQ ID NO 6)

5 S44 (SEQ ID NO 7)

S444 (SEQ ID NO 43)

S4444 (SEQ ID NO 8)

S5 (SEQ ID NO 9)

S55 (SEQ ID NO 10)

S555 (SEQ ID NO 39)
 S5555 (SEQ ID NO 40)
 S55C (SEQ ID NO 44)
 S55M (SEQ ID NO 45)
 5 S6 (SEQ ID NO 11)
 S66 (SEQ ID NO 12)

- (iv) detecting the hybrids formed in step (iii);
 (v) inferring the rifampicin susceptibility (sensitivity versus resistance) of M. tuberculosis present in the sample from the differential hybridization signal(s) obtained in step
 10 (iv).

The term 'susceptibility' refers to the phenotypic characteristic of the M. tuberculosis strain being either resistant or sensitive to the drug, as determined by *in vitro* culture methods, more specifically to rifampicin (and/or rifabutin). Resistance to rifampicin is revealed by the absence of hybridization with at least one of the S-probes.

15 Standard hybridization and wash conditions are for instance 3X SSC (Sodium Saline Citrate), 20% deionized FA (Formamide) at 50°C. Other solutions (SSPE (Sodium saline phosphate EDTA), TMACl (Tetramethyl ammonium Chloride), etc.) and temperatures can also be used provided that the specificity and sensitivity of the probes is maintained. If need
 20 be, slight modifications of the probes in length or in sequence have to be carried out to maintain the specificity and sensitivity required under the given circumstances. Using the probes of the invention, changing the conditions to 1.4X SSC, 0.07% SDS at 62°C lead to the same hybridisation results as those obtained under standard conditions, without the necessity to adapt the sequence or length of the probes.

Suitable primer pairs can be chosen from a list of primer pairs as described below.

25 In a more preferential embodiment, the above-mentioned polynucleic acids from step (i) or (ii) are hybridized with at least two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen or seventeen of the above-mentioned S-probes, preferably with 5 or 6 S-probes, which, taken together, cover the "mutation region" of the rpoB gene.

30 The term "mutation region" means the region in the rpoB-gene sequence where most, if not all of the mutations responsible for rifampicin resistance are located. This mutation region is represented in fig. 1.

In a more preferential embodiment, the above-mentioned polynucleic acids from step (i) or (ii) are hybridized with a selected set of rpoB-wild-type probes, with said set comprising at least one, and preferentially all of the following probes (see Table 2) :

S11 (SEQ ID NO 2)
 S2 (SEQ ID NO 3)
 S33 (SEQ ID NO 5)
 S4444 (SEQ ID NO 8)
 S55 or S5555 (SEQ ID NO 10 or 40)

In another particular embodiment the set of S-probes as described above, or at least one of them, can be combined with one or more SIL-probes, detecting silent mutations in the rpoB gene. A preferential SIL-probe is SIL-1 (SEQ ID NO 13, see Table 2).

In another embodiment of the invention, the set of S-probes and possibly SIL-probes, can be combined with at least one R-probe detecting a specific mutation associated with rifampicin-resistance.

R-probes are selected from the following group of probes (see Table 2) :

R1 (SEQ ID NO 46)
 R2 (SEQ ID NO 14)
 R2B (SEQ ID NO 47)
 R2C (SEQ ID NO 48)
 R3 (SEQ ID NO 49)
 R4A (SEQ ID NO 15)
 R44A (SEQ ID NO 16)
 R444A (SEQ ID NO 17)
 R4B (SEQ ID NO 18)
 R44B (SEQ ID NO 19)
 R444B (SEQ ID NO 20)
 R4C (SEQ ID NO 50)
 R4D (SEQ ID NO 51)
 R4E (SEQ ID NO 52)
 R5 (SEQ ID NO 21)
 R55 (SEQ ID NO 22)
 R5B (SEQ ID NO 53)

R5C (SEQ ID NO 54)

Preferably the set of S-probes and possibly SIL-probes can be combined with at least two, three, four, five, six, or more R probes.

Most preferably, the set of S-probes and possibly SIL-probes are combined with at least one R-probe from the following restricted group of probes :

R2 (SEQ ID NO 14)

R444A (SEQ ID NO 17)

R444B (SEQ ID NO 20)

R55 (SEQ ID NO 22)

In the case where S and R probes are combined, resistance to rifampicin is revealed by absence of hybridization with one of the S-probes and possibly by a positive hybridization signal with the corresponding R-probe.

Since some mutations may be more frequently occurring than others, e.g. in certain geographic areas (see e.g. Table 5) or in specific circumstances (e.g. rather closed communities) it may be appropriate to screen only for specific mutations, using a selected set of S and/or R probes. This would result in a more simple test, which would cover the needs under certain circumstances. According to Telenti et al. (1993a and b) most mutations described in his publication are relatively rare (3% or less): predominant mutations are S531L (51.6%), H526Y (12.5%), D516V (9.4%) and H526D (7.8%).

In a particular embodiment of the invention a selected set of two or three S-probes is used, the respective sets of probes being:

S4444 (SEQ ID NO 8)

S55 (or S5555) (SEQ ID NO 10 or 40)

or

S2 (SEQ ID NO 3)

S4444 (SEQ ID NO 8)

S55 (or S5555) (SEQ ID NO 10 or 40)

Using this restricted sets of S-probes the majority of rifampicin resistant cases can be detected, by an absence of hybridisation signal with at least one of these probes.

In another particular embodiment of the invention, at least one R probe is used, possibly combined with a selected set of two or three S probes as described above, said R probe being chosen from the following list of probes:

R2	(SEQ ID N0 14)
R444A	(SEQ ID N0 17)
R444B	(SEQ ID N0 20)
R55	(SEQ ID N0 22)

5 In this case, the specific mutation responsible for the rifampicin-resistant phenotype can be inferred from a positive hybridization signal with one of the R-probes and/or the absence of hybridization with the corresponding S-probe.

In another embodiment of the invention, the above-mentioned S, SIL or R probes may be combined with at least one species-specific probe for M. tuberculosis allowing simultaneous identification of Mycobacterium tuberculosis and detection of rifampicin resistance, with said species-specific probe being chosen from the following group of probes (see Table 2) :

MT-POL-1	(SEQ ID N0 23)
MT-POL-2	(SEQ ID N0 24)
15 MT-POL-3	(SEQ ID N0 25)
MT-POL-4	(SEQ ID N0 26)
MT-POL-5	(SEQ ID N0 27)

Most preferably the species-specific M. tuberculosis probe is:

MT-POL-1	(SEQ ID N0 23)
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20 In yet another embodiment of the invention, the above-mentioned S, SIL, R or MT-POL probes can be combined with at least one species-specific probe for M. paratuberculosis, M. avium, M. scrofulaceum, M. kansasii, M. intracellulare (and MAC-strains) or M. leprae, with said probes being respectively MP-POL-1 (SEQ ID N0 28), MA-POL-1 (SEQ ID N0 29), MS-POL-1 (SEQ ID N0 38), MK-POL-1 (SEQ ID N0 55), MI-POL-1 (SEQ ID N0 68) and ML-POL-1 (SEQ ID N0 57) (see table 2B) or any species-specific probe derived from the sequence of the relevant part of the rpoB gene of M. paratuberculosis (SEQ ID N0 35), M. avium (SEQ ID N0 36), M. scrofulaceum (SEQ ID N0 37), M. kansasii (SEQ ID N0 56) or MAC-strains (SEQ ID N0 69), as represented respectively in figs. 5, 6, 7, 8 and 11. It should be noted that the sequences represented in figs. 5-8 and 11 are new. The sequence of the rpoB-gene fragment of M. intracellulare and M. leprae has been described by others (Guerrero et al. 1994; Honore and Cole, 1993).

The term "MAC-strains" means "M. avium complex" strains known to the man

skilled in the art of mycobacteria taxonomy. This rather heterogeneous group of MAC-strains may however comprise strains which are genotypically rather like M. intracellulare. This is also the case for isolate ITG 926, of which the rpoB sequence is shown in fig. 11. The MI-POL-1 probe derived from SEQ ID NO 69 and the published M. intracellulare rpoB sequence is therefor specific for M. intracellulare and some MAC strains together.

It should be stressed that all of the above-mentioned probes, including the species-specific probes, are contained in the sequence of the rpoB gene, and more particularly in the sequence of the amplified rpoB gene fragment. Moreover, as illustrated further in the examples, the probes described above as "preferential", are designed in such a way that they can all be used simultaneously, under the same hybridization and wash conditions. These two criteria imply that a single amplification and hybridization step is sufficient for the simultaneous detection of rifampicin resistance and the identification of the mycobacterial species involved.

In a preferential embodiment, and by way of an example, a method is disclosed to detect M. tuberculosis and its resistance to rifampicin, comprising the steps of:

- (i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample;
- (ii) if need be amplifying the relevant part of the rpoB gene with at least one suitable primer pair;
- (iii) hybridizing the polynucleic acids of step (i) or (ii) with the following set of probes under appropriate hybridization and wash conditions

MT-POL-1

S11

S2

S33

S4444

S55 or S5555

R2

R444A

R444B

R55

- (iv) detecting the hybrids formed in step (iii):

(v) inferring the presence of M. tuberculosis and its susceptibility to rifampicin from the differential hybridization signal(s) obtained in step (iv).

In order to detect the mycobacterial organisms and/or their resistance pattern with the selected set of oligonucleotide probes, any hybridization method known in the art can be used (conventional dot-blot, Southern blot, sandwich, etc.).

However, in order to obtain fast and easy results if a multitude of probes are involved, a reverse hybridization format may be most convenient.

In a preferred embodiment the selected set of probes are immobilized to a solid support. In another preferred embodiment the selected set of probes are immobilized to a membrane strip in a line fashion. Said probes may be immobilized individually or as mixtures to delineated locations on the solid support.

A specific and very user-friendly embodiment of the above-mentioned preferential method is the LiPA method, where the above-mentioned set of probes is immobilized in parallel lines on a membrane, as further described in the examples.

The above mentioned R-probes detect mutations which have already been described in the prior art (Telenti et al., 1993a and 1993b). However, as illustrated further in the examples, four new mutations associated with rifampicin-resistance in M. tuberculosis, not yet described by others, are disclosed by the current invention. Using the S-probes of the current invention, new mutations as well as mutations already described in the prior art can be detected. The unique concept of using a set of S-probes covering the complete mutation region in the rpoB gene, allows to detect most, if not all of the mutations in the rpoB-gene responsible for rifampicin resistance, even those mutations which would not yet have been described until now.

The four new rpoB mutations (D516G, H526C, H526T and R529Q) are marked in Table 1 with an asterisk. By way of an example, the sequence of the rpoB-mutant allele H526C is represented in Fig. 2 (SEQ ID NO 34).

The invention also provides for any probes or primersets designed to specifically detect or amplify specifically these new rpoB gene mutations, and any method or kits using said primersets or probes.

In another embodiment, the invention also provides for a method for detection of rifampicin (and/or rifabutin) resistance of M. leprae present in a biological sample, comprising the steps of:

- (i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample:
- (ii) if need be amplifying the relevant part of the rpoB gene with at least one suitable primer pair:
- 5 (iii) hybridizing the polynucleic acids of step (i) or (ii) with a selected set of rpoB wild-type probes under appropriate hybridization and wash conditions, with said set comprising at least one of the following probes (see Table 2) :

ML-S1 (SEQ ID NO 58)

ML-S2 (SEQ ID NO 59)

10 ML-S3 (SEQ ID NO 60)

ML-S4 (SEQ ID NO 61)

ML-S5 (SEQ ID NO 62)

ML-S6 (SEQ ID NO 63)

- (iv) detecting the hybrids formed in step (iii):

- 15 (v) inferring the rifampicin susceptibility (sensitivity versus resistance) of M. leprae present in the sample from the differential hybridization signal(s) obtained in step (iv).

Resistance to rifampicin is revealed by the absence of hybridization with at least one of the ML-S-probes.

20 In another embodiment of the invention, the above-mentioned ML-S probes may be combined with a species specific probe for M. leprae, ML-POL-1, allowing simultaneous identification of M. leprae and detection of rifampicin resistance, with said species specific probe being represented in SEQ ID NO 57.

25 It is to be noted that the above-mentioned ML-S probes and ML-POL-1 probe are all contained within the same amplified rpoB-gene fragment of M. leprae, and are designed as such that they can all be used under the same hybridization and wash conditions.

The invention further provides for any of the probes as described above, as well as compositions comprising at least one of these probes.

30 The invention also provides for a set of primers, allowing amplification of the mutation region of the rpoB gene of M. tuberculosis. The sets of primers can be chosen from the following group of sets (see table 2):

P1 and P5 (SEQ ID NO 30 and 33)

P3 and P4 (SEQ ID N0 31 and 32)

P7 and P8 (SEQ ID N0 41 and 42)

P2 and P6, in combination with (P1 and P5) or (P3 and P4) or (P7 and P8)

Most preferably, the set of primers is the following:

5 P3 and P4 (SEQ ID N0 31 and 32).

The invention also provides for a set of primers allowing amplification of the mutation region of the rpoB gene in mycobacteria in general, i.e. in at least M. tuberculosis, M. avium, M. paratuberculosis, M. intracellulare, M. leprae, M. scrofulaceum. These general primers can be used e.g. in samples where the presence of mycobacteria other than M. tuberculosis is suspected, and where it is desirable to have a more general detection method.

The set of primers is composed of a 5'-primer, selected from the following set:

MGRPO-1 (SEQ ID N0 64)

MGRPO-2 (SEQ ID N0 65)

and a 3'-primer, selected from the following set:

15 MGRPO-3 (SEQ ID N0 66)

MGRPO-4 (SEQ ID N0 67).

The sequence of these primers is shown in Table 2B.

Primers may be labeled with a label of choice (e.g. biotine). Different primer-based target amplification systems may be used, and preferably PCR-amplification, as set out in the examples. Single-round or nested PCR may be used.

The invention also provides for a kit for inferring the antibiotic resistance spectrum of mycobacteria present in a biological sample, possibly coupled to the identification of the mycobacterial species involved, comprising the following components:

- 25 (i) when appropriate, a means for releasing, isolating or concentrating the polynucleic acids present in the sample;
- (ii) when appropriate, at least one of the above-defined set of primers;
- (iii) at least one of the probes as defined above, possibly fixed to a solid support;
- (iv) a hybridization buffer, or components necessary for producing said buffer;
- (v) a wash solution, or components necessary for producing said solution;
- 30 (vi) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization.

The term "hybridization buffer" means a buffer enabling a hybridization reaction to

occur between the probes and the polynucleic acids present in the sample, or the amplified products, under the appropriate stringency conditions.

The term "wash solution" means a solution enabling washing of the hybrids formed under the appropriate stringency conditions.

5 More specifically, the invention provides for a kit as described above, for the simultaneous detection of M. tuberculosis and its resistance to rifampicin.

In another specific case, the invention provides for a kit as described above, for the simultaneous detection of M. leprae and its resistance to rifampicin.

TABLE LEGENDS

Table 1 summarizes the nucleotide and amino acid changes (described by Telenti et al. (1993a and b), Kapur et al. 1994 and the present invention) in the rpoB gene fragment of rifampicin resistant M. tuberculosis isolates. Codon numbering is as in Fig. 1. New mutations described by the current invention are indicated with an asterisk (*).

Table 2 lists the sequences of the primers and probes selected from the rpoB gene.

2A: M. tuberculosis

2B: other mycobacterial species

Table 3 shows the hybridization results obtained with probe MT-POL-1, with DNA from different mycobacterial and non-mycobacterial species.

Table 4 shows some representative results obtained with LiPA for some M. tuberculosis isolates which have been sequenced and the interpretation of the different LiPA patterns.

Table 5 shows the occurrence of the different rpoB mutations in M. tuberculosis in different geographical areas. Abbreviations: Bel= Belgium, Bengla= Bangladesh, Bur-Fa= Burkina faso, Buru= Burundi, Can= Canada, Chi= Chili, Col= Colombia, Egy= Egypt, Gui= Guinea, Hon= Honduras, Pak= Pakistan, Rwa= Rwanda, Tun= Tunesia.

Table 6 shows a comparison of LiPA results versus rifampicin resistance determination in culture for M. tuberculosis. S= Sensitive, R= Resistant.

FIGURE LEGENDS

Fig. 1 represents the nucleotide sequence and the amino acid sequence of the mutation region of respectively the rpoB gene and the RNA polymerase β -subunit of a wild-type (i.e. not resistant) Mycobacterium tuberculosis strain (ITG 9081). The codon (amino acid) numbering is as in Telenti et al. (1993a). The nucleotides or amino acids involved in resistance-inducing changes are underlined. The observed mutations are boxed. The horizontal bars indicate the positions of some of the oligonucleotide probes (one probe per group is indicated).

Fig 2 shows the partial nucleotide sequence of the newly described rpoB mutant allele of M. tuberculosis strain ITG 9003 (SEQ ID N0 34).

Fig 3 shows results obtained on LiPA-strips with probes S44 and S4444 applied at different concentrations on the membrane strip. As target material, nucleic acid preparations of M. tuberculosis strains ITG 8872 and ITG 9081 were used.

Fig 4 gives a comparison of the performance of probes S44 and S4444 in a Line probe assay conformation. The probes on strip A and B are identical except for S44 and S4444. The hybridizations were performed using amplified material originating from M. tuberculosis strain ITG 8872.

Fig 5 shows the partial nucleotide sequence of the presumptive rpoB gene of M. paratuberculosis strain 316F (SEQ ID N0 35)

Fig 6 shows the partial nucleotide sequence of the presumptive rpoB gene of M. avium strain ITG 5887 (SEQ ID N0 36)

Fig 7 shows the partial nucleotide sequence of the presumptive rpoB gene of M. scrofulaceum strain ITG 4979 (SEQ ID N0 37)

Fig 8 shows the partial nucleotide sequence of the presumptive rpoB gene of M. kansasii strain ITG 4987 (SEQ ID N0 56)

Fig 9 shows some rpoB mutations in M. tuberculosis and their corresponding LiPA patterns. Nomenclature of the mutations is as described in Table 1. Nomenclature of the LiPA pattern is as follows:

wt= positive hybridization with all S-probes, and negative hybridization with all R-probes;

Δ S1-5= absence of hybridization with the respective S-probe;

R2, 4A, 4B, 5= positive hybridization with the respective R-probes and absence of

2192364

23

hybridization with the corresponding S-probe:

C = positive control line: should be positive when the test is carried out properly;

Mtb = MT-POL-probe.

Only one probe of each group is mentioned, but it stands for the whole group: e.g. S5 stands
5 for S5, S55, S555, S5555, S55C, S55M.

Fig. 10 shows the frequency of the different mutations encountered in the rifampicin-resistant M. tuberculosis strains analysed by LiPA. Nomenclature is as in Fig. 9. "Mix" means that a mix of strains was present in the sample. "Double" means the presence of two mutations in one strain.

10 Fig. 11 shows the partial nucleotide sequence of the presumptive rpoB gene of MAC strain ITG 926 (SEQ ID NO 69).

Example 1 : Amplification of the rpoB gene fragment in M. tuberculosis

After nucleic acid extraction from mycobacterial isolates (either cultured or present in body fluid or tissue) 2 μ l product was used to amplify the relevant part of the rpo B gene by using one or more combinations between the 5'-primers (P1 (SEQ ID N0 30), P2, P3 (SEQ ID N0 31) and P7 (SEQ ID N0 41)) and the 3'-primers (P4 (SEQ ID N0 32), P5 (SEQ ID N0 33), P6 and P8 (SEQ ID N0 42)).

The sequence of these primers is given in Table 2. P1, P3, P4, P5, P7 and P8 are new primer sequences, described by the current invention. P2 and P6 have been described before (Telenti et al. 1993a).

These primers may be labeled with a label of choice (e.g. biotine). Different primer-based target-amplification systems may be used. For amplification using the PCR, the conditions used are described hereafter. Single-round amplification with primers P1 and P5 involved 35 cycles of 45sec/94°C, 45sec/58°C, 45sec/72°C. If a nested PCR was preferable, in the second round primers P3 and P4 were used and 25 cycles (45sec/94°C, 60sec/68°C) were performed starting from 1 μ l of first round product.

If P3 and P4 were used in a single-round PCR, the following cycling protocol was used : 30 cycles of 1min/95°C, 1min/55°C, 1min/72°C. The same cycling protocol was used for set of primers P2/P6.

PCRs were usually performed in a total volume of 50 μ l containing 50mM KCl, 10mM Tris-HCl (pH 8.3), 2.2mM MgCl₂, 200 μ l each dNTP, 0.01% gelatine and 1 U Taq polymerase.

Primer concentrations ranged between 10 and 25 pmol/reaction.

Since the set of primers P3/P4 yielded significantly stronger signals after hybridization than the set of primers P2/P6, the first set of primers was used for all further hybridization experiments. Set of primers P2/P6 was used for sequence analysis. Nested PCR using P2 and P6 as outer primers and P3 and P4 as inner (biotinylated) primers was only used for direct detection in clinical samples (expectorations and biopsies).

The length of the amplified product, as monitored by agarose-gelelectrophoresis, is as follows :

Primer set P1/P5 :	379 basepairs
Primer set P3/P4 :	257 basepairs
Primer set P2/P6 :	411 basepairs
Primer set P7/P8 :	339 basepairs

Example 2 : Sequencing of rpoB gene fragments from M. tuberculosis strains

DNA extracted from mycobacterial isolates known to be resistant or sensitive to rifampicin was amplified using the set of primers P2/P6 (of which P6 was biotinylated at the 5' end). Direct sequencing of single stranded PCR-product was performed by using streptavidin coated beads and the Taq Dye Deoxy Terminator / Cycle Sequencing kit on an ABI373A DNA sequencer (Applied Biosystems, Forster City, CA, USA) as recommended by the manufacturer. The primers used for sequencing were the same than those used for amplification (P2 or P6).

As expected, all sensitive (= sensitive in culture and sensitive LiPA pattern) strains sequenced (7) yielded a wild-type sequence (no mutation). In most resistant strains a mutation could be identified. Most of these mutations have been previously described (Telenti et al., 1993a, 1993b, Kapur et al. 1994). However, 4 new mutations are described in the current invention: D516G, H526C, H526T and R529Q (see also Table 1 (*)). The full sequence of the amplified rpoB fragment of mutant allele H526C (isolate ITG 9003) is shown in Fig. 2 (SEQ ID NO 34) by way of an example.

A few strains (3/180, see table 6) were resistant to rifampicin in culture but showed a wild-type LiPA pattern. After sequencing, these isolates all showed a wild-type rpoB gene sequence, confirming the hybridization results. It is therefore possible that for these isolates a different molecular mechanism of rifampicin resistance applies.

Example 3 : Development of the Line Probe Assay (LiPA)-strip

The principle and protocol of the line probe assay was as described earlier (Stuyver et al. 1993) with a few exceptions. Instead of the incorporation of biotinylated dUTP, biotinylated primers were used and the hybridization and stringent wash were performed at 50°C in 3 x SSC/20% deionized formamide.

The mutations in the rpoB gene leading to resistance to rifampicin are mainly located in a small area of the gene spanning 67 nucleotides (23 amino acid codons). At least 17 nucleotides, evenly interspersed over this region, are involved in mutations leading to at least 28 different amino acid changes. Such a complexity of nucleotide changes poses problems for the detection of all these changes in a single hybridization step. In principle, per mutated site one wild-type probe would be needed and per nucleotide change one mutant-probe specifically detecting that particular mutation. Hence, a hybridization test would involve at least about 35 sequence specific oligonucleotide probes which would render this test complex to manufacture and use, and consequently commercially less attractive.

Therefore a particular approach was designed making use of carefully chosen wild-type (S-) probes, each spanning more than one polymorphic site and overlapping the complete area of relevance (see fig. 1). Doing so, the set of at least 10 wild-type probes could be reduced to a total of five or six probes. These probes were meticulously adjusted experimentally such that the presence of each resistance causing mutation in the rpoB mutation region resulted in a clearly detectable decrease in hybrid-stability between the target and at least one of these probes under the same hybridization and wash conditions.

Since with this combination of probes all relevant mutations described so far can be detected, rifampicin resistance can be monitored in Mycobacterium tuberculosis isolates. This set of probes is also able to detect the presence of non-described mutations, e.g. the TGC mutation at position 526 in strain ITG 9003.

Although strictly taken the addition of mutant (R-) probes to the selected panel of wild-type probes is obsolete, for scientific purposes it might be informative to detect the exact point mutation present. Therefore, 4 mutant probes (R2, R4A, R4B, R5) were designed which correspond to the most frequently occurring mutations and which, taken together, are able to positively identify more than 70% of all resistant cases (see fig. 10). Additional mutant probes (e.g. R1, R2B, R2C, R3, R4C, R4D, R4E, R5B, R5C) may be added to this set of 4 in order to positively identify most clinically relevant resistant cases. The addition

of these probes also adds to the reliability of the assay since the appearance of a mutant probe should inevitably result in the disappearance of a wild-type probe if one is not dealing with mixed infections. In some cases, it may be of clinical relevance to distinguish between the different mutations possibly present. This situation may occur for instance at amino-acid position 516. If at that position the normal (wild-type) amino acid present (Aspartic acid), changes into a Valine or Tyrosine, high level resistance or intermediate level resistance to rifampicin is induced; however, unpublished data seem to indicate that the sensitivity to rifabutin is maintained. Hence, for infections by strains with this mutation, rifabutin would still be effective where as rifampicin is not. The same may be true for codon 511 but, to our knowledge, these are the only mutation-sites for which the effect of rifampicin and rifabutin might be different: usually strains resistant to rifampicin are also resistant to rifabutin.

In order to develop a LiPA-strip to detect the presence of mutations generating resistance to rifampicin (and/or rifabutin) in M. tuberculosis a total of 36 oligonucleotide probes were synthesized and evaluated in a reverse hybridization test. The sequence of these probes is shown in Table 2A (wild-type and mutation probes). The first set of probes tested was : S1, S2, S3, S4, S5, R2, R4A, R4B and R5. Under the conditions used for reverse hybridization most probes did not perform as theoretically expected and modifications had to be introduced leading to the synthesis and evaluation of the following additional probes : S11, S33, S44, S444, S555, R44A, R444A, R44B, R444B, R55. From the total panel of probes, probes exhibiting the most optimal features with respect to specificity and sensitivity, under the same experimental conditions were selected for further use.

The preferred probes with wild-type sequences (S-probes) which together overlap the entire rpoB region of interest are : S11, S2, S33, S4444 and S55 or S5555 (see Table 2). Resistance will be detected by a loss of hybridization signal with any of these S-probes.

The preferred mutation probes (R-probes) are : R2, R444A, R444B and R55 (see table 2). In some cases of resistance a loss of hybridization signal with the S-probes may be accompanied by a positive hybridization signal with the corresponding R-probe.

By way of an example, some of the rpoB-mutations and their corresponding LiPA pattern are shown in Fig. 9.

Although the probes from the same group (e.g. probes S4, S44, S444 and S4444) are differing only slightly from each other, their hybridization characteristics may vary considerably. This is illustrated by way of example in an experiment in which the

performance of probes S44 and S4444 is compared using PCR products originating from two *M. tuberculosis* strains (ITG 8872 and ITG 9081) both displaying a wild-type sequence in the target region of probes S44 and S4444. The difference between both probes is shown below :

```

5   S44      GGTGACCCACAAGCGCCGA
      |||||
S4444      TTGACCCACAAGCGCCGACTGTC

```

Probe S44 was chosen on a theoretical basis from the known gene sequence (Telenti et al., 1993a). This probe would be theoretically the best probe for discriminating mismatches in the CAC-codon (underlined) since this codon is in the central part of the probe. However, in contrast to the general rules, the performance of probe S4444 proved to be significantly better (as described in the following experiment).

Both probes were applied to nitrocellulose strips in different amounts (2.4, 1.2 and 0.6 pmol/strip). After fixation and blocking of the strips, the probes were hybridized with biotinylated PCR fragments (from strains IGT 8872 and ITG 9081) as described earlier. The results are shown in Fig. 3.

Under the hybridization conditions used (3 x SSC, 20% deionized formamide, 50°C) the signals obtained with probe S44 are very weak. On the other hand probe S4444 generates very strong and reliable signals. Consequently, probe S4444 is preferred over S44 for use on the LiPA-strip. This dramatic effect cannot only be attributed to the difference in length of both probes, but also the location seems to be of importance. Most probably the secondary structure of the target region of the probes highly influences the hybridization characteristics.

In Fig. 4 the results of another experiment are shown in which the performance of both probes is compared in the context of the other probes. Both strips A and B are identically processed using amplified product originating from strain ITG 8872. Both strips are almost identical except that strip A contains probe S44 (0.6 pmol) and strip B probe S4444 (0.1 pmol) and the order of the probes applied to the strip is different. On strip A probe S44 is negative whereas on strip B probe S4444 is clearly positive, although in both cases there is a perfect match between the target and the probe and in both cases a positive result would have been expected.

These results clearly illustrate that probe design, especially under the fixed conditions of the reverse hybridization format (the same conditions for each probe) is not

2192364

29

straightforward and probes have to be evaluated meticulously before they can be used in a reverse hybridization format.

In general we can state that suitable probes cannot always be simply derived on a theoretical basis from a known gene sequence.

5 Although not detected among the isolates tested in the current invention, the presence of a silent mutation may give rise to a resistant pattern on the strips (one wild-type probe missing) while the strain is sensitive. Up to now only one silent substitution has been described (in codon 528 : CGC → CGT; Telenti et al., 1993a). This mutation would result in a destabilization of the hybrid with probe S-4444. However, by adding to the strip a probe
10 specific for the silent mutation (Probe SIL-1, Table 2), this silent mutation can be discriminated from resistance inducing mutations and would prevent misinterpretation of the pattern observed. Moreover, the SIL-probes can be applied on the strip at the same location than the corresponding S-probes (mixed probes). Doing so, no loss of hybridisation signal would be observed as a result of the silent mutation.

15 In order to detect the insertion mutations conferring rifampicin resistance, 514insF and 514insFM (see Fig. 1), two new wild-type probes were designed, S6 and S66, the sequence of which is represented in Table 2. Hybridization with nucleic acids originating from strains harbouring these insertion mutations, results in the absence of a hybridization signal with S6 and S66 (see also Table 1).

20 In order to *positively* identify more mutations than those detected by the set of R2, R4A, R4B and R5, a number of additional R-probes were designed, which may be added to the LiPA strip, using the same hybridization and wash conditions. Together with the above-described R-probes, the additional R-probes (R1, R2B, R2C, R3, R4C, R4D, R4E, R5B, R5C) allow a positive identification of the mutations most frequently encountered in the strain
25 collection tested in the current application.

Example 4 : Probe specific for the M. tuberculosis complex

A line probe assay was developed in order to enable the simultaneous detection of the mutations causing resistance to rifampicin directly coupled to detection of the pathogen in casu M. tuberculosis.

5 Since it is extremely advantageous to be able to detect *simultaneously* the presence of M. tuberculosis and the presence or absence of a gene or mutation causing drug-resistance, the aim was to develop a M. tuberculosis probe contained in the same PCR fragment as at least one of the relevant resistance markers to be identified. Therefore rpoB genes of non-M. tuberculosis isolates were sequenced. These organisms were : M.
10 paratuberculosis 316F, M. avium ITG5887, M. scrofulaceum ITG4979 and M. kansasii ITG4987. The sequences of the corresponding rpoB gene fragments are shown in Fig. 5 to 8, respectively. In addition, the sequence of the rpoB gene fragments of M. leprae and M. intracellulare were known from the literature (Honoré and Cole, 1993; Guerrero et al., 1994). Comparison of these sequences with the rpoB gene sequence of M. tuberculosis
15 enabled the delineation of a specific region (outside the region responsible for resistance) in the rpoB gene fragment from which the development of probes, potentially specific for M. tuberculosis appeared feasible. An oligonucleotide probe (further referred to as MT-POL-1) derived from that region with the following sequence :

GGA CGT GGA GGC GAT CAC ACC (SEQ ID NO 23)

20 was further evaluated with respect to its sensitivity and specificity by hybridization on LiPA strips. The amplification and hybridization conditions were as described above. The results are summarized in Table 3. In addition to the four M. tuberculosis strains listed in Table 3, 521 more M. tuberculosis clinical isolates were tested: all isolates gave a positive hybridization signal. Evidently, also M. bovis strains hybridize with the probe, but it is
25 clinically not relevant to distinguish M. tuberculosis from M. bovis for this purpose. In fact, throughout the application the term "M. tuberculosis" can be replaced by "M. tuberculosis complex", referring to M. tuberculosis s.s., M. bovis, M. africanum and M. microti, without affecting the significance of the results.

Although, using the sets of primers described in example 1, a PCR product may be
30 obtained with DNA of some other Mycobacterium species and even some genetically

unrelated micro-organisms which might also be present in the respiratory tract. none of these bacteria showed hybridization with the selected MT-POL-1 probe. In conclusion we can state that the selected probe is highly specific for M. tuberculosis complex strains and 100% sensitive for M. tuberculosis. Also other probes from this region might be useful for the specific detection of M. tuberculosis complex strains, such as : MT-POL-2, MT-POL-3, MT-POL-4 and MT-POL-5 (see Table 2).

In a similar way, the following probes can be used to differentiate M. avium, M. paratuberculosis, M. scrofulaceum , M. kansasii, M. intracellulare and M. leprae strains from each other and from other mycobacteria : MA-POL-1, MP-POL-1, MS-POL-1, MK-POL-1, MI-POL-1 and ML-POL-1 respectively (see table 2B).

Example 5 : Evaluation of LiPA strips for M. tuberculosis

LiPA strips were prepared carrying the following probes (in addition to a positive control line) : MT-POL-1, S11, S2, S33, S4444, S5555, R2, R444A, R444B, R55.

These strips were hybridized with PCR products from M. tuberculosis strains for which the relevant rpoB gene sequence was determined.

Some representative results are summarized in Table 4.

The hybridization results completely correlated with the sequencing results, indicating that the probes used can discriminate at the level of a single mismatch. Each mutation screened for could be detected either by the absence of one of the wild-type probes (S-probes) or by the absence of a wild-type probe together with the presence of the corresponding mutant probe (R-probe). In the latter case, the exact mutation present can be derived from the hybridization results. However, the knowledge of the exact mutation present is not necessary to determine whether or not one is dealing with a strain resistant to rifampicin since each mutation screened for confers resistance. Sensitive strains, i.e. strains without mutations in the relevant part of the rpoB gene, give positive reactions on all S-probes while all R-probes score negative (= wt-pattern).

Example 6 : Direct detection of M. tuberculosis strains and rifampicin-resistance in clinical samples

Sixty-eight clinical specimens from different geographical origins (13 and 35 sputum specimens from Belgium and Rwanda respectively, and 20 lymph node biopsies from Burundi) all positive in culture for M. tuberculosis and kept at -20°C were analyzed. Sample preparation for amplification was based on the procedure of Boom et al. (1990) modified by De Beenhouwer et al. (submitted). A nested PCR approach for the relevant region of the rpoB gene was carried out with biotinylated inner primers (P3 and P4). After thermal cycling, the amplified product was incubated with the LiPA strip. Rifampicin resistance was determined on Löwenstein-Jensen using the proportion method of Canetti et al. (1963). For resistant strains, the MIC (Minimal Inhibitory Concentration) of rifampicin was determined on 7H10 agar (Heifets, 1988).

Microscopically, 20 (29.4%) samples were negative, 15 (22.1%) weakly positive (1+ or less according to the scale of the American Thoracic Society) and 33 (48.5%) strongly positive ($\geq +2$) upon Ziehl-Neelsen staining.

The LiPA detected rifampicin sensitivity in 49 specimens (only *M. tuberculosis* and wild-type probes positive) and resistance in 19 specimens (M. tuberculosis probe positive, one of the wild-type probes missing, and eventually one of the mutation probes positive). *In vitro* rifampicin susceptibility testing confirmed these results with three exceptions. For all the sensitive strains, a sensitive probe pattern was observed indicating that possible silent mutations were not detected in this series. All strains displaying a resistance pattern were found to be resistant by conventional techniques. For three specimens (from three multidrug resistant patients from Rwanda) a sensitive pattern was obtained by PCR-LiPA while culture revealed resistance (MIC > 2 µg/ml on 7H10). Sequencing of the rpoB region of these strains confirmed the wild-type gene sequence, possibly implying a different mechanism of rifampicin resistance in these cases or a mutation in another part of the rpoB-gene. It is also interesting to note that the nested PCR system gave positive results for all the tested specimens positive in culture including 20 Ziehl-Neelsen negative specimens. In another experiment (data not shown) including 17 smear negative sputum specimens from clinically suspected tuberculosis cases negative in culture, no signal at all was obtained with the LiPA system, indicating that the infections were most probably not caused by M. tuberculosis.

In a subsequent experiment, a large collection of clinical specimens from different geographical origin were tested in LiPA. Results are shown in Table 5. Of the 137 resistant strains found, quite a large number could be attributed to one of the mutations represented by the R-probes (R2, R4A, R4B, R5). Interestingly, some mutations seem to be more frequent in some countries as compared to others (e.g. in Tunisia and Egypt: R4B (=H526D), in Rwanda: R5 (S531L)). This may eventually lead to different test formats for different countries.

On a total of 213 rifampicin-resistant strains analysed by LiPA in the current application, 151 (71%) could be attributed to the mutations S531L, H526D, D516V or H526Y, and were thus detectable by a positive signal with respectively probes R5, R4B, R2 and R4A (see fig. 10).

On a total of 180 strains analysed both by culture and by LiPA, correct identification (sensitive/resistant) was made in 164 (=91.1%) of the strains (see Table 6). In three resistant strains, LiPA results and sequencing showed a wild-type rpoB-gene fragment, which indicates that the mechanism for rifampicin resistance could not be attributed to mutations in the investigated part of the rpoB gene.

Thirteen of the 180 analyzed strains were resistant in LiPA but seemed to be sensitive in culture. However, after reculturing 2 of these 13 strains in synthetic 7H11 medium instead of the traditional Löwenstein Jensen medium, they turned out to be rifampicin resistant anyway. This until now unpublished finding shows that the conventional Löwenstein Jensen medium is not recommended for determination of antibiotic susceptibility of mycobacteria (possibly due to the presence of traces of antibiotics found in commercially available eggs used to prepare Löwenstein Jensen medium). Therefore, the percentage of discrepant strains which are resistant in LiPA but sensitive in culture (in this case $13/180 = 7.2\%$) is expected to be much lower (and possibly 0%) when culturing is done on a synthetic medium like 7H11.

Interestingly, most of the rifampicin resistant isolates (>90%) examined in the current application were in addition resistant to isoniazid, and thus multidrug-resistant (definition of multidrug resistance = resistant to at least isoniazid and rifampicin). Rifampicin resistance can thus be considered as a potential marker for multidrug resistance, and the above-described LiPA test may therefore be an important tool for the control of multidrug resistant tuberculosis.

In conclusion, the above-described method permits correct identification of M. tuberculosis and simultaneous detection of rifampicin resistance directly in clinical specimens without preculturing. It enables easy detection of rifampicin resistance directly in a clinical specimen in less than 24 hours.

Example 7 Detection of Rifampicin resistance in M. leprae

The above-described detection approach can also be applied to the detection of M. leprae present in biological samples coupled to the detection of its resistance to rifampicin. The sequence of the rpoB gene of M. leprae was described earlier by Honoré and Cole (1993). They only identified a limited number of mutations responsible for rifampicin resistance in M. leprae. It can be reasonably expected that, similar to M. tuberculosis, a lot of other mutations may cause rifampicin resistance in M. leprae, and that most of these mutations will be localized in a rather restricted area of the rpoB gene, corresponding to the "mutation region" described earlier in this application.

Therefore, a set of wild-type probes is selected overlapping the putative mutation region in the rpoB-gene of M. leprae (see table 2B):

ML-S1	(SEQ ID NO 58)
ML-S2	(SEQ ID NO 59)
ML-S3	(SEQ ID NO 60)
ML-S4	(SEQ ID NO 61)
ML-S5	(SEQ ID NO 62)
ML-S6	(SEQ ID NO 63).

Rifampicin resistance is revealed by an absence of hybridization with at least one of these ML-S probes. This set of ML-S probes will detect rifampicin-resistance causing mutations in this region, even though the sequence of these mutations has not yet been specified.

The above-mentioned ML-S probes have been carefully designed in such a way that they can all be used under the same hybridization and wash conditions. The same holds for the species specific ML-POL-1 probe, with which the ML-S probes can be combined to allow a simultaneous detection of M. leprae and its resistance to rifampicin.

All the probes mentioned in this example are contained in the same rpoB gene fragment of M. leprae, which can be obtained by PCR using a set of primers chosen from MGRPO-1 or MGRPO-2 (5' primers) and MGRPO-3 or MGRPO-4 (3' primers).

Table 1 :

Abbr.	Position		Wild-type sequence			Mutation sequence			Probe detection	
	Nucl.	Codon	Nucl.	Codon	AA	Nucl.	Codon	AA	Δ Wt probe	Mutant probe
L511P	9	511	T	CTG	Leu	C	CCG	Pro	S11	-
L511R	9	511	T	CTG	Leu	G	CGG	Arg	S11	-
S512T	12	512	G	AGC	Ser	C	ACC	Thr	S11	-
Q513L	15	513	A	CAA	Gln	T	CTA	Leu	S11	-
Q513K	14	513	C	CAA	Gln	A	AAA	Lys	S11	-
514 ins F	16	514	-	-	-	TTC	TTC	Phe	S6	-
514 ins FM	16	514	-	-	-	TTC ATG	TTC ATG	Phe Met	S6	-
Δ514-516	15-23	514-516	AA TTC ATG G	AA TTC ATG G	Gln Phe Met Asp	Δ	Δ	His	S1-S2-S6	-
Δ516-517	23-28	516-517	GAC CAG	GAC CAG	Asp Gln	Δ	Δ	Δ	S2-S6	-
D516Y	23	516	G	GAC	Asp	T	TAC	Tyr	S2	-
D516V	24	516	A	GAC	Asp	T	GTC	Val	S2	R2
D516E	25	516	C	GAC	Asp	G	GAG	Glu	S2	-

D516G(')	24	516	A	GAC	Asp	G	GGC	Gly	S2	-
Δ517-518	26-31	517-518	CAG AAC	<u>CAG AAC</u>	Gln Asn	Δ	Δ	Δ	S2	-
Δ518	29-31	518	AAC	<u>AAC</u>	Asn	Δ	Δ	Δ	S2	-
S522L	42	522	C	<u>TCG</u>	Ser	T	<u>TTG</u>	Leu	S33	-
H526Y	53	526	C	<u>CAC</u>	His	T	<u>TAC</u>	Tyr	S4444	R444A
H526D	53	526	C	<u>CAC</u>	His	G	<u>GAC</u>	Asp	S4444	R444B
H526N	53	526	C	<u>CAC</u>	His	A	<u>AAC</u>	Asn	S4444	-
H526C(')	53-54	526	CA	<u>CAC</u>	His	TG	<u>TGC</u>	Cys	S4444	-
H526R	54	526	A	<u>CAC</u>	His	G	<u>CGC</u>	Arg	S4444	-
H526P	54	526	A	<u>CAC</u>	His	C	<u>CCC</u>	Pro	S4444	-
H526Q	55	526	C	<u>CAC</u>	His	^A G	<u>CA^A_G</u>	Gln	S4444	-
H526L	54	526	A	<u>CAC</u>	His	C	<u>CTC</u>	Leu	S4444	-
H526T(')	53-54	526	CA	<u>CAC</u>	His	AC	<u>ACC</u>	Thr	S4444	-
R529Q(')	63	529	G	<u>CGC</u>	Arg	A	<u>CAA</u>	Gln	S4444	-
S531Q	68-69	531	TC	<u>TCG</u>	Ser	CA	<u>CAG</u>	Gln	S5555	-
S531L	69	531	C	<u>TCG</u>	Ser	T	<u>TTG</u>	Leu	S5555	R55
S531W	69	531	C	<u>TCG</u>	Ser	G	<u>TGG</u>	Trp	S5555	-
S531Y	69-70	531	CG	<u>TCG</u>	Ser	^A T _C	<u>TA^T_C</u>	Tyr	S5555	-

S531C	69-70	531	CG	TCG	Ser	GT	TGT	Cys	S5555	-
L533P	75	533	T	CTG	Leu	C	CCG	Pro	S5555	-

Table 2 :
Primers and probes selected from the rpoB gene

2A: M. tuberculosis

Primers

		<u>SEQ ID NO</u>
P1	GAGAATTCGGTCGGCGAGCTGATCC	30
P2	TACGGTCGGCGAGCTGATCC	
P3	GGTCGGCATGTCGCGGATGG	31
P4	GCACGTCGCGGACCTCCAGC	32
P5	CGAAGCTTGACCCGCGCTACACC	33
P6	TACGGCGTTTCGATGAACC	
P7	CGGCATGTCGCGGATGGAGCG	41
P8	CGGCTCGCTGTCGGTGTACGC	42

Probes

species-specific probes

MT-POL-1	GGACGTGGAGGCGATCACACC	23
MT-POL-2	CGATCACACCGCAGACGTTGATC	24
MT-POL-3	CATCCGGCCGGTGGTCGC	25
MT-POL-4	CTGGGGCCCCGGCGGTCT	26
MT-POL-5	CGGTCTGTCACGTGAGCGTG	27

wild-type probes

S1	CAGCCAGCTGAGCCAATTCATG	1
S11	CAGCCAGCTGAGCCAATTCAT	2
S2	TTCATGGACCAGAACAACCCGC	3
S3	AACCCGCTGTCGGGGTTGA	4
S33	AACCCGCTGTCGGGGTTGACC	5
S4	GTTGACCCACAAGCGCCGA	6
S44	GGTTGACCCACAAGCGCCGA	7
S444	TTGACCCACAAGCGCCGACTGT	43
S4444	TTGACCCACAAGCGCCGACTGTC	8
S5	GACTGTCGGCGCTGGGG	9
S55	CGACTGTCGGCGCTGGGGC	10
S555	GACTGTCGGCGCTGGGGCC	39
S5555	GACTGTCGGCGCTGGGGC	40
S55C	GCCCCAGCGCCGACAGTCG	44
S55M	CGACTGTCGGCGTTGGGGC	45
S6	TGAGCCAATTCATGGACCAGAA	11
S66	CTGAGCCAATTCATGGACCAGA	12
SIL-1	CCACAAGCGTCGACTGTCTG	13

mutant probes

R2	AATTCATGGTCCAGAACAACCCG	14
R4A	GGTTGACCTACAAGCGCCGA	15
R44A	GGGTTGACCTACAAGCGCCGA	16
R444A	TTGACCTACAAGCGCCGACTGTC	17
R4B	GTTGACCGACAAGCGCCGA	18
R44B	GGTTGACCGACAAGCGCCGA	19
R444B	TTGACCGACAAGCGCCGACTGTC	20
R5	CGACTGTTGGCGCTGGGG	21
R55	CGACTGTTGGCGCTGGGGC	22
R1	CAGCCAGCCGAGCCAATTCAT	46
R2B	AATTCATGTACCAGAACAACCCG	47
R2C	ATGGACCAGAACCCGCTGTCTG	48
R3	AACCCGCTGTTGGGGTTGACC	49
R4C	TTGACCCGCAAGCGCCGACTGTC	50
R4D	TTGACCCCCAAGCGCCGACTGTC	51
R4E	TTGACCTGCAAGCGCCGACTGTC	52
R5B	CGACTGTGGGCGCTGGGGC	53
R5C	ACTGTGGGCGCCGGGGCCC	54

2B: other mycobacterial species**primers**

MGRPO-1	CCAAAACCAGATCCGGGGTCGG	64
MGRPO-2	GTCCGGGAGCGGATGACCAC	65
MGRPO-3	GGGTGCACGTCGCGGACCTC	66
MGRPO-4	GGGCACATCCGGCCGTAGTG	67

probes

MP-POL-1	CATCCGTCCCGTCGTGGC	28
MA-POL-1	CATCCGTCCAGTCGTGGCG	29
MS-POL-1	GCCGGTCGTGGCCGCG	38
MK-POL-1	AGCGCCGGCTTTCGGCGC	55
ML-POL-1	GACGCTGATCAATATCCGTCCGG	57
MI-POL-1	ATCCGGCCGGTCGTGCGCC	68
ML-S1:	CAGCCAGCTGTCTGCAGTTCATG	58
ML-S2:	GTTCATGGATCAGAACAACCCTC	59
ML-S3:	AACCCTCTGTCTGGGCCTGACC	60
ML-S4:	ACCCACAAGCGCCGGCTGTC	61
ML-S5:	GGCTGTCTGGCGCTGGGC	62
ML-S6:	CTGTCTGCAGTTCATGGATCAGA	63

Table 3 : Hybridization results obtained with probe MT-POL-1

	Species	Strain	MT-POL-1
1	M. tuberculosis	ATCC 27294	+
2	M. tuberculosis	NCTC 7417	+
3	M. tuberculosis	ITG 8017	+
4	M. tuberculosis	ITG 9173	+
5	M. bovis	BCG (Kopen)	+
6	M. bovis	patient isolate	+
7	M. avium	ITG 5872	-
8	M. avium	ITG 5874	-
9	M. intracellulare	ITG 5913	-
10	M. intracellulare	ITG 5918	-
11	M. paratuberculosis	2 E	-
12	M. kansasii	ITG 4987	-
13	M. marinum	ITG 7732	-
14	M. scrofulaceum	ITG 4988	-
15	M. gordonae	ITG 4989	-
16	Bordetella pertussis	NCTC 8189	-
17	Bordetella parapertussis	NCTC 7385	-
18	Bordetella bronchiseptica	NCTC 8761	-
19	Moraxella catarrhalis	LMG 5128	-
20	Moraxella catarrhalis	LMG 1133	-
21	Moraxella catarrhalis	LMG 4200	-
22	Moraxella catarrhalis	LMG 4822	-
23	Haemophilus influenzae	NCTC 8143	-
24	Haemophilus influenzae	ITG 3877	-
25	Streptococcus pneumoniae	H90-11921	-
26	Streptococcus pneumoniae	H91-04493	-
27	Streptococcus pneumoniae	H90-11780	-
28	Pseudomonas cepacia	ATCC 25609	-
29	Acinetobacter calcoaceticus	ATCC 23055	-
30	Staphylococcus aureus	6420	-
31	Staphylococcus aureus	6360	-
32	Pseudomonas aeruginosa	5682	-
33	Pseudomonas aeruginosa	5732	-

Table 4: Interpretation of LiPA-results for rifampicin resistance detection in M. tuberculosis

MT- POL-1	Hybridization results with probes								LiPA pattern	Mutation (verified by sequencing)	Interpretation
	S11	S2	S33	S4444	S55	R2	R444A	R444B			
+	+	+	+	+	+	-	-	-	wt	-	Sensitive
+	-	+	+	+	+	-	-	-	ΔS1	in S1	Resistant
+	+	-	+	+	+	-	-	-	ΔS2	in S2	Resistant
+	+	-	+	+	+	+	-	-	R2	D516V	Resistant
+	+	+	-	+	+	-	-	-	ΔS3	in S3	Resistant
+	+	+	+	-	+	-	-	-	ΔS4	in S4	Resistant
+	+	+	+	-	+	-	+	-	R4A	H526Y	Resistant
+	+	+	+	-	+	-	-	+	R4B	H526D	Resistant
+	+	+	+	+	-	-	-	-	ΔS5	in S5	Resistant
+	+	+	+	+	-	-	-	-	R5	S531L	Resistant

Table 5: Occurrence of different mutations in *M. tuberculosis* strains originating from different countries

#	Mutation	Bel	Bengla	Benin	Bur-Fa	Buru	Can	Chi	Col	Egy	Gui	Hon	Pak	Rwa	Tun
2	ΔS1		1											1	1
2	ΔS2		1											1	
4	ΔS3	1			3										
19	ΔS4	1	1				2	2	1	1			1	8	2
8	ΔS5		1					1			2		1	2	1
11	R2	2							1				2		6
15	R4a	4	1	1	2					2			1	2	2
14	R4b				1			1		3				1	8
56	R5	9	1				1	1	1		1		6	26	10
1	R2+?														1
2	R4a+R5												1		1
1	R4b+R5													1	
1	ΔS1/R2							1							1
1	ΔS1/ΔS2														
137	Totaal	17	6	1	6	0	3	6	3	6	3	0	12	41	33

Table 6 : Comparison of LiPA results versus rifampicin resistance determination in culture for M. tuberculosis.

		Culture	
		S	R
LiPA	S	71	3
	R	13	93

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29233-1

48a

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Innogenetics N.V.
- (B) STREET: Industriepark Zwijnaarde 7 Bus 4
- (C) CITY: Gent
- (E) COUNTRY: Belgium
- (F) POSTAL CODE (ZIP): 9052
- (G) TELEPHONE: 00-32-09.241.07.11
- (H) TELEFAX: 00-32-09.241.07.99

(ii) TITLE OF INVENTION: Probes, methods and kits for detection of mycobacterial nucleic acids in biological samples

(iii) NUMBER OF SEQUENCES: 69

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM™ PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS™
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CAGCCAGCTG AGCCAATTCA TG

22

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAGCCAGCTG AGCCAATTCA T

21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

48b

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
 TTCATGGACC AGAACAACCC GC 22

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 19 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
 AACCCGCTGT CGGGGTTGA 19

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
 AACCCGCTGT CGGGGTTGAC C 21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 19 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
 GTTGACCCAC AAGCGCCGA 19

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
 GGTTGACCCA CAAGCGCCGA 20

48c

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TTGACCCACA AGCGCCGACT GTC

23

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GACTGTCGGC GCTGGGG

17

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CGACTGTCGG CGCTGGGGC

19

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TGAGCCAATT CATGGACCAG AA

22

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs

48d

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CTGAGCCAAT TCATGGACCA GA

22

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CCACAAGCGT CGACTGTCG

19

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

AATTCATGGT CCAGACAAC CCG

23

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGTTGACCTA CAAGCGCCGA

20

(2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

A

48e

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGGTTGACCT ACAAGCGCCG A

21

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTGACCTACA AGCGCCGACT GTC

23

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GTTGACCGAC AAGCGCCGA

19

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGTTGACCGA CAAGCGCCGA

20

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TTGACCGACA AGCGCCGACT GTC

23

48f

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CGACTGTTGG CGCTGGGG

18

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 19 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CGACTGTTGG CGCTGGGGC

19

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGACGTGGAG GCGATCACAC C

21

(2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 23 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CGATCACACC GCAGACGTTG ATC

23

(2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid

48g

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CATCCGGCCG GTGGTCGC

18

(2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CTGGGGCCCG GCGGTCT

17

(2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CGGTCTGTCA CGTGAGCGTG

20

(2) INFORMATION FOR SEQ ID NO: 28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CATCCGTCCC GTCGTGGC

18

(2) INFORMATION FOR SEQ ID NO: 29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

48h

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:
CATCCGTCCA GTCGTGGCG 19

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:
GAGAATTCGG TCGGCGAGCT GATCC 25

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:
GGTCGGCATG TCGCGGATGG 20

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:
GCACGTCGCG GACCTCCAGC 20

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:
CGAAGCTTGA CCCGCGCTAC ACC 23

48i

(2) INFORMATION FOR SEQ ID NO: 34:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 329 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GATCCGGGTC GGCATGTCGC GGATGGAGCG GGTGGTCCGG GAGCGGATGA CCACCCAGGA	60
CGTGGAGGCG ATCACACCGC AGACGTTGAT CAACATCCGG CCGGTGGTCG CCGCGATCAA	120
GGAGTTCTTC GGCACCAGCC AGCTGAGCCA ATTCATGGAC CAGAACAACC CGCTGTCGGG	180
GTTGACCTGC AAGCGCCGAC TGTCGGCGCT GGGGCCCGN GGTCTGTCAC GTGAGCGTGC	240
CGGGCTGGAG GTCCGCGACG TGCACCCGTC GCACTACGN CGGATGTGCC CTATCGAAAC	300
CCCTGAGGGG GCCAACATCG NTCTTTATC	329

(2) INFORMATION FOR SEQ ID NO: 35:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 319 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

TCCGGGTCGG CATGTCCCGG ATGGAGTGTG TCGTCCNGA GCGGATGACC ANCCAGGACG	60
TNGAGGCCAT CACGCCGAG ACCCTGATCA ACATCCGTCC CGTCGTGGCG GCGATCAAGG	120
AGTTCTTCGG NACCAGCCAG TTGTCCAGT TCATGGACCA GAACAACCCG CTGTGCGGGC	180
TCACCCACAA GCGCCGCCTG TCGGCGNTGG GCCCGGGTGG TCTGTCCCGG GAGCGGGCCG	240
GGCTGGAGGT CCGNGACGTG NACCCGTCCC ACTACGGCCG GATGTGCCCC ATCGAGACCC	300
CGGAGGGTCC CAACATCGG	319

(2) INFORMATION FOR SEQ ID NO: 36:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 324 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

GGATGGAGCG CTCCGTCCGC GAGCGGATGA CCACCCAGGA CGTCGAGGCC ATCACGCCGC	60
---	----

48j

AGACCCTGAT CAACATCCGT CCAGTCGTGG CGGCGATCAA GGAGTTCTTC GGCACCAGCC 120
 AGCTGTCCCA GTTCATGGAC CAGAACAACC CGCTGTCGGG GCTCACCCAC AAGCGCCGCC 180
 TGTCGGCGCT GGGCCCGGGT GGTCTGTCCC GGGAGCGGGC CGGGCTGGAG GTCCGCGACG 240
 TGCACCCGTC CCACTACGGC CGGATGTGCC CGATCGAGAC CCCGGAGGGT CCCAACATCG 300
 GTCTGATCGG CTCGCTGTCG GTGT 324

(2) INFORMATION FOR SEQ ID NO: 37:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 254 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

CGGGTCGGCA TGTCCCGCAT GGAGCGGGTC GTCCGCGAGC GGATGACCAC GCAGGACGTC 60
 GAGGCGATCA CGCCGCAGAC CCTGATCAAC ATCCGGCCGG TCGTGGCCGC GATCAAGGAG 120
 TTCTTCGGCA CCAGCCAGCT CTCGCAGTTC ATGGACCAGA ACAACCCGNT GTCGGGCCTG 180
 ACCCACAAGC GCCGCCTGTC GGTGCTGGGC CCGGTTGGTC TGTCCCGCGA GCGGGCCGGG 240
 TTGGAGGTCC GGAG 254

(2) INFORMATION FOR SEQ ID NO: 38:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 16 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

GCCGGTCGTG GCCGCG 16

(2) INFORMATION FOR SEQ ID NO: 39:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 19 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

GACTGTCGGC GCTGGGGCC 19

48k

(2) INFORMATION FOR SEQ ID NO: 40:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

GACTGTCGGC GCTGGGGC

18

(2) INFORMATION FOR SEQ ID NO: 41:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

CGGCATGTCTG CGGATGGAGC G

21

(2) INFORMATION FOR SEQ ID NO: 42:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

CGGCTCGCTG TCGGTGTACG C

21

(2) INFORMATION FOR SEQ ID NO: 43:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

TTGACCCACA AGCGCCGACT GT

22

(2) INFORMATION FOR SEQ ID NO: 44:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs

481

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

GCCCCAGCGC CGACAGTCG

19

(2) INFORMATION FOR SEQ ID NO: 45:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

CGACTGTCGG CGTTGGGGC

19

(2) INFORMATION FOR SEQ ID NO: 46:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

CAGCCAGCCG AGCCAATTCA T

21

(2) INFORMATION FOR SEQ ID NO: 47:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

AATTCATGTA CCAGAACAAAC CCG

23

(2) INFORMATION FOR SEQ ID NO: 48:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

A

48m

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

ATGGACCAGA ACCCGCTGTC G

21

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

AACCCGCTGT TGGGGTTGAC C

21

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

TTGACCCGCA AGCGCCGACT GTC

23

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

TTGACCCCCA AGCGCCGACT GTC

23

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

TTGACCTGCA AGCGCCGACT GTC

23

48N

(2) INFORMATION FOR SEQ ID NO: 53:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

CGACTGTGGG CGCTGGGGC

19

(2) INFORMATION FOR SEQ ID NO: 54:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

ACTGTGGGCG CCGGGGCC

19

(2) INFORMATION FOR SEQ ID NO: 55:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

AGCGCCGGCT TTCGGCGC

18

(2) INFORMATION FOR SEQ ID NO: 56:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

AGCGCCGGCT TTCGGCGC

18

(2) INFORMATION FOR SEQ ID NO: 57:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs

480

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

GACGCTGATC AATATCCGTC CGG

23

(2) INFORMATION FOR SEQ ID NO: 58:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

CAGCCAGCTG TCGCAGTTCA TG

22

(2) INFORMATION FOR SEQ ID NO: 59:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

GTTCATGGAT CAGAACAACC CTC

23

(2) INFORMATION FOR SEQ ID NO: 60:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

AACCCTCTGT CGGGCCTGAC C

21

(2) INFORMATION FOR SEQ ID NO: 61:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

48p

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:
ACCCACAAGC GCCGGCTGTC 20

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:
GGCTGTCCGC GCTGGGC 17

(2) INFORMATION FOR SEQ ID NO: 63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63:
CTGTCGCAGT TCATGGATCA GA 22

(2) INFORMATION FOR SEQ ID NO: 64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64:
CCAAAACCAG ATCCGGGTCG G 21

(2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65:
GTCCGGGAGC GGATGACCAC 20

48q

(2) INFORMATION FOR SEQ ID NO: 66:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66:

GGGTGCACGT CGCGGACCTC

20

(2) INFORMATION FOR SEQ ID NO: 67:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 67:

GGGCACATCC GGCCGTAGTG

20

(2) INFORMATION FOR SEQ ID NO: 68:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

ATCCGGCCGG TCGTCGCC

18

(2) INFORMATION FOR SEQ ID NO: 69:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 226 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

GGCATTACG GATGGAACGC GTGGTCCGCG ACGGATGACC ACGCAGGACG TCGAGGCCAT 60

CACGCCGAG ACCCTGATCA ACATCCGGCC GGTCGTCGCC GCGATCAAGG AGTTCTTCGG 120

GACCAGCCAG CTGTGCGAGT TCATGGACCA GAACAACCCG CTGTGCGGTC TGACCCACAA 180

GCGTCGCCTG TCGGCGCTGG GTCCCGGCGG TCTGTCCCGT GAGCGC 226

CLAIMS:

1. Method for the detection of at least one of rifampicin resistance and rifabutin resistance of Mycobacterium species present in a sample, comprising the
5 steps of:

(i) hybridizing polynucleic acids in said sample with a set of wild-type probes, at least one of which is an S-probe from Table 2, each probe of said set spanning more than one polymorphic site of the polynucleic acid, and said
10 set overlapping a complete mutation region of a rpoB gene delimited in Figure 1, under appropriate hybridization and wash conditions;

(ii) detecting the hybrids formed in step (i); and

(iii) determining the rifampicin susceptibility
15 (sensitivity versus resistance) of the Mycobacterium species present in the sample from the differential hybridization signal(s) obtained in step (ii).

2. The method according to claim 1 further comprising one or both of the following additional steps prior to the
20 hybridization step (i):

releasing, isolating or concentrating the polynucleic acids present in the sample; and

amplifying the relevant part of the rpoB gene with at least one suitable primer pair.

25 3. The method according to claim 1 or 2 wherein said set of wild-type probes consists of 2, 3, 4, 5, 6, 7, 8 or 9 wild-type probes.

4. The method according to any one of claims 1 to 3 wherein the set of wild-type probes in step (i) is further combined with at least one species-specific probe for M. tuberculosis, M. paratuberculosis, M. avium, M. scrophi
5 scrophulaceum, M. kansasii, M. intracellulare (and MAC-strains) or M. leprae.

5. The method according to claim 4 wherein the species-specific probe(s) for M. tuberculosis is (are) selected from the group consisting of:

10 MT-POL-1 (SEQ ID NO 23);
MT-POL-2 (SEQ ID NO 24);
MT-POL-3 (SEQ ID NO 25);
MT-POL-4 (SEQ ID NO 26); and
MT-POL-5 (SEQ ID NO 27).

15 6. The method according to claim 4 wherein the species-specific probe(s) for M. paratuberculosis, is (are) selected from the group consisting of:

MP-POL-1 (SEQ ID NO 28); and

any M. paratuberculosis specific probe derived
20 from the sequence of the relevant part of the rpoB gene of M. paratuberculosis (SEQ ID NO 35).

7. The method according to claim 4 wherein the species-specific probe(s) for M. avium, is (are) selected from the group consisting of:

25 MA-POL-1 (SEQ ID NO 29); and

any M. avium specific probe derived from the sequence of the relevant part of the rpoB gene of M. avium (SEQ ID NO 36).

8. The method according to claim 4 wherein the
5 species-specific probe(s) for M. scrofulaceum, is (are) selected from the group consisting of:

MS-POL-1 (SEQ ID NO 38); and

any M. scrofulaceum specific probe derived from the sequence of the relevant part of the rpoB gene of M.
10 scrofulaceum (SEQ ID NO 37).

9. The method according to claim 4 wherein the species-specific probe(s) for M. kansasii, is (are) selected from the group consisting of:

MK-POL-1 (SEQ ID NO 55); and

15 any M. kansasii specific probe derived from the sequence of the relevant part of the rpoB gene of M. kansasii (SEQ ID NO 56).

10. The method according to claim 4 wherein the species-specific probe(s) for M. intracellulare (and MAC-
20 strains), is (are) selected from the group consisting of:

MI-POL-1 (SEQ ID NO 68); and

any MAC-strain specific probe derived from the sequence of the relevant part of the rpoB gene of a MAC-strain (SEQ ID NO 69).

25 11. The method according to claim 4 wherein the species-specific probe for M. leprae consists of:

ML-POL-1 (SEQ ID NO 57).

12. The method according to any one of claims 1 to 11 wherein said set of wild-type probes in step (i) is further combined with at least one rpoB silent mutation probe.

13. The method according to any one of claims 1 to 12
5 wherein said set of wild-type probes in step (i) is further combined with at least one rpoB mutation probe from Table 2.

14. The method according to any one of claims 1 to 11 wherein the Mycobacterium species are M. tuberculosis.

15. The method according to claim 14 wherein said set
10 of wild-type probes in step (i) comprises at least one (S-)probe selected from the group consisting of:

S1 (SEQ ID NO 1);

S11 (SEQ ID NO 2);

S2 (SEQ ID NO 3);

15 S3 (SEQ ID NO 4);

S33 (SEQ ID NO 5);

S4 (SEQ ID NO 6);

S44 (SEQ ID NO 7);

S444 (SEQ ID NO 43);

20 S4444 (SEQ ID NO 8);

S5 (SEQ ID NO 9);

S55 (SEQ ID NO 10);

S555 (SEQ ID NO 39);

S5555 (SEQ ID NO 40);

53

S55C (SEQ ID NO 44);

S55M (SEQ ID NO 45);

S6 (SEQ ID NO 11); and

S66 (SEQ ID NO 12).

5 16. The method according to claim 14, wherein said set of wild-type probes comprises at least one (S-)probe selected from the group consisting of:

S11 (SEQ ID NO 2);

S2 (SEQ ID NO 3);

10 S33 (SEQ ID NO 5);

S4444 (SEQ ID NO 8);

S55 (SEQ ID NO 10); and

S5555 (SEQ ID NO 40).

17. The method according to claim 14, wherein said set
15 of wild-type probes comprises at least the following (S-)probes:

S4444 (SEQ ID NO 8); and

S55 or S5555 (SEQ ID NO 10) or (SEQ ID NO 40).

18. The method according to claim 17, wherein said set
20 of wild-type probes consists of all of the following (S-)probes:

S11 (SEQ ID NO 2);

S2 (SEQ ID NO 3);

54

S33 (SEQ ID NO 5);

S4444 (SEQ ID NO 8); and

S55 or S5555 (SEQ ID NO 10) or (SEQ ID NO 40).

19. The method according to any one of claims 14 to 18
5 wherein said set of wild-type probes in step (i) is combined
with at least one rpoB silent mutation probe from Table 2.

20. The method according to claim 19 wherein the rpoB
silent mutation probe consists of:

SIL-1 (SEQ ID NO 13).

10 21. The method according to any one of claims 14 to 20
wherein said set of wild-type probes in step (i) is further
combined with at least one rpoB mutation probe from Table 2.

22. The method according to claim 21 wherein the rpoB
mutation probe(s), or R-probe(s), is (are) selected from the
15 group consisting of:

R1 (SEQ ID NO 46);

R2 (SEQ ID NO 14);

R2B (SEQ ID NO 47);

R2C (SEQ ID NO 48);

20 R3 (SEQ ID NO 49);

R4A (SEQ ID NO 15);

R44A (SEQ ID NO 16);

R444A (SEQ ID NO 17);

R4B (SEQ ID NO 18);

55

R44B (SEQ ID NO 19);

R444B (SEQ ID NO 20);

R4C (SEQ ID NO 50);

R4D (SEQ ID NO 51);

5 R4E (SEQ ID NO 52);

R5 (SEQ ID NO 21);

R55 (SEQ ID NO 22);

R5B (SEQ ID NO 53); and

R5C (SEQ ID NO 54).

10 23. The method according to claim 21, wherein the R-probe(s) is (are) selected from the group consisting of:

R2 (SEQ ID NO 14);

R444A (SEQ ID NO 17);

R444B (SEQ ID NO 20); and

15 R55 (SEQ ID NO 22).

24. The method according to any one of claims 14 to 23 further comprising use of a probe or a set of primers designed to specifically detect or amplify the following new rpoB gene mutations :

20 D516G, representing a mutation in codon 516 of the rpoB gene, resulting in a change of amino acid D to G;

H526C, representing a mutation in codon 526 of the rpoB gene, resulting in a change of amino acid H to C;

H526T, representing a mutation in codon 526 of the rpoB gene, resulting in a change of amino acid H to T; or

R529Q, representing a mutation in codon 529 of the rpoB gene, resulting in a change of amino acid R to Q;

5 as specified in Table 1.

25. The method according to any one of claims 1 to 11 wherein the Mycobacterium species is M. leprae.

26. The method according to claim 25 wherein said set of wild-type probes in step (i) comprises at least one
10 (S-)probe selected from the group consisting of:

ML-S1 (SEQ ID NO 58);

ML-S2 (SEQ ID NO 59);

ML-S3 (SEQ ID NO 60);

ML-S4 (SEQ ID NO 61);

15 ML-S5 (SEQ ID NO 62); and

ML-S6 (SEQ ID NO 63).

27. The method according to any one of claims 1 to 26 wherein the probes are immobilized on a solid support.

28. Set of wild type probes, at least one of which is
20 an S-probe from Table 2, for the detection of at least one of rifampicin resistance and rifabutin resistance of Mycobacterium species characterized in that each probe spans more than one polymorphic site and that said set overlaps a complete rpoB mutation region delimited in Figure 1.

29. The set of wild type probes according to claim 28, said set comprising 2, 3, 4, 5, 6, 7, 8 or 9 wild type probes.

30. The set of wild type probes according to claim 28 or 29 for the detection of at least one of rifampicin resistance and rifabutin resistance of M. tuberculosis, said set comprising at least one of the wild type probes selected from the group consisting of:

	S1	(SEQ ID NO 1);
10	S11	(SEQ ID NO 2);
	S2	(SEQ ID NO 3);
	S3	(SEQ ID NO 4);
	S33	(SEQ ID NO 5);
	S4	(SEQ ID NO 6);
15	S44	(SEQ ID NO 7);
	S444	(SEQ ID NO 43);
	S4444	(SEQ ID NO 8);
	S5	(SEQ ID NO 9);
	S55	(SEQ ID NO 10);
20	S555	(SEQ ID NO 39);
	S5555	(SEQ ID NO 40);
	S55C	(SEQ ID NO 44);
	S55M	(SEQ ID NO 45);

58

S6 (SEQ ID NO 11); and

S66 (SEQ ID NO 12).

31. The set of wild type probes according to claim 28 for the detection of at least one of rifampicin resistance
5 and rifabutin resistance of M. leprae, said set comprising at least one of the wild type probes selected from the group consisting of:

ML-S1 (SEQ ID NO 58);

ML-S2 (SEQ ID NO 59);

10 ML-S3 (SEQ ID NO 60);

ML-S4 (SEQ ID NO 61);

ML-S5 (SEQ ID NO 62); and

ML-S6 (SEQ ID NO 63).

32. A kit for determining the rifampicin and/or
15 rifabutin resistance spectrum of mycobacteria present in a biological sample, comprising the following components:

(i) at least one of the set of wild-type probes as defined in claim 28, possibly fixed to a solid support;

(ii) a hybridization buffer, or components
20 necessary for producing said buffer; and

(iii) a wash solution, or components necessary for producing said solution.

33. The kit according to claim 32, further comprising one or more components selected from the group consisting
25 of:

(iv) a means for releasing, isolating or concentrating the polynucleic acids present in the sample;

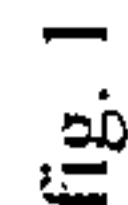
(v) a set of primers for the amplification of the rpoB gene; and

5 (vi) a means for detecting the hybrids resulting from hybridization.

34. The kit according to claim 32 or 33 for the detection of at least one of rifampicin resistance and rifabutin resistance of M. tuberculosis, wherein the set of
10 wild type probes in step (i) consists of the set defined in claim 30.

35. The kit according to claim 32 or 33 for the detection of at least one of rifampicin resistance and rifabutin resistance of M. leprae, wherein the set of wild
15 type probes in step (i) consists of the set defined in claim 31.

111



2/11

ITG 9003

GATCCGGGTC GGCATGTTCG GATGGAGCG GGTGGTCCGG GAGCGGATGA CCACCCAGGA CGTGGAGGCG
ATCACACCGC AGACGTTGAT CAACATCCGG CCGGTGGTCG CCGCGATCAA GGAGTTCTTC GGCACCAGCC
AGCTGAGCCA ATTCAATGGAC CAGAACAAAC CGCTGTCGGG GTTGACCTGC AAGCGCCGAC TGTGCGCGCT
GGGGCCCGGN GGTCTGTAC GAGAGCGTGC CGGGCTGGAG GTCCGCGACG TGCACCCGTC GCACTACGGN
CGGATGTGCC CTATCGAAAC CCTGAGGGG GCCAACATCG NTCTTTATC

FIG. 2

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3/11

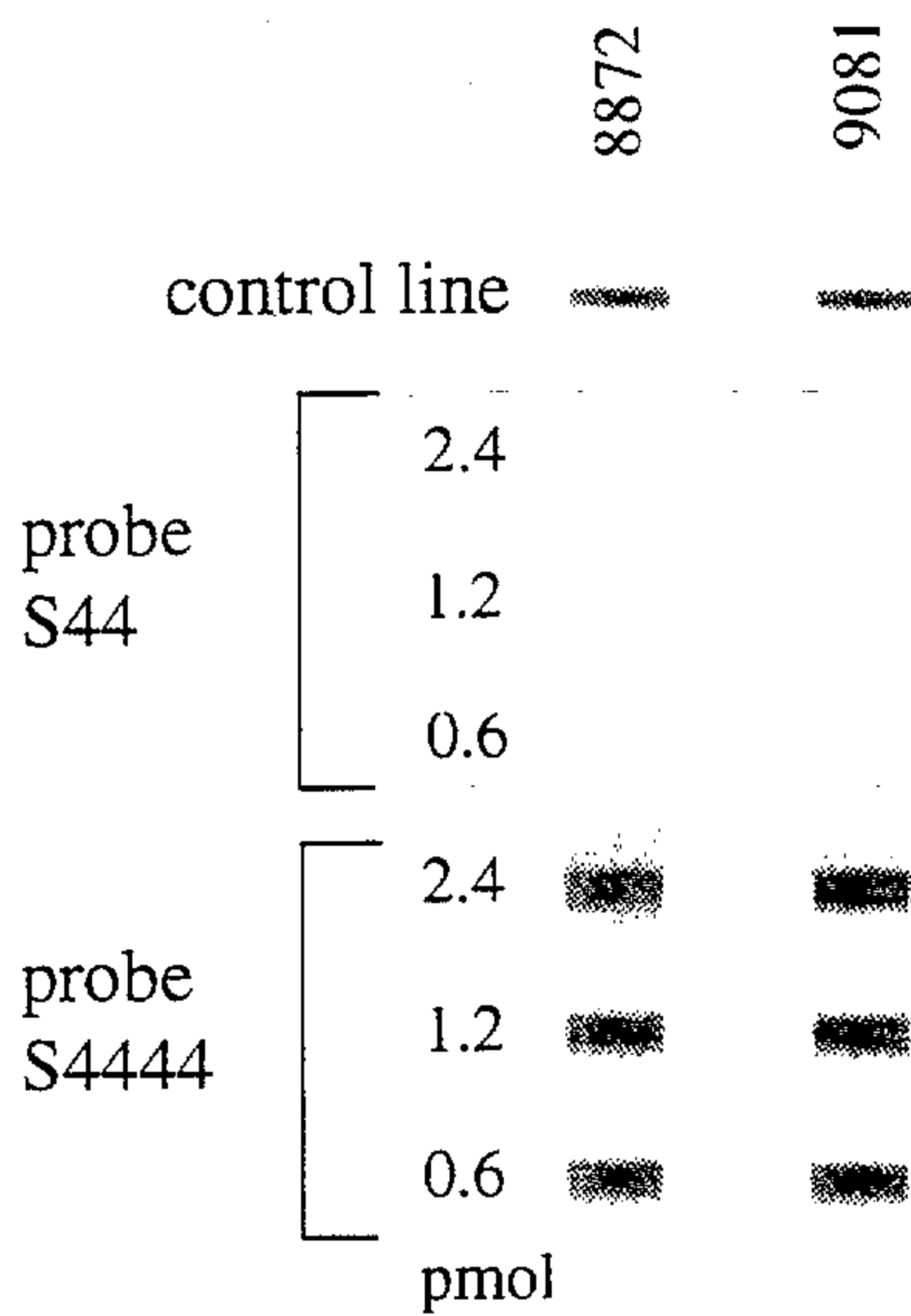


Fig. 3

4/11

	A	B
	C	C
MT-POL-1	MT-POL-1	MT-POL-1
S1	S1	S1
S2	S2	S2
R2		S33
S33	S33	S4444
S44	S44	S55
R44A	R44A	R2
R44B	R44B	R44A
S55	S55	R44B
R55	R55	R55

Fig. 4

5/11

M. paratuberculosis 316F

```
TCCGGGTCGG CATGTCCC GG ATGGAGTGTG TCGTCCGNGA GCGATGACC ANCCAGGACG TNGAGGCCAT
CACGCCGCAG ACCCTGATCA ACATCCGTCC CGTCGTGGCG GCGATCAAGG AGTTCTTCGG NACCAGCCAG
TTGTCCCAGT TCATGGACCA GAACAACCCG CTGTCGGGGC TCACCCACAA GCGCCGCCCTG TCGGCGNTGG
GCCCGGTTGG TCTGTCCC GG GAGCGGGCCG GGCTGGAGGT CCGNGACGTG NACCCGTCCC ACTACGGCCG
GATGTGCCCC ATCGAGACCC CGGAGGGTCC CAACATCGG
```

FIG. 5

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6/11

M. avium ITG 5887

GGATGGAGCG CTCCTGTCCGC GAGCGGATGA CCACCCAGGA CGTCGAGGCC ATCACGCCGC AGACCCCTGAT
CAACATCCGT CCAGTCGTGG CGGCGATCAA GGAGTTCCTC GGCACAGCC AGCTGTCCCA GTTCATGGAC
CAGAACACC CGCTGTCGGG GCTCACCCAC AAGCGCCGCC TGTGGGCGCT GGGCCCGGGT GGTCTGTCCC
GGAGCGGGC CGGGCTGGAG GTCCGCGGACG TGCACCCGTC CCACTACGGC CGGATGTGCC CGATCGAGAC
CCCGGAGGGT CCCAACATCG GTCTGATCGG CTCGCTGTCTG GTGT

FIG. 6

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7/11

M. scrofulaceum ITG 4979

CGGGTCGGCA TGTCCCGCAT GGAGCGGGTC GTCCGCGAGC GGATGACCAC GCAGGACGTC GAGGCGATCA
CGCCGCAGAC CCTGATCAAC ATCCGGCCCG TCGTGGCCCG GATCAAGGAG TTCTTCGGCA CCAGCCAGCT
CTCGCAGTTC ATGGACCAGA ACAACCCGNT GTCGGGCCCTG ACCCACAAGC GCCGCCCTGTC GGTGCTGGGC
CCGGTTGGTC TGTCCCGCGA GCGGGCCCGG TTGGAGGTCC GGAG

FIG. 7

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8/11

M. kansasii ITG 4987

GGATGGAACG GGTGGTCCGG GNNNNGGATG ACCACTCAGG ACGTCGAGGC GATCACGCCG
AGACACTGAT CAACATCCGC CCGGTGGTCG CCGCCATCAA GGAGTCTTC GGCACCAAGCC
AGCTCTCCCA GTTCATGGAC CAGAACAAAC CGCTGTCGGG CCTCACCCAC AAGCGCCGGC
TTTCGGCGCT GGGGCCGGGC GTCTGTCCC GGGAGCGTGC CGGGCTGGAG GTCCGCGATG
CTC

FIG. 8

PCT/EP95/02230

9/11

rpoB mutations and corresponding LiPA patterns

Mutation	M C tb S S S S S S R R R R R 2 4 4 5 A B	LiPA Pattern
wt		wt
L511P		Δ S1
D516Y		Δ S2
Δ D516-Q517		Δ S2
Δ N518		Δ S2
D516V		R2
S522L		Δ S3
H526P		Δ S4
H526T		Δ S4
H526R		Δ S4
H526N		Δ S4
H526C		Δ S4
H526Y		R4A
H526D		R4B
S531W		Δ S5
L533P		Δ S5
S531L		R5
L511P/D516G		Δ S1/ Δ S2
D516G/R529Q		Δ S2/ Δ S4
D516V/H526Q		R2/ Δ S4
S531L + ...		wt + R5
D516V + H526Y + ...		wt + R2 + R4A
No Mtb		

Fig. 9

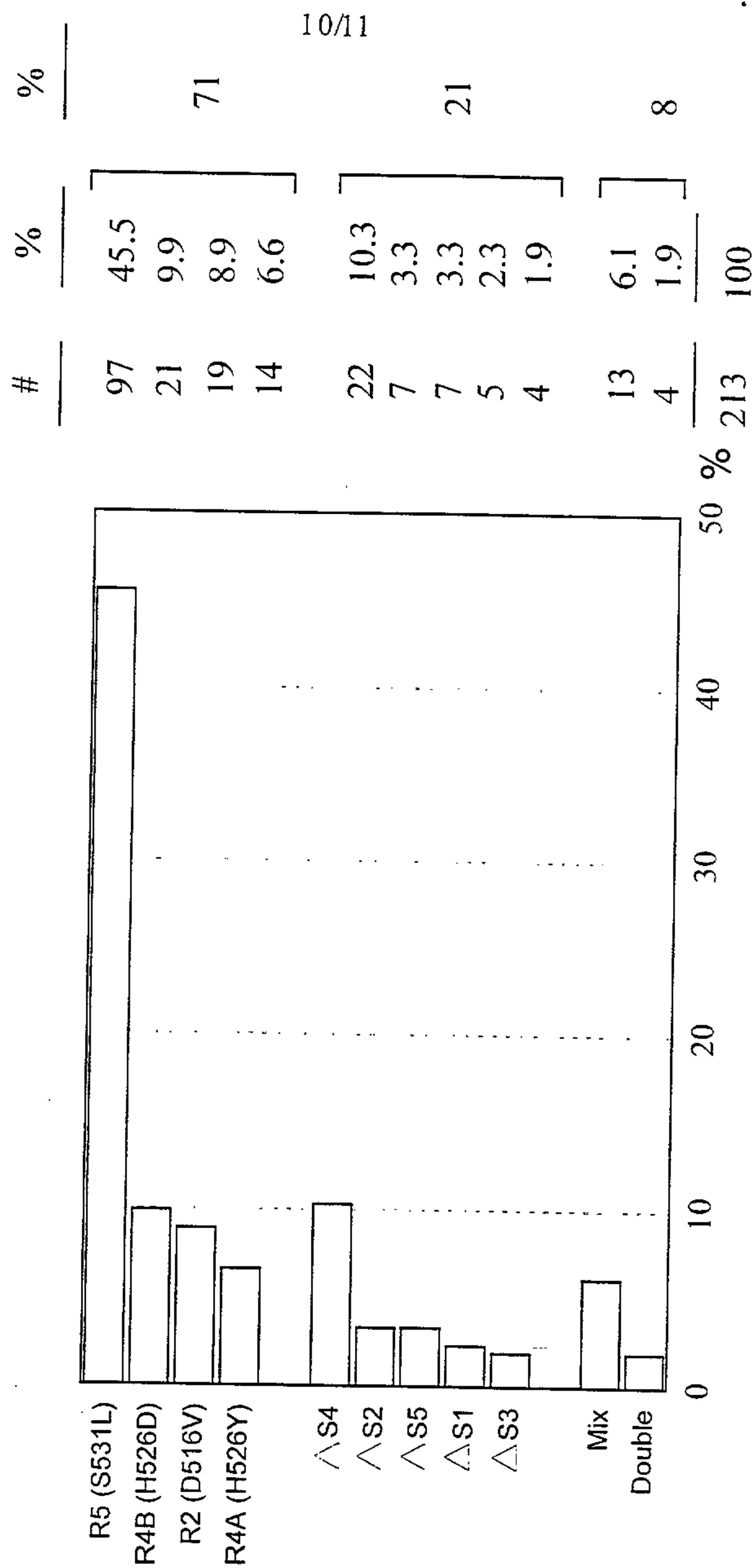


Fig. 10

11/11

MAC strain ITG 926

```
GGCATTTNAC  GGATGGAACG  CGTGGTCCGC  GANCGGATGA  CCACGCAGGA  CGTCGAGGCC  ATCACGCCGC  
AGACCCCTGAT  CAACATCCGG  CCGGTCGTG  CCGCGATCAA  GGAGTTCTTC  GGGACCAGCC  AGCTGTCGCA  
GTTTCATGGAC  CAGAACAAAC  CGCTGTCGG  TCTGACCCAC  AAGCGTCGCC  TGTGGGCGCT  GGGTCCCCGC  
GGTCTGTCCC  GTGAGCCG
```

FIG. 11

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