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## **MOLECULAR LINE PROBE ASSAYS FOR RAPID SCREENING OF PATIENTS AT RISK OF MULTI-DRUG RESISTANT TUBERCULOSIS (MDR-TB)**

### **EXPERT GROUP REPORT**

This report contains the collective views of an international group of experts, and does not necessarily represent the decisions or the stated policy of the World Health Organization or individual institutions

**May 2008**

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## EXECUTIVE SUMMARY

Multidrug-resistant tuberculosis (MDR-TB) poses a formidable challenge to TB control due to its complex diagnostic and treatment challenges. The annual global MDR-TB burden is estimated at around 490 000 cases, or 5% of the global TB burden; however, less than 5% of existing MDR-TB patients are currently being diagnosed, as a result of serious laboratory capacity constraints. Alarming increases in MDR-TB, the emergence of extensively drug-resistant TB (XDR-TB), potential institutional transmission, and rapid mortality of MDR-TB and XDR-TB patient with HIV co-infection, have highlighted the urgency for rapid screening methods.

Conventional methods for mycobacteriological culture and drug susceptibility testing (DST) are slow and cumbersome, requiring sequential procedures for isolation of mycobacteria from clinical specimens, identification of *M. tuberculosis* complex, and *in vitro* testing of strain susceptibility in the presence of anti-TB drugs. During this time patients may be inappropriately treated, drug resistant strains may continue to spread, and amplification of resistance may occur. Novel technologies for rapid detection of anti-TB drug resistance has therefore become a priority in TB research and development, and molecular line probe assays focused on rapid detection of rifampicin resistance (alone or in combination with isoniazid) are most advanced. Apart from the impact on morbidity, mortality and transmission of MDR-TB, introduction of these assays in screening and diagnostic algorithms could significantly reduce the need for sophisticated and costly conventional laboratory infrastructure, still vastly inadequate in most high-burden countries.

An Expert Group was convened by the World Health Organization (WHO) and the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) in March 2008, to assess available data on line probe assays with a view towards policy recommendations on their use. Data from published literature, laboratory validation studies, and investigator-driven laboratory and field demonstration studies were used to assess assay performance and feasibility of programmatic implementation. The associated laboratory infrastructure, human resource requirements and research gaps were also defined. Detailed cost-effectiveness and patient impact data will only be available once large-scale field demonstration projects (coordinated by the Foundation for Innovative New Diagnostics, FIND) have been completed.

An extensive literature search resulted in published information from two line probe assays with proven efficacy: the INNO-LiPA Rif.TB kit (Innogenetics, Zwijndrecht, Belgium), labeled for use on *M. tuberculosis* isolates grown on solid culture, and the Genotype MTBDR and Genotype MTBDR*plus* assays (Hain Lifescience, GmbH, Germany), labeled for use on isolates from solid and liquid culture as well as directly on smear-positive pulmonary specimens. Both assays are complete, PCR-based, hybridization assays simultaneously detecting *M. tuberculosis* complex and specific mutations in the *rpoB* gene conferring rifampicin resistance. The Genotype MTBDR*plus* assay also simultaneously detects specific mutations in the *katG* gene conferring high-level isoniazid resistance as well as those in the *inhA* gene conferring low-level isoniazid resistance.

Data from systematic reviews and meta-analyses were used to evaluate assay performance results against conventional DST methods: For the INNO-LiPA Rif.TB assay, 12 of 14 studies that applied the line probe assay to *M. tuberculosis* isolates had sensitivity greater than 95% and specificity of 100%. The pooled sensitivity was 0.97 (95%CI 0.95 - 0.98) and the pooled specificity 0.99 (95%CI 0.98 - 1.00). Overall discriminatory ability of the assay was 99% and overall accuracy 97%, with all studies yielding consistently high test performance values. Four studies that applied INNO-LiPA Rif.TB directly to clinical specimens showed 100% specificity and sensitivity ranging from 80% to 100%. The Expert Group concluded that INNO-LiPA Rif.TB was a highly sensitive and specific test for the detection of rifampicin resistance in isolates of *M. tuberculosis* complex.

Performance data for the Genotype MTBDR and MTBDR<sub>plus</sub> assays compared to conventional DST from 14 studies on rifampicin and 15 studies on isoniazid confirmed excellent accuracy for rifampicin resistance, both on *M. tuberculosis* isolates and on clinical specimens: The pooled sensitivity was 0.98 (95%CI 0.96 - 0.99) and the pooled specificity was 0.99 (95% CI 0.97 - 0.99), consistent across subgroups, assay versions and clinical specimen types. Overall discriminatory ability of the assay was 99% and overall accuracy 97%, with all studies yielding consistently high performance values. The accuracy estimates for isoniazid were variable, with sensitivity highly heterogeneous (57% - 100%) across studies. The pooled sensitivity for isoniazid resistance detection was 0.85 (95%CI 0.77 - 0.90) and the pooled specificity (fairly consistent across studies) was 0.99 (95%CI 0.98 - 1.00). Stratified analyses (by test version and specimen type) indicated no differences in specificity, but sensitivity for isoniazid resistance detection was higher (as expected) in Genotype MTBDR<sub>plus</sub> studies, increasing to almost 90%. The proportion of isoniazid resistance due to mutations in *katG* and *inhA* (and thus the sensitivity of the MTBDR<sub>plus</sub> assay) also varied considerably in different geographic locations.

Laboratory validation studies on Genotype MTBDR<sub>plus</sub> compared to conventional DST yielded consistently high performance data, as did results from field implementation studies. A large validation study conducted by FIND in Cape Town, South Africa showed sensitivity, specificity, positive and negative predictive values (PPV and NPV) of 98% or higher for detection of rifampicin resistance; 94% or higher for detection of isoniazid resistance, and 99% of higher for detection of MDR, when compared to conventional results. Similar results were reported from small field implementation projects in Lima, Peru and Samara, Russia.

The large-scale field Demonstration Project in South Africa, jointly conducted by FIND, the SA Medical Research Council (SAMRC) and the SA National Health Laboratory Service (NHLS), confirmed consistent and excellent performance of the Genotype MTBDR<sub>plus</sub> assay in high-volume public health laboratory settings, both on *M. tuberculosis* isolates and when used directly on smear-positive sputum specimens - overall accuracy for rifampicin resistance and MDR detection was 99% and 98% for isoniazid resistance detection, when tested on both *M. tuberculosis* isolates and on smear-positive sputum specimens. The MTBDR<sub>plus</sub> assay provided interpretable results in 84% of specimens in which conventional DST results were unavailable (due to contamination of the primary liquid culture), and detected MDR in 31 additional cases.

Using rifampicin resistance as a marker for MDR on all specimens, the sensitivity, specificity, PPV and NPV of the Genotype MTBDR<sub>plus</sub> assay were 99.3%, 97.0%, 76.5% and 99.9% respectively. For smear-positive specimens the corresponding values were 100%, 97.5%, 80.3% and 100%. The slight variation in results were due to strains with true rifampicin mono-resistance (confirmed by conventional DST in 31 of 44 strains) or with additional *rpoB* mutations.

For resistant isolates, the MTBDR<sub>plus</sub> assay showed five false-positive results and five false-negative results when compared with conventional DST. In four of the five false-negative results by MTBDR<sub>plus</sub>, rifampicin resistance alone was detected by the MTBDR<sub>plus</sub> assay. Of the five false-positive MTBDR<sub>plus</sub> results, all exhibited additional *rpoB* mutations as well as *CatG* or *InhA* mutations, making it very likely that these were true MDR-TB strains.

Detailed costing data from South Africa showed that the cost reduction in using the Genotype MTBDR<sub>plus</sub> assay under routine diagnostic algorithms amounted to between 30% and 50% when compared to conventional DST methods. As expected, the cost was lowest when the Genotype MTBDR<sub>plus</sub> assay was directly applied to smear-positive specimens (around USD17-19) and highest when the assay was used on isolates from liquid primary culture (around USD25 - 35). Cost-effectiveness and cost-benefit of line probe assays remain to be assessed, and will be dependent on screening and diagnostic algorithms in different epidemiological settings.

The Expert Group concluded that there was sufficient generalisable evidence to justify a recommendation on the use of line probe assays for rapid detection of MDR-TB, within country-specific settings, with further operational research to address country-specific implementation needs. The recommended use of line probe assays is currently limited to *M. tuberculosis* isolates and direct testing of smear-positive sputum specimens. Line probe assays are not a complete replacement for conventional culture and DST, as mycobacteriological culture is still

required for smear-negative specimens while conventional DST is still necessary to confirm XDR-TB, nevertheless, the implementation of line probe assays in MDR-TB screening algorithms may significantly reduce the demand on conventional culture and DST laboratory capacity. Commercial line probes are recommended over in-house assays, as the latter have not been adequately validated and their use for clinical care of patients is not recommended.

A range of implementation issues was identified, without which line probe assays would not be useful. These include requirements for specimen collection, storage and transport, laboratory design and biosafety, electrical supply and back-up power, product-specific and general laboratory equipment, quality and shelf-life of reagents, human resources and training, technical support, and quality assurance.

Issues requiring further research were also identified. While these should not prevent or delay the implementation of line probe assays, priorities for research include the evaluation of line probe assays in screening and diagnostic algorithms in different epidemiological settings, the role of these assays in combination with conventional culture in smear-negative specimens, the impact of specimen decontamination procedures on assay performance, and methods to optimize DNA extraction, especially from specimens with low numbers of organisms.

**The Expert Group recommended that WHO endorse the use of line probe assays,** with the following guiding principles:

- 5.1 Adoption of line probe assays for rapid detection of MDR-TB should be decided by Ministries of Health within the context of country plans for appropriate management of MDR-TB patients, including the development of country-specific screening algorithms and timely access to quality-assured second-line anti-tuberculosis drugs;
- 5.2 Line probe assay performance characteristics have been adequately validated in direct testing of sputum smear-positive specimens and on isolates of *M. tuberculosis* complex grown from smear-negative and smear-positive specimens. Direct use of line probe assays on smear-negative clinical specimens is not recommended;
- 5.3 The use of commercial line probe assays rather than in-house assays is recommended to ensure reliability and reproducibility of results, as in-house assays have not been adequately validated or used outside limited research settings. Any new or generic line probe assays should be subject to adequate validation, ie. published laboratory validation studies, adequate data to allow systematic review and meta-analysis (including assessment of data quality), and results from field demonstration projects documenting feasibility and consistent performance equal to conventional methods and commercial line probe assays. New or generic line probe assays for MDR-TB should have the following characteristics:
  - 5.3.1 A specific probe to identify *M. tuberculosis* complex;
  - 5.3.2 Multiple probes to detect the most common mutations in *rpoB* (codons 531, 526 and 516);
  - 5.3.3 Multiple overlapping wild-type (susceptible) probes covering the RRDR region of *rpoB*;
  - 5.3.4 Preferably, multiple probes to detect both high-level (*catG* mutations) and low-level isoniazid resistance (*inhA* mutations);
  - 5.3.5 Strip technology, with appropriate assay procedure controls, allowing visual detection of results;
  - 5.3.6 Line probe test production under ISO 13485:2003 standards;
  - 5.3.7 Performance characteristics equal to those of conventional DST methods;

- 5.3.8 Performance characteristics equal to those of current commercial line probe assays.
- 5.4 Adoption of line probe assays does not eliminate the need for conventional culture and DST capability; culture remains necessary for definitive diagnosis of TB in smear-negative patients, while conventional DST is required to diagnose XDR-TB. However, the demand for conventional culture and DST capacity may change, based on the local epidemiological situation and the use of line probe assays in country-specific screening algorithms;
- 5.5 As current line probe assays only detect resistance to rifampicin and/or isoniazid, countries with documented or suspected cases of XDR-TB should establish or expand conventional culture and DST capacity for quality-assured susceptibility testing of second-line drugs, based on current WHO policy guidance;
- 5.6 Adoption of line probe assays for rapid detection of MDR-TB should be phased in, starting at national/central reference laboratories or those with proven capability to conduct molecular testing. Once this has been accomplished, expansion could be considered, within the context of country laboratory strengthening plans, and considering availability of suitable personnel in peripheral centres, quality of specimen transport systems, and country capability to provide appropriate treatment and management of MDR-TB patients once diagnosed;
- 5.7 Adequate and appropriate laboratory infrastructure and equipment should be provided, ensuring that required precautions for biosafety and prevention of contamination are met:
- 5.7.1 Specimen processing for culture must be performed in biological safety cabinets (BSCs) in at least Biosafety Level (BSL) 2 facilities;
- 5.7.2 Procedures for manipulation of cultures (conventional identification, subculture for DNA extraction and conventional DST) must be performed in BSL3 facilities;
- 5.7.3 Laboratory facilities for line probe assays require at least three separate rooms - one each for DNA extraction, pre-amplification procedures, and amplification and post-amplification procedures. Restricted access to molecular facilities, uni-directional work flow, and stringent cleaning protocols must be established to avoid amplicon contamination leading to false-positive results;
- 5.7.4 Successful establishment, staffing, and maintenance of BSL2, BSL3 and molecular laboratories are demanding. Upgrading of facilities and establishment of the required infrastructure for molecular assays should be carefully planned and adequately financed;
- 5.8 Appropriate laboratory staff should be trained to conduct line probe assay procedures, especially those relating to amplification and interpretation of results. Supervision of staff by a senior individual with adequate training and experience in molecular assays is strongly recommended;
- 5.9 A detailed commercial sales contract and customer support plan should be negotiated with manufacturers, guaranteeing ample and continuous supply of materials, appropriate shipment conditions, customs clearance, equipment installation, maintenance, repair and replacement, and provision of training and ongoing technical support;
- 5.10 Stringent laboratory protocols, standard operating procedures for molecular line probe assays, and internal quality control mechanisms must be implemented and enforced. A programme for external quality assessment of laboratories involved in line probe assays should be developed as a matter of priority.

- 5.11 Mechanisms for rapid reporting of line probe assays results to clinicians must be established to provide patients with the benefit of an early diagnosis;
- 5.12 WHO and partners should assist countries with operational plans to introduce line probe assays within the appropriate epidemiological and resource availability context.

# MOLECULAR LINE PROBE ASSAYS FOR RAPID SCREENING OF PATIENTS AT RISK OF MULTI-DRUG RESISTANT TUBERCULOSIS (MDR-TB)

## EXPERT GROUP REPORT

### 1. BACKGROUND

The spread of drug resistant strains of *Mycobacterium tuberculosis* and the management of patients diagnosed with drug resistant disease is proving to be one of the most formidable obstacles faced by national tuberculosis control programmes. Multi-drug resistant TB (MDR-TB)\* is significantly more difficult to treat than drug susceptible TB, in large part because the necessary second-line drugs are less effective, associated with more severe adverse effects, must be administered for a prolonged period of time, and are much more expensive, than conventional first-line anti-tuberculosis drugs.

Recent data from the World Health Organization (WHO) estimated that 489 139 MDR-TB cases (95% CI 455 093 - 614 215) were prevalent in 2006, arising from a global proportion of 4.8% of all TB cases (95% CI 4.6 - 6.0).<sup>1</sup> Despite the formidable burden, it is estimated that less than 5% of existing MDR-TB cases are currently being detected.<sup>1</sup>

Alarming increases in MDR-TB have been documented globally, compounded by the emergence of extensively-drug resistant TB (XDR-TB)\*\* which is virtually untreatable in many settings. Lethal outbreaks of MDR-TB and XDR-TB in association with HIV infection have been described in South Africa<sup>2</sup> and linked to institutional transmission, particularly in the absence of adequate infection control.

In response to the XDR-TB crisis, WHO and partners constituted a Global XDR-TB Task Force in October 2006 and subsequently issued a Global MDR-TB and XDR-TB Response Plan.<sup>3</sup> Among a number of measures, the Plan calls for urgent evaluation and wide-scale implementation of rapid methods to screen patients at risk of MDR-TB in order to enable prompt and appropriate treatment, decrease morbidity and mortality, and interrupt transmission. However, lack of appropriate diagnostics and inadequate laboratory capacity currently constitute key barriers preventing an effective response.

Conventional culture and drug susceptibility testing (DST) is a slow process, requiring isolation of mycobacteria from clinical specimens, identification of *M. tuberculosis* complex and testing of the susceptibility pattern of strains in the presence of anti-tuberculosis drugs. Depending on the methodology, culture and DST can take up to four months to be completed. During this time patients may be inappropriately treated, drug resistant strains may continue to spread, and amplification of resistance may occur.

Rapid diagnosis of drug resistance will have obvious patient- as well as public health benefits, including better prognosis, increased survival, prevention of acquisition of further drug resistance, and reduced spread of drug resistant strains to vulnerable populations. It is

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\*MDR-TB: Isolates of *Mycobacterium tuberculosis* complex showing in vitro resistance to isoniazid and rifampicin, with or without resistance to other first-line anti-TB drugs

\*\*XDR-TB: Isolates of *Mycobacterium tuberculosis* complex defined as multidrug-resistant, with additional resistance to a fluoroquinolone and one or more of the following injectable drugs: kanamycin, amikacin, capreomycin

estimated that more than 90% of rifampicin-resistant strains of *M. tuberculosis* are also resistant to isoniazid;<sup>4</sup> The WHO surveys on anti-TB drug resistance have shown that, globally, rifampicin mono-resistance occurs at low prevalence.<sup>1</sup> In most settings, therefore, rifampicin resistance can reliably be used as a surrogate marker for MDR-TB.

Novel technologies for rapid detection of anti-TB drug resistance are in development phase, undergoing laboratory validation, or are in early stages of large-scale field studies to assess their feasibility, cost-effectiveness and cost-benefit. Molecular line probe assays focused on rapid detection of rifampicin resistance alone or in combination with isoniazid resistance are most advanced. Apart from the impact on morbidity, mortality and transmission of MDR-TB, introduction of these assays in screening and diagnostic algorithms could significantly reduce the need for sophisticated and costly conventional laboratory infrastructure, still vastly inadequate in most high-burden countries.

## **2. EXPERT GROUP PROCESS**

An Expert Group was convened by WHO on 31 March 2008 to assess available data on rapid line probe assays, with the aim towards development of policy recommendations on their use.

An extensive literature search resulted in published information from two line probe assays with proven efficacy: the INNO-LiPA Rif.TB kit (Innogenetics, Zwijndrecht, Belgium), and the Genotype MTBDR and Genotype MTBDR*plus* assays (Hain Lifescience, GmbH, Germany). Both of these are design-locked, completed products, for which published data exist on testing of *M. tuberculosis* isolates and/or clinical specimens.

The INNO-LiPA Rif.TB assay is labeled for use on isolates grown on solid medium.<sup>5</sup> The Genotype MTBDR*plus* is registered for direct use on smear-positive pulmonary clinical specimens, and on isolates grown on solid or in liquid medium after specimen processing with NaOH-NALC.<sup>6</sup>

The objectives of the Expert Group meeting were as follows:

- To review and evaluate data from published literature and laboratory validation studies on the performance characteristics of line probe assays for MDR-TB screening;
- To evaluate preliminary data from field demonstration projects on line probe assays for MDR-TB screening, coordinated by the Foundation for Innovative New Technologies (FIND);
- To define the laboratory infrastructure, human resource requirements, and further operational research data needed for programmatic implementation of line probe assays.

The Expert Group consisted of laboratory experts with technical experience in TB molecular assays, scientists/researchers, epidemiologists and national TB programme representatives. The meeting agenda and list of participants are presented in Annexure 1. All Expert Group members signed a conflict of interest statement in line with WHO policies.

Data evaluated by the Expert Group included:

- Meta-analyses of studies on the two assays, either previously published (INNO-LiPA Rif.TB) or commissioned by WHO for this purpose (Genotype MTBDR and MTBDR*plus*);
- Input and unpublished data from the manufacturers of the two commercial line probe assays;
- Results from investigator-driven laboratory validation, field studies and costing analyses;

- Preliminary results from the large scale field demonstration project on the Genotype MTBDR<sub>plus</sub> assay in South Africa conducted by FIND, the SA Medical Research Council (SAMRC) and the SA National Health Laboratory Service (NHLS).

This document reflects the findings and consensus of the Expert Group and is presented to the WHO Strategic and Technical Advisory Group on Tuberculosis (STAG-TB) for consideration and endorsement. Detailed cost-effectiveness and patient impact data will only be available in future, given that field demonstration projects have not yet been completed; however, the Expert Group believes that the recommendations given in this report are fully supported by the currently available data.

### 3. INTRODUCTION

#### 3.1 Molecular basis of drug resistance

Drug resistance in TB is a natural phenomenon, due to random mutations in *M. tuberculosis*, typically occurring at rates ranging from  $3 \times 10^{-7}$  to  $1 \times 10^{-9}$  per organism per generation for first-line anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol, streptomycin).<sup>4</sup> Thus, the *M. tuberculosis* population within a given human host is likely to contain at least a small proportion of naturally occurring drug resistant mutants which are selected for under conditions of inadequate or ineffective chemotherapy. This process not only expands the drug resistant population within the infected individual, but also allows for resistance to additional drugs to develop, creating a cycle of amplification of drug resistance.

Rifampicin acts by binding to the beta-subunit of the RNA polymerase (coded for by the *rpoB* gene), inhibiting RNA transcription.<sup>7</sup> Telenti *et al*<sup>8</sup> first reported on the molecular mechanism of rifampicin resistance in *M. tuberculosis*. Subsequent DNA sequencing studies have shown that more than 95% of rifampicin resistant strains have mutations in an 81-base pair region (codons 507 - 533) of the *rpoB* gene.<sup>9-11</sup> More than 50 mutations within this region have been characterized by automated DNA sequencing; however, the majority are point mutations in codons 516, 526, or 531.<sup>4,7</sup> Mutations in other regions of the *rpoB* gene have also been reported, but much less commonly. In addition, a few silent mutations infrequently occur which do not seem to confer rifampicin resistance.

Isoniazid inhibits InhA, enoyl-ACP-reductase, which is involved in mycolic acid biosynthesis.<sup>4</sup> Mutations causing isoniazid resistance are located in different regions of several genes. Isoniazid is a so-called 'pro-drug' which is converted to its active form by the catalase-peroxidase enzyme KatG. Therefore resistance can be due to several factors, including the binding of the pro-drug to its *InhA* target, the activation of the pro-drug by KatG (encoded by the *katG* gene), or by increased expression of the target *InhA*.<sup>12,13</sup>

Mutations in codon 315 of the *katG* gene have been found in 50-90% of isoniazid resistant strains, while 20-35% of isoniazid-resistant strains have been reported to have mutations in the *inhA* regulatory region, and 10-15% of strains have had mutations reported in the *ahpC-oxvR* intergenic region (often together with *katG* mutations in other regions).<sup>4,7,12,13</sup> Deficiency in catalase activity leads to high-level resistance to isoniazid. Most commonly, mutations in the *katG* gene are single point mutations at codon 315 involving a serine-to-threonine amino acid substitution. Other mutations in *katG* occur less commonly. Low-level resistance to isoniazid is most commonly caused by *inhA* and *ahpC-oxvR* mutations.<sup>13</sup>

Although the molecular mechanisms of resistance to the other main anti-tuberculosis drugs (including isoniazid, pyrazinamide, streptomycin, ethambutol and fluoroquinolones) have been elucidated, the molecular basis for resistance is still not fully understood and is beyond the scope of this document.

### 3.2 Line probe assays for tuberculosis drug resistance

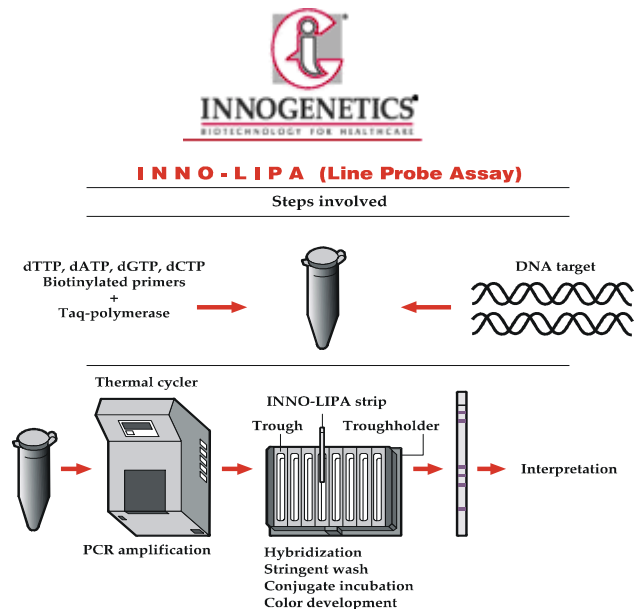
Line probe assay technology involves the following steps: First, DNA is extracted from *M. tuberculosis* isolates or directly from clinical specimens. Next, polymerase chain reaction (PCR) amplification of the resistance-determining region of the gene under question is performed using biotinylated primers. Following amplification, labeled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip. Captured labeled hybrids are detected by colorimetric development, enabling detection of the presence of *M. tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance.

If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe. Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations. The post-hybridization reaction leads to the development of coloured bands on the strip at the site of probe binding and is observed by eye.

A number of investigators have developed in-house reverse line hybridization assay protocols for detection of resistance to rifampicin and other drugs. These methods commonly detect the hybridization of PCR products to membrane-oligonucleotide probes through chemoluminescence using light-sensitive film. As none of these assays have been adequately validated, their use outside of research settings is not recommended.

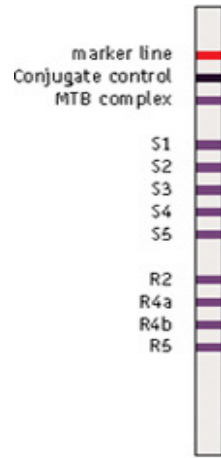
#### 3.2.1 INNO-LiPA Rif.TB (Innogenetics, Zwijndrecht, Belgium)<sup>5</sup>

The INNO-LiPA Rif.TB test is a line probe assay able to identify *M. tuberculosis* complex and simultaneously detect genetic mutations in the *rpoB* gene region related to rifampicin resistance.



The INNO-LiPA Rif.TB kit contains 10 oligonucleotide probes, ie. one specific for the *M. tuberculosis* complex, five overlapping wild-type (susceptible) probes, and four probes for detecting specific mutations (D516V, H526Y, H526D and S531L) associated with rifampicin resistance, immobilized on nitrocellulose paper strips. The region from codon 509 to 534 is covered by the wild-type probes.

Figure 1.2. Position of the oligonucleotide probes on the INNO-LiPA Rif.TB strip.



A red Marker line is drawn at the top of the INNO-LiPA Rif.TB strip for orientation. The conjugate control (Conj. Control) provides an internal control for the colour development reaction.

The MTB complex line is a specific probe for *M.tuberculosis* complex.

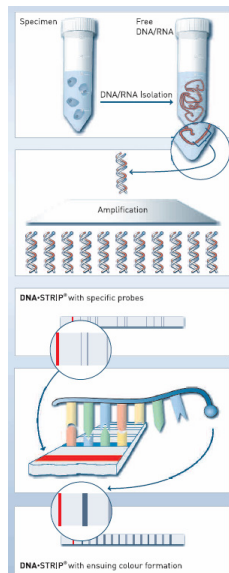
S1-S5 are wild-type probes for the *rpoB* gene, and R2, R4a, R4b and R5 are specific *rpoB* mutation probes.

The INNO-LiPA Rif.TB assay is validated for use on bacteria grown on solid media.

The INNO-LiPA Rif.TB assay has been labeled for use on isolates of *M. tuberculosis* complex grown on solid media. Information on expanded certification for use of INNO-LiPA Rif.TB on clinical specimens is pending.

### 3.2.2 Genotype MTBDR and MTBDRplus (Hain Lifescience, GmbH, Germany)<sup>6</sup>

The GenoType MTBDR assay, introduced in 2004, identifies *M. tuberculosis* complex and simultaneously detects mutations in the *rpoB* gene as well as mutations in the *katG* gene for high-level isoniazid resistance.



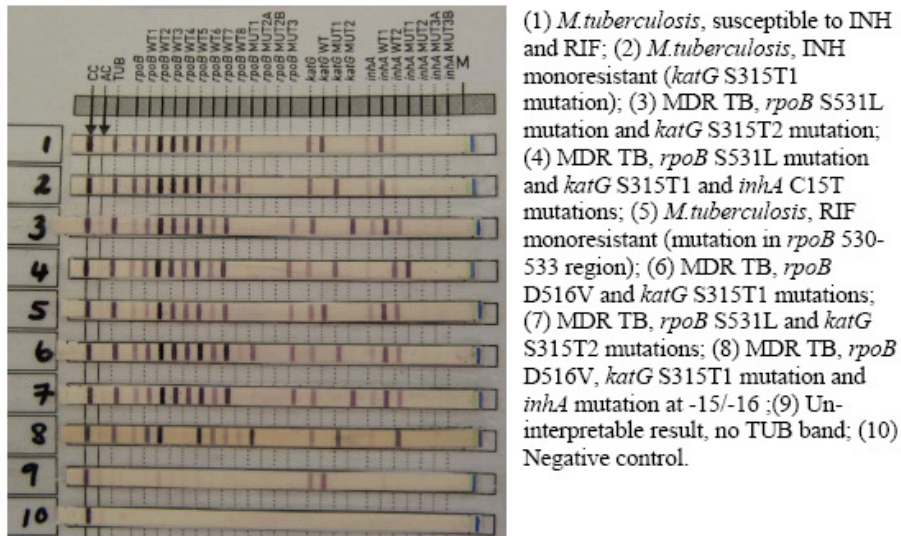
	<i>katG</i>				sensitive	resistant	
	Tub	<i>rpoB</i> WT	<i>rpoB</i> MUT	<i>katG</i> WT		<i>katG</i> MUT	R
CC	+	+	-	+	+		
UC	+	-	-	+		+	
<i>M. tub.</i> complex	+	+	+	+		+	
<i>rpoB</i> Uni	+	+	-	+			
<i>rpoB</i> MUT 1	+	-	-	+			
<i>rpoB</i> WT 2	+	+	-	+			
<i>rpoB</i> WT 3	+	+	-	+			
<i>rpoB</i> WT 4	+	+	-	+			
<i>rpoB</i> WT 5	+	+	-	+			
<i>rpoB</i> MUT D516V	+	+	-	+			
<i>rpoB</i> MUT H526Y	+	+	-	+			
<i>rpoB</i> MUT H526D	+	+	-	+			
<i>rpoB</i> MUT S531L	+	+	-	+			
<i>katG</i> Uni	+	+	-	+			
<i>katG</i> MUT 1	+	+	-	+			
<i>katG</i> MUT 2	+	+	-	+			
	+	+	-	+			
	+	-	-	+			+

Genotype MTBDRplus, the second-generation assay, also detects mutations in the *inhA* gene that confers resistance to low-levels of isoniazid.

For rifampicin resistance, four specific *rpoB* mutations are detected, ie. D516V, H526Y, H526D, and S531L. Eight wild-type probes are also present which cover the region from codon 505 to 533.

For isoniazid resistance, S315T1 and S315T2 *katG* mutation probes are detected, together with four *inhA* mutation probes, ie. C15T, A16G, T8C and T8A.

**Figure 1.1. Examples of GenoType® MTBDRplus strips (from Barnard 2008).<sup>29</sup>**



The GenoType MTBDRplus assay has been validated for use directly on smear-positive pulmonary specimens, as well as on isolates of *M. tuberculosis* grown on solid medium or in liquid medium, after specimen processing by NaOH-NALC.

### 3.3 Use of line probe assays in resource-rich settings

The Mycobacterium Reference Unit of the United Kingdom Health Protection Agency has operated a national rapid molecular service for detection of rifampicin resistance using the INNO-LiPA Rif.TB assay since 1999.<sup>14</sup>

The GenoType MTBDRplus assay is in use in many industrialized countries including Australia, Belgium, Denmark, Finland, France, Germany, Ireland, Italy, Latvia, Netherlands, Portugal, Spain, United Kingdom, Sweden, and Switzerland.

### 3.4 Use of line probe assays in resource-limited settings

The INNO-LiPA Rif.TB assay has been implemented in the Latvian national TB programme for clinical management of patients at high risk of MDR-TB since October 2004.<sup>15</sup>

GenoType MTBDRplus assays have been implemented in Nepal as part of the MDR-TB programme, and in Samara Oblast, Russian Federation under operational conditions. Preliminary data from both sites are presented in this report.

### **3.5 FIND's demonstration projects on Genotype MTBDR<sub>plus</sub>**

Following the Global XDR-TB Task Force meeting in October 2006, which recommended implementation of available technologies for rapid MDR screening, FIND accelerated a large-scale demonstration project involving the Genotype MTBDR<sub>plus</sub> line probe assay in South Africa, in collaboration with the SAMRC, the NHLS, and National and Provincial Departments of Health. FIND Genotype MTBDR<sub>plus</sub> demonstration projects in India, Thailand, Turkey, Uganda, China, Russia, and Vietnam are planned for implementation in 2008.

The overall aim of FIND's demonstration projects is to investigate the performance of the Genotype MTBDR<sub>plus</sub> under large scale implementation conditions in programmatic settings in high burden countries. A summary of preliminary data from the South African Demonstration Project is presented in this report. The detailed report is available from FIND.

## **4. SYSTEMATIC REVIEWS AND META-ANALYSES OF PERFORMANCE DATA FOR COMMERCIAL LINE PROBE ASSAYS**

Data from systematic reviews and meta-analyses from studies involving the two commercial line probes assays were used to assess the evidence base on performance characteristics of these assays and are summarized below. Full articles are attached in Annex 2 (INNO-LiPA Rif.TB) and Annex 3 (Genotype MTBDR and Genotype MTBDR<sub>plus</sub>).

### **4.1 Methodology**

Published methods for systematic review and meta-analysis of diagnostic tests to evaluate overall test accuracy and performance characteristic were followed. In summary, structured search strategies of several databases were used for retrieving citations of primary studies. Titles and/or abstracts of all citations found were screened independently by two reviewers using a standardized questionnaire and predefined inclusion criteria, with consensus on articles warranting full text review.

Articles eligible for inclusion were assessed by a primary reviewer who extracted data using a standardized data extraction form. A second reviewer independently extracted data from a subset of included studies, with discrepancies reconciled by consensus.

Study quality was assessed by using QUADAS criteria<sup>16</sup> for assessment of quality of diagnostic studies, ie. comparison of the index test with an appropriate reference standard, blinded interpretation between test and reference results, complete verification of test results with the reference standard, consecutive or random recruitment of patients, and cross-sectional vs case-control design.<sup>16</sup> Meta-analysis was conducted using appropriate statistical software.

Sensitivity and specificity, with 95% confidence intervals, were used as primary measures of diagnostic accuracy, using conventional DST results as reference standard:

*Sensitivity (true positive rate)*

Proportion of resistant results correctly identified by the assay under investigation.

*Specificity (true negative rate or 1-false positive rate)*

Proportion of susceptible results correctly identified by the assay under investigation.

Accuracy results were summarized in forest plots. Summary receiver operating characteristic (SROC) curve analysis was used to explore the effect of diagnostic thresholds on the results. Bivariate random effects regression analyses were performed to generate pooled accuracy estimates. Heterogeneity between studies was explored by stratified analyses to determine if accuracy estimates varied across subgroups.

Overall discriminatory ability of assays was assessed by the SROC area under the curve (AUC) and test accuracy (maximum joint sensitivity and specificity) summarized by the Q\* index, with an AUC of 1.00 representing perfect discriminatory ability and a Q\* index of 1.00 representing perfect accuracy.

## 4.2 Summary of results

### 4.2.1 INNO-LiPA Rif.TB

Fifteen studies included in the meta-analysis involved 1 738 specimens (mean 91; range 20 - 411), of which 1 164 (67%) were rifampicin resistant. All had complete verification of INNO-LiPA Rif.TB with a reference standard (conventional proportion method, minimum inhibitory concentration method, or BACTEC 460 liquid system).

Twelve of 14 studies that applied INNO-LiPA Rif.TB to *M. tuberculosis* isolates had sensitivity greater than 95% and specificity of 100%. The pooled sensitivity was 0.97 (95%CI 0.95 - 0.98) and the pooled specificity 0.99 (95%CI 0.98 - 1.00). The SROC curve had an AUC of 0.99 and Q\* index of 0.97. Despite variations in patient populations, heterogeneity was not a concern, all 15 studies yielding consistently high estimates of sensitivity and specificity. Four studies that applied INNO-LiPA Rif.TB directly to clinical specimens (sputum, bronchial aspirates, urine, tissue biopsy, cerebrospinal fluid, faeces, skin exudates, gastric fluid aspirate) had 100% specificity, and sensitivity that ranged between 80% and 100%.

Strengths of the review included compliance with published guidelines, comprehensiveness of the review, independent processes for study selection, data extraction, and assessment of data quality. Study limitations included possible language bias (limited to English or Spanish literature), possible publication bias (due to studies with positive results being more likely to be accepted for publication), possible inflation of accuracy estimates due to exclusion of indeterminate results (a common practice in assessing test performance), and underlying MDR-TB epidemiology (studies reporting a high prevalence of MDR-TB).

The meta-analysis concluded that INNO-LiPA Rif.TB was a highly sensitive and specific test for the detection of rifampicin resistance in isolates of *M. tuberculosis* complex. The test appeared to have relatively lower sensitivity when used directly on clinical specimens, although studies were limited. Assuming different underlying MDR-TB prevalence estimates, the meta-analysis concluded that the accuracy of INNO-LiPA Rif.TB could be maintained even in low-prevalence MDR-TB regions if used judiciously (ie. in patients suspected of having MDR-TB, thereby raising the pretest probability).

### 4.2.2 Genotype MTBDR and MTBDRplus

Ten articles with 14 comparisons for detection of rifampicin resistance and 15 for detection of isoniazid resistance were included in the meta-analysis, involving 3 349 specimens (mean 116; range 36 to 470). All had complete verification of the two generations of the assay with a reference standard (conventional proportion method, minimum inhibitory concentration method, BACTEC 460 system, or MGIT 960 system).

GenoType MTBDR assays demonstrated excellent accuracy for rifampicin resistance, both on *M. tuberculosis* isolates and on clinical specimens. The pooled sensitivity (98.1%; 95% CI 95.9 - 99.1) and pooled specificity (98.7%; 95% CI 97.3 - 99.4) estimates for rifampicin resistance were very high and consistent, across all subgroups, assay versions and clinical specimen types. The AUC of the SROC curve was 0.99 and the Q\* index was 0.97.

The accuracy estimates for isoniazid were variable and the sensitivity highly heterogeneous across studies, ranging from 57% to 100%. The pooled sensitivity was 84.3% (95%CI 0.77 - 0.90). The pooled specificity estimate was high (99.5%; 95%CI 0.98 - 1.00) and fairly consistent across studies. Stratified analyses (by test version and specimen type) indicated no differences in specificity, but sensitivity was higher (as expected) when only MTBDR*plus* studies were pooled, increasing to almost 90%. The proportion of isoniazid resistance due to mutations in *katG* and *inhA* (and thus the sensitivity of the MTBDR*plus* assay) also varied considerably in different geographic locations.

Strengths of the review included compliance with published guidelines, comprehensiveness of the review, independent processes for study selection, data extraction, and assessment of data quality. Study limitations included possible language bias (limited to English literature), possible publication bias (due to positive results being more likely to be accepted for publication), possible inflation of accuracy estimates due to exclusion of indeterminate results, and underlying MDR-TB epidemiology (studies reporting a high prevalence of MDR-TB).

The meta-analysis concluded that Genotype MTBDR assays were highly sensitive and specific for the detection of rifampicin resistance, both in culture isolates and clinical specimens. The accuracy for isoniazid resistance was variable, with sensitivity lower and more inconsistent than specificity. As expected, the newer generation Genotype MTBDR*plus* assay retained excellent discriminatory ability to rule in and rule out rifampicin resistance while showing higher sensitivity for detecting isoniazid resistance.

## 5. SUMMARY OF UNPUBLISHED PERFORMANCE DATA

### 5.1 INNO-LiPA Rif.TB

An evaluation of the INNO-LiPA Rif.TB assay in comparison with indirect DST on Löwenstein Jensen (LJ) solid medium was conducted between 2004 and 2005 as part of a larger study on MDR-TB in Lima, Peru, funded by WHO/TDR and monitored by FIND. The study involved 926 newly-diagnosed and 68 retreatment smear-positive patients recruited from TB diagnostic and treatment sites. Of these, 907 (97.9%) had an interpretable INNO-LiPA Rif.TB result and 857 (92.5%) a valid result for rifampicin DST. 852 specimens had valid results by both methods and were used for calculation of performance parameters (Table 5.1):

**Table 5.1. Performance of the INNO-LiPA Rif.TB assay compared with indirect DST on LJ solid medium**

	<b>Rifampicin</b>	<b>MDR</b>
Sensitivity % (n)	94.7 (107/113)	95.2 (98/103)
Specificity % (n)	99.2 (733/739)	98.0 (734/749)
Overall accuracy % (n)	98.6 (840/852)	97.7 (832/852)
PPV % (n)	94.7 (107/113)	86.7 (98/113)
NPV % (n)	99.2 (733/739)	99.3 (734/739)

## 5.2 Genotype MTBDRplus

### 5.2.1 Samara Oblast, Russian Federation

Findings from a study on the performance of the MTBDRplus assay compared to MGIT culture and DST, undertaken by Nikolayevskyy V, Balabanova Y, Simak T, Malomanova, Fedorin I, and Drobniowski F in collaboration with Hain Lifescience, were as follows:

A total of 168 smear-positive sputum specimens (79 from new and 89 from previously treated patients) were collected at three participating sites in 2007. Specimens were divided into two portions, one of which was tested by the MTBDRplus assay while the other underwent MGIT culture and DST for rifampicin and isoniazid, backed up by LJ solid medium where MGIT results were un-interpretable. Performance parameters are shown in Table 5.2:

**Table 5.2 Performance of MTBDRplus assay compared to MGIT/LJ culture and DST on sputum specimens**

	<b>Rifampicin</b>	<b>Isoniazid</b>
Sensitivity %, (n)	96.2 (102/106)	97.3 (110/113)
Specificity %, (n)	90.7 (39/43)	83.3 (30/36)
Overall accuracy %, n	94.6 (141/149)	94.0 (140/149)
PPV %, (n)	96.2 (102/106)	94.8 (110/113)
NPV %, (n)	90.7 (39/43)	90.9 (30/33)
Proportion interpretable results	89.9 (151/168)*	89.9 (151/168)*

\*MTBDRplus strips for the remaining 17 samples were unreadable with 14 strips having either no bands or very weak/unreadable bands in *rpoB*, *katG* and *inhA* sections and 3 more strips having TB band missing probably indicating presence of non-TB mycobacteria (later confirmed as *M. kansasii*).

Amplicon contamination was reported to be a prolonged problem, generally seen as weak bands (primarily *katG* and *inhA* bands) on the negative control strips when testing was done directly on sputum specimens (which uses a higher number of PCR cycles than testing on cultures). This was rectified by strict procedures for cleaning and separation of PCR work areas.

### 5.2.2 Nepal

Suspected MDR-TB patients in the Nepal DOTS-Plus Program were tested by Genotype MTBDRplus assay in the GENETUP (German Nepal Tuberculosis Project) laboratory in Kathmandu. Results were as follows:

A total of 281 culture isolates were evaluated. Of these, 257 (91.5%) were true MDR-TB cases. Conventional LJ culture and DST detected 98.1% of these while MTBDRplus detected 89.5%. Using rifampicin resistance only as a surrogate for MDR, the MTBDRplus assay detected 98.1% (254/257) of the true MDR-TB cases. Detailed analysis of the performance of the Genotype MTBDRplus relative to conventional DST is pending; however, it was shown that using *katG* alone for detection of INH resistance resulted in detection of only 79.1% resistance. Addition of *inhA* probes led to detection of an additional 15.7% isoniazid resistance.

## **6. FIND/SAMRC/NHLS DEMONSTRATION PROJECT, SOUTH AFRICA**

### **6.1 Laboratory validation studies**

As a prelude to the large-scale FIND/SAMRC/NHLS demonstration project in South Africa, validation of the Genotype MTBDR*plus* assay was undertaken in two of the NHLS laboratories (Cape Town and Johannesburg), to investigate the feasibility of routine implementation in high-volume laboratories and to evaluate assay performance compared to conventional culture and DST (MGIT-960 or proportion method on Middlebrook 7H11 solid agar medium).

Detailed results from the validation study in Cape Town are described in the article by Barnard et al.<sup>17</sup> In summary, the assay was performed directly on 536 consecutive smear-positive sputum specimens from patients at increased risk of MDR-TB. Overall, 97% of smear-positive specimens gave interpretable results within one to two days using the molecular assay. Sensitivity, specificity, and positive and negative predictive values were 98.9, 99.4, 97.9, and 99.7, respectively, for detection of rifampicin resistance; 94.2, 99.7, 99.1, and 97.9, respectively, for detection of isoniazid resistance; and 98.8, 100, 100, and 99.7, respectively, for detection of MDR compared to conventional results. The assay performed well on specimens that were contaminated on conventional culture. Smear-negative sputum specimens from 100 high-risk MDR-TB suspects were also tested. Of these, interpretable results were obtained in 20 out of 25 specimens, with all MTBDR*plus* results correlating with those from conventional DST.

The study concluded that Genotype MTBDR*plus* was a highly accurate screening tool for MDR-TB and achieved a substantial reduction in diagnostic delay. Overall performance characteristics were thought to be superior to conventional culture and DST (given the problem of contamination on primary culture), and the potential for high throughput with substantial cost savings was highlighted.

In the Johannesburg validation study, testing was performed on leftover portions of routine clinical specimens submitted for culture and DST. Results were available for 460 smear-positive sputum specimens. Overall, 85.9% of specimens gave interpretable results by the MTBDR*plus* test. Performance characteristics for rifampicin resistance detection were as follows: sensitivity 90.7%, specificity 99.7%, positive predictive value 98.3%, negative predictive value 98.0%. Corresponding values for isoniazid resistance detection were: sensitivity 91.2%, specificity 98.7%, positive predictive value 92.9%, negative predictive value 99.5%.

### **6.2 Demonstration Project**

#### *6.2.1 Aims*

The Demonstration Project aims to investigate the feasibility, impact and cost-effectiveness of implementation of rapid screening for MDR-TB using the Genotype MTBDR*plus* assay in patients at high risk of MDR-TB in a high-burden TB setting.

#### *6.2.2 Project status*

The preliminary data analysis summarized below includes patients enrolled between June and 31 December 2007, and all laboratory results that were available on or before 31 January

2008. Preliminary data were reviewed by the Demonstration Project Data and Safety Monitoring Committee in February 2008, who advised continuation of the study according to the protocol.

### 6.2.3 Preliminary performance results

A total of 10 793 sputum specimens from patients at risk for MDR-TB were enrolled for testing during this period (a total of 11 981 specimens were received; 40 were without patient consent forms and therefore not tested, and 1 148 were duplicates). Patients were enrolled in four provinces (Gauteng, Northern Cape, Northwest and Western Cape), and line probe assay testing was performed at two NHLS laboratories (Johannesburg, Gauteng and Cape Town, Western Cape).

Specimens from patients in Northwest province and Northern Cape province were referred to the Johannesburg laboratory for line probe assay testing. Patients have also been enrolled in KwaZulu-Natal province (testing performed at the Johannesburg laboratory) but are not included in the preliminary analysis as patient enrollment only started in November 2007.

The reference culture test was MGIT 960 in both laboratories, with MGIT 960 DST at the Johannesburg laboratory and indirect proportion method on Middlebrook 7H11 medium at the Cape Town laboratory.

#### Detection of resistance on all specimens

2 246 specimens were tested by the MTBDR*plus* assay; of these 1 080 (48.1%) were smear-positive and 1 166 (51.9%) were smear-negative/culture-positive. 803 specimens were tested directly (801 smear-positive and two smear-negative specimens) and 1 443 specimens (1 164 smear-negative and 279 smear-positive specimens) were tested on the *M. tuberculosis* isolates from MGIT culture.

A summary of performance characteristics of the MTBDR*plus* assay for detection of isoniazid, rifampicin and MDR compared with MGIT culture and conventional DST in all specimens is presented in Table 6.1:

**Table 6.1 Performance of MTBDR*plus* assay compared to conventional culture and DST (all specimens)**

	Rifampicin	Isoniazid	MDR
Sensitivity % (95% CI)	98.4 (95.3 – 99.7)	91.4 (87.2 – 94.6)	96.5 (92.0 – 98.9)
Specificity % (95% CI)	99.1 (98.5 – 99.5)	99.7 (99.3 – 99.9)	99.7 (99.2 – 99.9)
Overall accuracy % 95% CI)	99.0 (98.5 – 99.5)	98.4 (97.8 – 99.0)	99.4 (98.9 – 99.7)
PPV % (95% CI)	93.2 (88.7 – 96.3)	98.2 (95.6 – 99.5)	96.5 (92.0 – 98.9)
NPV % (95% CI)	99.8 (99.4 – 100.0)	98.5 (97.7 – 99.1)	99.7 (99.2 – 99.9)

A total of 163 MDR-TB cases were detected by MGIT culture and conventional DST; of these 76 (46.6%) were smear-positive. A total of 189 MDR-TB cases were detected by the MTBDR*plus* assay; of these 87 (46.6%) were smear-positive.

Overall, results of 92.0% (2 032/2 208) specimens were available by MTBDR*plus* assay, whilst 77.5% (1 712/2 208) of results were available by MGIT culture and conventional DST. There was no significant difference in the proportion of interpretable results obtained by the MTBDR*plus* assay when tested directly on sputum specimens compared to positive MGIT cultures ( $p=0.1112$ ). However, there was a significantly lower proportion of interpretable results by the conventional method in smear-positive compared to smear-negative/culture-positive specimens ( $p<0.0001$ ). Overall, non-tuberculous mycobacteria were isolated from 23/2 246 (1%) specimens enrolled in the study.

The MTBDR*plus* assay provided interpretable results in 399 (84.4%) specimens in which no conventional DST result was available due to contamination of the primary MGIT culture. In 31 additional cases, the MTBDR*plus* assay detected the presence of MDR-TB.

No susceptible isolates were mistakenly reported as MDR by the MTBDR*plus* assay, nor were any MDR isolates falsely reported as susceptible.

Using rifampicin resistance as a marker for MDR on all specimens, the sensitivity, specificity, PPV and NPV were 99.3%, 97.0%, 76.5% and 99.9% respectively. For smear-positive specimens the corresponding values were 100%, 97.5%, 80.3% and 100%. The lower sensitivity was ascribed to strains with true rifampicin mono-resistance (confirmed by conventional DST in 31 of 44 strains).

For resistant isolates, the MTBDR*plus* assay showed five false-positive results and five false negative results when compared with conventional DST. In four of the five false-negative results by MTBDR*plus*, rifampicin resistance alone was detected by the MTBDR*plus* assay and in one case no rifampicin resistance was found, indicating that only the latter case would have been missed by the MTBDR*plus* assay if rifampicin resistance alone were considered indicative of presumptive MDR-TB.

Of the five false-positive MTBDR*plus* results, two strains had the same banding pattern, with an *rpoB* mutation between codon 526 and 529, and S315T1 mutation in *katG*. A further two strains had mutations between *rpoB* 516 and 519 (rare mutations detected *in silico*) as well as S315T1 mutation in *katG*. One of these strains exhibited an additional mutation at *rpoB* 513 - 517, possibly indicating the presence of Del515, D516V or D516Y mutations. The fifth strain had the common S531L mutation in *rpoB* as well as a common *inhA* mutation (-15C15T). It is therefore likely that these were true MDR-TB strains.

#### Detection of resistance on smear-negative/culture-positive specimens

92.7% (1 338/1 443) results of the MTBDR*plus* assay for MDR-TB were available from positive MGIT cultures, compare to 79.8% (1 151/1 443) of specimens with an interpretable conventional DST result ( $p<0.0001$ ).

Performance characteristics of the MTBDR*plus* assay for detection of isoniazid, rifampicin and MDR-TB in smear-negative/culture-positive specimens are summarized in Table 6.2:

**Table 6.2. Performance of Genotype MTBDR*plus* assay compared to conventional culture and DST (smear-negative/culture-positive specimens)**

	Rifampicin	Isoniazid	MDR
Sensitivity % (95% CI)	98.4 (94.2 – 99.8)	92.2 (87.0 – 95.8)	94.7 (88.1 – 98.3)
Specificity % (95% CI)	99.2 (98.4 – 99.7)	99.8 (99.2 – 100.0)	99.7 (99.1 – 99.9)
Overall accuracy % (95% CI)	99.1 (98.4 – 99.6)	98.7 (97.8 – 99.2)	99.3 (98.6 – 99.7)
PPV % (95% CI)	93.8 (88.1 – 97.2)	98.7 (95.4 – 99.8)	96.8 (90.9 – 99.3)
NPV % (95% CI)	99.8 (99.3 – 100.0)	98.6 (97.7 – 99.3)	99.5 (98.8 – 99.8)

Detection of resistance directly from smear-positive sputum specimens

93.3% (749/803) results of the MTBDR*plus* assay for MDR-TB were available on direct sputum testing while 69.6% (559/803) had an interpretable conventional DST result ( $p < 0.0001$ ). A significant association ( $p < 0.001$ ) between smear-positivity grading and the proportion of interpretable results was found for the MTBDR*plus* assay: low positive, 1+, 2+ and 3+ positive specimens resulted in 58.9%, 93.3%, 96.6% and 98.1% of interpretable results respectively.

Performance characteristics of the MTBDR*plus* assay for detection of isoniazid, rifampicin and MDR-TB in smear-positive specimens tested directly are summarized in Table 6.3:

**Table 6.3. Performance of Genotype MTBDR*plus* assay compared to conventional culture and DST in direct testing of smear-positive sputum specimens**

	Rifampicin	Isoniazid	MDR
Sensitivity % (95% CI)	98.3 (90.9 – 100.0)	89.9 (81.0 – 95.5)	100.0 (92.7 – 100.0)*
Specificity % (95% CI)	99.0 (97.7 – 99.7)	99.6 (98.4 – 99.9)	99.6 (98.5 – 99.9)
Overall accuracy % (95% CI)	98.9 (97.7 – 99.6)	98.1 (96.6 – 99.1)	99.6 (98.6 – 100.0)
PPV % (95% CI)	92.1 (82.4 – 97.4)	97.2 (90.4 – 99.7)	96.1 (86.5 – 99.5)
NPV % (95% CI)	99.8 (98.9 – 100.0)	98.1 (96.6 – 99.1)	100.0 (99.2 – 100.0)*

\*one-sided, 97.5% confidence interval

#### 6.2.4 Turnaround time for laboratory results

Achieving an overall laboratory turnaround time (TAT) of less than seven days is a primary objective of the Demonstration Project. TAT was calculated monthly from the start of patient enrolment to assess the effect of time of routine implementation of the MTBDR*plus* assay, ie. whether increasing experience with running the assay led to a reduction in laboratory TAT. This preliminary analysis reports the TAT for smear-positive specimens only (as the TAT on positive cultures is highly dependent on the time to positivity in MGIT primary culture). Specimens with interpretable results both on the Genotype MTBDR*plus* assay and on conventional DST results were included in the analysis.

At the Cape Town laboratory the median time from specimen collection to receipt was 2 days (range 0 - 15 days), decreasing from a median of 10 days at the start to 5 days at the end of the assessment period. The median TAT from specimen collection to reporting of the assay result was 8 days overall, decreasing as the study progressed. Extreme values were reported in a few instances and the reasons are being investigated. One potential explanation relates to high workload in the laboratory which may have affected the flow of specimens indicated for Genotype MTBDR*plus* assay. In comparison, the median TAT from receipt of the specimen at the laboratory to reporting of results by conventional MGIT culture and DST was 41 days (range 21 - 69 days), consistent over the period of analysis.

At the Johannesburg laboratory the median time from specimen collection to receipt was 0 days (range 0 - 31 days), remaining consistent over the period of analysis. The median TAT for the assay result varied greatly per month, at 12 - 36 days (range 6 - 168 days), and remained unacceptably long (median 16 days) even after six months' implementation of the assay. The reasons for the long TAT are being investigated. In comparison, the median TAT for MGIT culture and DST was 46 days (range 25 - 105 days).

#### 6.2.5 Turnaround time for initiation of MDR-TB treatment

Results are limited and should be interpreted with caution. Of 99 MDR-TB patients for whom preliminary data were available, 29 patients started treatment before the assay result was reported, 28 patients started treatment on or after the date of the assay report but before the conventional DST results were reported, and 42 patients started treatment only after the conventional DST results were reported.

Patients starting treatment upon receipt of the assay result, before conventional DST results were available, were initiated on treatment a median of 24 days (range 8 - 56) after collection of the specimen and a median of 8 days (range 0 - 33 days) after the assay result was reported.

Patients starting treatment once the conventional DST result was reported were initiated on treatment after a median of 72 days (range 31 - 62) from the date of sputum collection, and a median of 21 days (range 2 - 128) after the conventional DST results were reported.

It is not possible to conclude from the preliminary findings whether the delay in initiation of treatment was due to clinicians waiting for conventional DST confirmation of the assay result or whether other factors were responsible (such as long waiting lists at MDR-TB treatment facilities). These aspects are currently being investigated.

### 6.2.6 *Data from qualitative assessment*

A semi-structured questionnaire was administered to laboratory personnel involved in implementing the MTBDR*plus* assay in the Johannesburg and Cape Town laboratories to explore qualitative issues related to training, technical support and test implementation.

Almost all laboratory staff reported being 'satisfied' or 'very satisfied' with use of the Genotype MTBDR*plus* assay, noting that the assay fitted well with other laboratory procedures and was flexible in terms of the ability to stop and re-start the assay procedure at several points.

Several staff reported that interpretation of unusual results of the line probe assay deserved more emphasis and required time and experience to acquire sufficient proficiency skills. Re-training at regular intervals was considered necessary, particularly with regard to interpretation of assay results and troubleshooting.

Laboratory personnel highlighted the need for meticulous cleaning and good work practices to reduce the risk of amplicon contamination.

### 6.2.7 *Experiences and lessons learnt*

Training at the time of assay implementation generally constituted four to five days in each laboratory and covered testing of clinical specimens and cultures, troubleshooting and theoretical aspects. Scientists with BSc, MSc or PhD qualifications in molecular biology were responsible for most of the molecular work, particularly for procedures involving amplification and hybridization. Involvement of less qualified staff was limited to specimen processing, conventional culture and DST procedures, and DNA extraction procedures.

Technical support was readily available in South Africa from the test distributor, and was required on a number of occasions to address hardware problems and to assist with troubleshooting related to unusual results and contamination.

Separate batches of tests had to be performed for smear-positive specimens and smear-negative/culture-positive specimens due to the requirement for a different number of PCR cycles. Testing of large numbers of specimens per day was facilitated by the automated (GT Blot) system; however, the manual (Twincubator) system was useful in allowing flexibility in testing smaller batches of specimens. Assays were performed five days per week, with decontamination and microscopy of specimens being done at weekends in some instances.

Problems with contamination were experienced, especially when newly-trained staff performed the assay.

Issues experienced during implementation of the assay consisted of adjusting laboratory workflow to triage appropriate specimens for rapid assay testing, explaining discrepancies between rapid assay results and conventional DST to clinicians and advising on patient management in such cases, as well as fitting daily rapid assay testing into an already high workload environment.

### 6.2.6 *Discussion and preliminary conclusions*

The performance of the Genotype MTBDR*plus* assay compared very favourably with conventional culture and DST in the Demonstration Project, with overall sensitivity, specificity, PPV and NPV of 96.5%, 99.7%, 96.5% and 99.7% respectively. The overall proportion of interpretable results with the Genotype MTBDR*plus* assay (92%) was significantly higher when compared to conventional methods (78%). The assay gave interpretable results in a considerable number of specimens in which no conventional DST result was available (mostly

due to contamination of the primary MGIT culture), and detected an additional 31 MDR-TB cases. The assay performed equally well when performed directly on smear-positive sputum specimens or on *M. tuberculosis isolates* from MGIT cultures.

The assay has been successfully implemented in two very high-volume public health laboratories in South Africa, with similar performance achieved in both laboratories. Less than half (47%) of MDR-TB cases detected in this setting were smear-positive, probably related to the high burden of HIV in South Africa. Although assay performance was associated with the level of smear positivity when direct testing on sputum specimens was done (very similar to the findings in Samara), the excellent performance characteristics of the assay were retained on isolates for which the original smear results were negative - a very encouraging finding for use of the Genotype MTBDR*plus* assay in combination with conventional culture in high HIV-burden settings.

A favourable turnaround time for reporting of assay results from smear-positive specimens was achieved in the Cape Town laboratory within two months of implementations; however, the same result has not yet been achieved in the Johannesburg laboratory despite six months of implementation. Both laboratories receive an exceedingly high volume of specimens, with more than 600 samples processed for culture each day. Implementation of the Genotype MTBDR*plus* assay in high-throughput laboratories may therefore require adjustments to the workflow as well as additional staff.

Preliminary data do not yet allow conclusions to be drawn about the reduction in TAT to initiate treatment in patients rapidly detected by the molecular assay. The impact of reduced TAT on patient outcomes such as sputum culture conversion, cure, mortality and treatment failure also remains to be assessed once the Demonstration project has been completed: however, it is conceivable that early diagnosis of MDR-TB together with early treatment initiation will have a significant impact at both the patient- and public health level.

## **7. ECONOMIC DATA**

### **7.1 Costing analyses**

#### *7.1.1 INNO-LipA Rif.TB*

Limited information was available on the cost of the INNO-LiPA Rif.TB assay. The meta-analysis by Morgan *et al*<sup>18</sup> quoted the cost of the kit at USD45 per sample tested. When additional costs for import and transport were taken into account, the actual cost per sample tested increased to USD116.<sup>18</sup>

#### *7.1.2 Genotype MTBDRplus*

Information on the cost of the Genotype MTBDR*plus* assay was available from Latvia and from the FIND Demonstration Project in South Africa:

In Latvia the cost of performing the MTBDR*plus* test was dependent on the number of specimens tested per run, varying between EUR85.86 for one specimen per run to EUR41.60 per test for 11 samples per run (Vaira Leimane *et al*, unpublished data).

In South Africa, extensive data on the cost of conventional culture and DST as well as the Genotype MTBDR*plus* assay have been collected from the post-validation phase of the FIND

demonstration studies in Johannesburg and Cape Town. A summary analysis is provided below.

*Although it is expected that FIND-negotiated prices will be extended to many high-burden settings, it will be important to ensure that pricing structures are negotiated within individual countries to achieve similar cost savings.*

#### Costing assumptions

- Unit costs for diagnostic methods evaluated were based on the routine diagnostic algorithm implemented at each site, with DST costs limited to isoniazid and rifampicin;
- Capital and recurrent costs were collated from the time of specimen arrival at the laboratory until time of the test result being reported (ie. limited to actual laboratory-related costs);
- Unit costs were calculated using the 'ingredients' approach, ie. multiplying the quantity of inputs used by price;
- Capital costs related to laboratory space, infrastructure and equipment were annualized based on estimated expected life-span;
- Overhead costs were calculated by allocating overall laboratory expenditure to each test based on the number of staff, staff time and building space utilized by each diagnostic system;
- Prices were expressed in USD 2007 values and based on the average 2007 exchange rate with the local currency (ZAR, South African Rand);
- Costs for conventional culture (MGIT) and DST (7H11 agar medium or MGIT) were based on South African pricing and local laboratory algorithms, while Genotype MTBDR*plus* costs were based on FIND-negotiated prices.

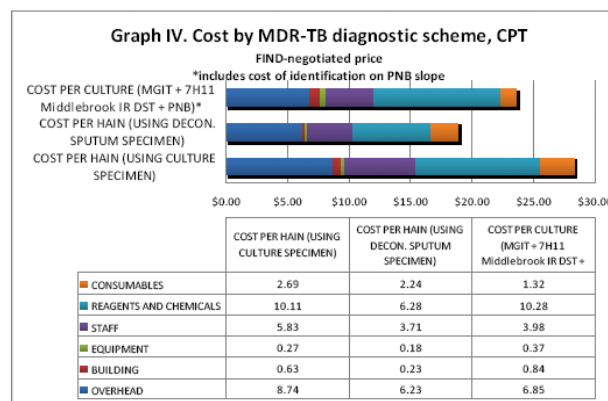
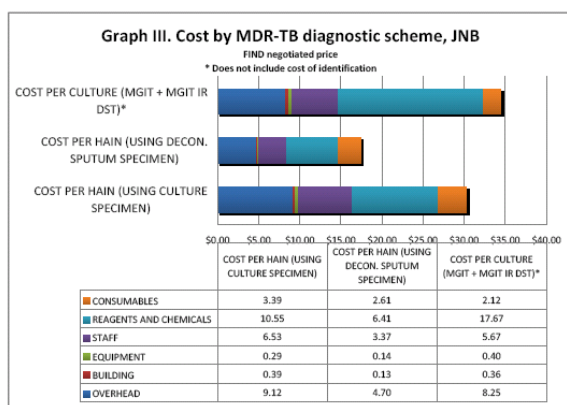
Relative costs per specimen are summarized in Table 7.1:

**Table 7.1. Costs of conventional culture and DST relative to Genotype MTBDRplus in NHLS laboratories, South Africa**

Unit cost (USD)	Cape Town	Johannesburg*
<i>Conventional culture and DST</i>		
Decontamination	2.26	3.18
Primary culture	7.82	11.35
Acid-fast bacilli (AFB) microscopy confirmation	2.57	2.99
DST (isoniazid and rifampicin)	12.01	16.96
<b>Total</b>	<b>24.66</b>	<b>34.50</b>
<i>Genotype MTBDRplus</i>		
Decontamination	2.26	3.18
Acid-fast bacilli (AFB) microscopy confirmation	1.00	1.44
Resistance detection (isoniazid and rifampicin)	15.61	12.74
<b>Total</b>	<b>18.90</b>	<b>17.40</b>

\*excludes species identification cost by Genotype Mycobacterium CM assay which is routinely done at approximately USD17 per test

As expected, the cost of liquid culture and DST (MGIT) was higher than liquid culture followed by DST on solid medium as illustrated by the graphs below. Different laboratory algorithms explain the differences in microscopy confirmation of acid-fastness of the primary culture when conventional methods were compared to the line probe assay.



Test-only variations in the cost of performing the Genotype MTBDRplus assay in the two sites were around USD3 per test, as illustrated in the graph below.

Variations were associated with differences in overhead and staff costs between the two laboratories, particularly related to the number of staff involved in pre-DNA extraction preparations (registration, labeling, aliquoting of specimens) and utilization of a manual



MTBDR*plus* assay is performed directly on smear-positive sputum specimens. Nevertheless, even when performed on cultures, the cost per valid test result for MTBDR*plus* is still lower than the cost per valid conventional DST result.

Subsequent analyses will be undertaken at other laboratories involved in the Demonstration Project, in order to explore differences in costs of implementing the assay in various laboratory-volume and resource settings.

The above analyses did not include costs associated with establishing the required laboratory infrastructure for molecular assays, or those associated with training. These will be assessed in a subsequent, more detailed analysis.

## **7.2 Cost-effectiveness analyses**

### *7.2.1 INNO-LipA Rif.TB*

A comparative cost-effectiveness analysis of five DST methods, undertaken in Peru as part of a clinical trial comparing rapid and conventional DST methods, indicated that the INNO-LipA Rif.TB test had the highest cost, largely due to kit cost (USD84.50 including consumables). Medical supplies and personnel costs were major determinants of overall cost for this and other commercial methods; however, all methods were reported as cost-saving in patient populations with 20% MDR-TB prevalence or higher.

All methods were reported to be cost-effective when the average cost per daily adjusted life year (DALY) was compared with gross national income in Peru. The authors concluded that concessionary pricing or a cheaper version of the test would substantially increase the cost-effectiveness of the line probe assay.

### *7.2.2 Genotype MTBDRplus*

A complete cost-effectiveness analysis (utilizing test effectiveness and relative prevalence of MDR-TB in the target population), as well as a detailed cost-benefit analysis (utilizing patient outcome data), will be conducted at the conclusion of the FIND/SAMRC/NHLS Demonstration Project.

## **8. IMPLEMENTATION CONSIDERATIONS**

### **8.1 Specimen collection, storage and transport**

The quality of sputum specimens submitted to the laboratory is critical in obtaining reliable results with line probe assays, as with other tests. Although contamination of specimens due to inappropriate storage and long transport times of specimens to the laboratory is less of a concern than with conventional culture-based approaches, a reliable specimen transport system will ensure that the full benefit is gained from use of a rapid assay, by reducing diagnostic delay times.

Current WHO recommendations call for MDR strains to be screened for XDR, both during surveys and in clinical settings where XDR-TB patients are suspected or confirmed.<sup>19</sup> Refrigerated transport of specimens and rapid delivery systems are essential for conventional culture and DST procedures; therefore, strict adherence to standard operating procedures for

specimen collection, storage and transport will be necessary if laboratories wish to implement second-line culture-based DST on specimens found to be MDR-TB by line probe assay.

## **8.2 Biosafety**

Line probe assays require the digestion, decontamination and concentration of clinical specimens prior to DNA extraction. These processes involve aerosol-producing methods such as homogenization and centrifugation which pose a considerable risk of infection as well as cross-contamination of specimens. The processing of specimens for line probe assays should therefore be performed in a laboratory with adequate and appropriate biosafety level precautions.

Current WHO recommendations specify that specimen processing for mycobacterial culture be performed in a biological safety cabinet (BSC) under at least biosafety level 2 (BSL2) conditions.<sup>20</sup> Procedures involving manipulation of *M. tuberculosis* cultures (identification, sub-culturing and DST) must be performed in laboratories complying with BSL3 standards.<sup>20</sup> Applying these recommendations to line probe assays, processing of smear-positive specimens for direct testing should be performed in a BSL2 level laboratory, whereas performing the assay on positive cultures would require BSL3 facilities.

Conceivably, sputum specimens could be rendered non-infectious before shipping to the referral laboratory, obviating the need for BSL2 facilities; however, while both line probe assays are likely to perform well on specimens inactivated after collection, there are currently no sufficient data to recommend this practice. It should also be kept in mind that inactivation/decontamination of specimens result in loss of viability of organisms. Therefore, no subsequent culture (eg. for smear-negative specimens) and DST (eg. for second-line anti-TB drugs to detect XDR) will be possible.

Once the decontaminated specimens have been denatured (by heating), organisms present in the specimen are rendered non-viable. Subsequent steps may therefore be performed outside of the BSC; however, due consideration needs to be given to preventing amplicon contamination through stringent cleaning and working practices.

## **8.3 Laboratory design**

Precautions to reduce the risk of cross-contamination of DNA molecular procedures are critical. This is achieved by using different rooms for DNA extraction, preparation of reagents for PCR (pre-amplification), PCR amplification and hybridization, and interpretation of results (post-amplification). Restricted access and uni-directional workflow through the rooms further reduce the likelihood of amplicon contamination. Careful cleaning of all rooms after each use is also critical.

Due to space constraints it may not be possible to provide separate rooms for each step of the process in all settings. As a minimum requirement, three separate rooms for the different molecular steps should be established – one for DNA extraction, one for pre-amplification procedures, and one for amplification and post-amplification processes. As large equipment is not required for running the assays, the rooms can be fairly small in size. Restricted access, attention to the direction of workflow, and meticulously followed procedures for cleaning, are critical in attaining satisfactory results.

In most settings, renovations may be required to provide adequate facilities for performing line probe assays and the time, space and resources required for such renovations should be carefully considered.

## **8.4 Electrical supply and back-up power**

Reagents used in line probe assays must be refrigerated or frozen, while amplification and hybridization procedures must be conducted under closely monitored temperature conditions. Uninterrupted power supply (UPS) connection is required during PCR amplification and use of the automated hybridization systems to avoid interruption of the procedure and subsequent loss of results.

Use of line probe assays therefore poses challenges in settings where interruption of the electrical power supply is common. Connection of laboratory power supply to a back-up generator and UPS is strongly recommended in such settings.

## **8.5 Commercial vs in-house line probes assays**

Tests used to inform patient care can only be ethically justifiable if performed with a product that has met pre-defined performance targets in carefully controlled evaluation studies, and which have been registered for a given indication. Both commercial assays evaluated in this report are manufactured under ISO certification standards, offering the advantage of quality-assured reagents and test kits.

No in-house line probe assays have been adequately validated or evaluated outside of limited research settings, and their use for clinical care of patients is not recommended. In addition, consistent supply of the multiple reagents necessary for line in-house probe assays may be difficult in many resource-constrained settings.

Other, similar commercial assays, if/when developed, would need to be subjected to careful evaluation studies and registration before their wide-spread implementation can be recommended.

The Genotype MTBDR*plus* assay includes detection of both rifampicin and INH resistance, whereas the INNO-LiPA Rif.TB detects rifampicin resistance only. The choice of technology for line probe assays should take into account the prevalence of MDR-TB and rifampicin mono-resistance as well as country guidelines for management of rifampicin mono-resistant and MDR-TB patients.

## **8.6 Registration**

Registration for diagnostic tests is not required in most high-burden countries. The Genotype MTBDR*plus* assay is registered in Australia, Europe, Thailand and Canada, with registration in Russia in process. Information on registration of the INNO-LiPA Rif.TB assay is pending.

Both commercial line probe assays are manufactured under ISO certification and are CE marked, although neither has FDA approval. Information on expanded CE marking to include the use of INNO-LiPA Rif.TB on clinical specimens is pending.

## **8.7 Shipping/customs clearance**

No specific issues regarding shipping or customs clearance of commercial line probe assays have been identified thus far (Hain Lifescience, personal communication).

## **8.8 Reagent quality and shelf-life**

Molecular grade water and Taq polymerase are required for PCR amplification. The quality of these reagents may critically affect the performance of line probe assays, and locally available reagents should be validated prior to use.

Short expiration dates of reagents are a concern for laboratories, especially in relatively inaccessible areas with complex customs clearance procedures. Management of inventory based on usage, shelf-life and lead time for deliver of orders is therefore needed. FIND has developed a checklist (Annex 4) to facilitate ordering, shipment and importation/customs clearance.

The shelf life of the Genotype MTBDR*plus* assay is 18 months from date of manufacture. Information on the shelf-life of the INNO-LiPA Rif.TB assay is pending.

## **8.9 Equipment**

In addition to the equipment required for initial digestion-decontamination of sputum specimens (such as BSCs and safety centrifuges), line probe assays require specific equipment for molecular procedures such as a thermal cycler, shaking platform and water bath, heating block, sonicator, micro centrifuge and tubes, fridge, freezer, micropipettes and pipette tips, and PCR tubes. These are available from various commercial suppliers.

Correct specifications of equipment should be confirmed with line probe assay manufacturers and adequate lead time for procurement of such items must be allowed for when implementing these assays.

Certain equipment such as incubators and automated hybridization systems are product-specific. Both commercial line probe assays can be used in automated, product-specific hybridization systems, ie. Twincubator and GT Blot 48 for the Genotype MTBDR*plus* assay; Auto-LipA 48 for INNO LipA Rif.TB.

Manual line probe assay systems cost total approximately USD16 000 (FIND, personal communication) and are appropriate for use in laboratories processing small numbers of specimens. Automated systems require a much larger initial capital outlay but can process up to 48 samples per run, taking between 2 and 3.5 hours, and are therefore an option to consider for high-throughput laboratories. The additional cost of the automated GT Blot system (Hain Lifescience) is EUR14 352. Information on the price of the Auto-LipA 48 (Innogenetics) is pending.

## **8.10 Human resources and training**

Successful implementation and interpretation of line probe assays is highly dependent on the skill of laboratory staff performing the testing and the quality of supervision. The FIND/SAMRC/NHLS Demonstration Project has shown that these assays can be successfully implemented in high-volume laboratories; however, this is heavily reliant on the quality and training of personnel and their adherence to strict working practices, including cleaning and uni-directional workflow.

Since skilled and highly trained personnel are required for performing line probe assays, the human resource requirements need to be carefully assessed prior to implementation. It should be noted that supervision in most of the published studies was performed by scientists with postgraduate training in molecular technology.

Interpretation of results of line probe assays must be done with care due to the complexity of interpreting the banding patterns. A high level of skill is required to interpret banding patterns in cases of unusual mutations or mixed mycobacterial populations. These issues must be covered in initial training, with access to ongoing access to technical support when unusual results are obtained. Post-training supervision and monitoring (*ad hoc* or remote) of staff by a senior person with expertise in molecular assays is therefore strongly recommended.

### **8.11 Technical support**

Coordination between commercial suppliers and customers with regard to ordering, shipping and customs clearance is critical to ensure smooth delivery of reagents and equipment and to avoid customs delays which may cause product deterioration due to inadequate storage conditions in transit.

A detailed plan for training, based on country-specific human resource needs, must be developed. Training may be provided directly by the manufacturer, by a nominated local distributor, or by an accredited third-party, depending on the location and circumstances. Agreements as to responsibilities for training should be made in advance of supply of equipment and reagents.

In addition, ongoing technical support and continuous supply of consumables and reagents is essential, best provided for in a formal service contract between the supplier and customer. Such a contract should cover the following aspects:

- Maintenance of equipment and provision of a servicing contract, including the repair or replacement of faulty equipment at short notice;
- Supply of consumables and reagents with at least six months expiry after arrival at the laboratory;
- A detailed plan for provision of ongoing technical support and the channels through which this will be provided, eg. a local distributor, via helpline, or internet-based support;

### **8.12 Quality assurance**

The exquisite sensitivity of nucleic acid amplification assays such as PCR is also a draw-back, since even the smallest amount of DNA can be amplified. Target amplification methodologies such as line probe assays therefore require strict adherence to a number of procedures to minimize the risk of contamination leading to false-positive results.

Sources of contamination occurs when unwanted DNA is introduced to the assay through water, reagents, laboratory disposables and equipment, or through the environment, such as sample carry-over between tests or introduction of nonspecific amplification products through unrelated activities in neighbouring laboratories.

Providing each room with separate sets of equipment and supplies substantially reduces the risk of carry-over contamination. Working areas, equipment and everything that is routinely touched by hand (including doorknobs, telephones, handles of fridges and freezers, etc.) must be cleaned on a regular basis using appropriate cleaning agents and strategies. Additionally, the risk of contamination can be reduced by careful waste disposal.

From a clinical perspective, prevention of false-negative results is equally important. In nucleic acid amplification assays false-negative results are mostly due to the presence of inhibitors (often arising from laboratory surfaces), sub-optimal assay conditions or omission of key steps, or the absence of positive controls and internal process controls.

Aside from strict adherence to process and cleaning protocols and appropriate use of positive- and negative controls, monitoring of results based on expected outcomes is very useful to detect false-positive and false-negative trends. Knowledge of the underlying prevalence of MDR-TB in the populations from which the specimens are obtained is particularly useful.

Although data are limited, multi-centre PCR quality assurance studies have shown alarmingly high false-positive rates, but also indicating that procedural problems rather than specific assays were responsible.<sup>21</sup> While internal quality control should be executed continuously by laboratory staff, external quality assurance through blinded rechecking of subsets of specimens or proficiency testing by an independent external organization is strongly recommended.

Standardized external quality assurance programmes for line probe assays are not yet available. Development of such systems is therefore an urgent priority.

### **8.13 Recording and reporting**

In order to gain full benefit from implementation of line probe assays, systems must be implemented to ensure that results are reported rapidly to clinicians and patients to ensure that appropriate treatment is initiated. Furthermore, where conventional DST is used to confirm rapid assay results, the possibility of discrepant results must be considered, and a mechanism for explanation of implications of discrepancies to clinicians should be established.

## **9. CONCLUSIONS**

While the need remains for enhanced smear microscopy and for conventional systems for culture and drug susceptibility testing, the urgency created by MDR-TB and HIV-associated TB necessitates a paradigm shift in increasing the speed of treatment initiation. This requires expanded access to rapid diagnostics, including widespread utilization of molecular assays should their performance be shown to warrant their use.

A multitude of laboratory validation studies and implementation projects in diverse geographical and epidemiological settings have confirmed that available commercial line probe assays show excellent correlation with conventional DST methods and are highly sensitive and specific for the detection of rifampicin resistance. While specificity is excellent for isoniazid resistance, sensitivity estimates are modest and highly variable, albeit considerably improved in newer generation line probe assays. Using resistance to rifampicin as a proxy for MDR has been shown to be highly reliable, with sensitivity (and consequently PPV) only marginally affected. The slightly reduced sensitivity is in all likelihood due to strains with true rifampicin mono-resistance and/or strains with *rpoB* mutations that are defined as susceptible in phenotypical DST tests (given the rather robust cutoff points used in phenotypic methods to define resistance). In either case, treatment with an appropriate MDR regimen (strengthened by the addition of isoniazid where relevant) would be highly preferable.

Although line probe assays were originally designed for drug resistance detection in isolates, a number of recent studies, including the large-scale FIND/SAMRC/NHLS demonstration project in South Africa, have shown excellent performance characteristics when line probe assays are performed directly on smear-positive sputum specimens. A high proportion of interpretable results have been achieved, with sensitivity exceeding 97% and specificity exceeding 98%. These findings confirm the value of line probe assays in rapid screening of patients suspected of MDR-TB and warrant formal policy recommendations by WHO on their use in screening and diagnostic algorithms.

As expected, the sensitivity of line probe assays in smear-negative/culture-positive specimens was lower than in smear-positive specimens. Although reduced to around 80% in such

specimens, the value of line probe assays after rapid conventional culture warrants urgent further investigation, especially in high HIV-burden settings. In addition, wider application of molecular tests would benefit greatly from procedures that maximize DNA extraction.

While molecular methods are not yet sufficiently developed to fully replace conventional methods for culture and DST, implementation of line probe assays may significantly reduce the demand for sophisticated and expensive conventional laboratory infrastructure, especially in high MDR-TB burden settings. Experience to date with implementation of line probe assays has been in laboratories already performing conventional culture and DST, ie. with established core capacity to conduct complex and sophisticated laboratory procedures. It should be kept in mind that line probe assays are equally complex to perform and require skilled and well-trained laboratory personnel, as well as adequate laboratory space and -design to reduce the risk of false-positive results.

As with any new technology, several factors must be considered when implementing line probe assays, particularly in high-burden TB settings. Without these, implementation of line probe assays is likely to be problematic and lead to poor-quality results. The most critical factor relates to the risk of amplicon contamination, which must be controlled by appropriate and adequate laboratory design, restricted access to molecular facilities, fastidious cleaning and working practices, and highly trained and motivated laboratory personnel.

Line probe assays could potentially be used with less stringent biosafety and specimen transport requirements if sputum processing methods are applied that inactivate *M. tuberculosis*, provided that these do not affect assay sensitivity. Since this would considerably enhance scale-up of programmatic management of MDR-TB, research on the impact of different inactivation/decontamination methods on assay performance, linked to procedures to maximize DNA extraction as mentioned above, are priority areas.

Interpretation of line probe assays results has been shown to be generally easy and straightforward. However, unusual patterns of mutation and wild-type bands can occur on occasion, which require experience in interpretation and troubleshooting. Hetero-resistance, the presence of a mixed population of resistant and susceptible sub-populations in a clinical isolate, may be a hindrance to successful molecular drug resistance testing, and the sensitivity of such methods may be affected where a particularly low percentage of resistant organisms is present. Technical support from manufacturers or experienced users, as well as establishment of an external quality assurance programme, is also critical in maintaining the quality of the results.

Geographical variation in prevalence of mutations associated with rifampicin - and in particular with isoniazid resistance - may result in varying performance of line probe assays in different epidemiological settings. In addition, discrepancies between results from line probe assays and conventional DST may be due to silent mutations and/or mutations not present in the core regions detected by the line probe assays. Screening and diagnostic algorithms incorporating line probe assays will therefore need to be developed for different epidemiological settings. Introduction of line probe assays should ideally be preceded by an assessment of sensitivity and specificity of these assays in a representative collection of MDR- and non-MDR isolates at country or at regional level. Research on the prevalence and clinical relevance of rare mutations will be equally important.

Limited data are available on the cost-effectiveness and cost-benefit of rapid TB diagnostics. Nevertheless, data from a few settings point towards the potential for substantial savings in overall TB diagnostic cost if line probe assays are judiciously introduced in screening algorithms for detection of MDR-TB. As shown by the results from South Africa, performing the Genotype MTBDR*plus* assay directly from sputum may result in a cost-saving of up to 50% when compared to the conventional procedures currently used. Initiatives such as those by FIND and UNITAID to make new diagnostics affordable and accessible to the public sector in developing countries have a crucial role to play.

WHO and partners have called for urgent expansion of culture and DST capacity in response to the emergency of MDR-TB and XDR-TB. The cost and complexity of establishing laboratory capacity for conventional culture and DST to meet the anticipated need present a formidable challenge. Recent experience in a number of countries has shown that it is feasible to implement line probe assays in high TB-burden settings, with data from South Africa, Nepal, Peru, Russia (Samara) and Latvia adding to a growing body of evidence which supports the feasibility of implementation of line probe assay technology in public health laboratory facilities in low- and middle income settings. The high degree of accuracy, substantial reduction in reporting time for results, and the possibility for high throughput with substantial cost savings suggest the potential of molecular testing to revolutionize the diagnosis of MDR-TB. However, strengthening of all laboratory aspects of TB control, including infrastructure, human resource capacity, specimen transport systems, and systems for rapid patient follow-up will be necessary to enable full benefit to be drawn from these exciting and newly emerging TB diagnostic technologies.

## 10. RECOMMENDATIONS

*The Expert Group on molecular line probe assays for rapid screening of patients at risk of MDR-TB considers that there is sufficient generalisable evidence to justify a recommendation on the use of line probe assays for rapid detection of MDR-TB, within country-specific settings, with further operational research to address country-specific implementation needs.*

**WHO should endorse the use of line probe assays, with the following guiding principles:**

- 5.1 Adoption of line probe assays for rapid detection of MDR-TB should be decided by Ministries of Health within the context of country plans for appropriate management of MDR-TB patients, including the development of country-specific screening algorithms and timely access to quality-assured second-line anti-tuberculosis drugs;
- 5.2 Line probe assay performance characteristics have been adequately validated in direct testing of sputum smear-positive specimens and on isolates of *M. tuberculosis* complex grown from smear-negative and smear-positive specimens. Direct use of line probe assays on smear-negative clinical specimens is not recommended;
- 5.3 The use of commercial line probe assays rather than in-house assays is recommended to ensure reliability and reproducibility of results, as in-house assays have not been adequately validated or used outside limited research settings. Any new or generic line probe assays should be subject to adequate validation, ie. published laboratory validation studies, adequate data to allow systematic review and meta-analysis (including assessment of data quality), and results from field demonstration projects documenting feasibility and consistent performance equal to conventional methods and commercial line probe assays. New or generic line probe assays for MDR-TB should have the following characteristics:
  - 5.3.9 A specific probe to identify *M. tuberculosis* complex;
  - 5.3.10 Multiple probes to detect the most common mutations in *rpoB* (codons 531, 526 and 516);
  - 5.3.11 Multiple overlapping wild-type (susceptible) probes covering the RRDR region of *rpoB*;

- 5.3.12 Preferably, multiple probes to detect both high-level (*catG* mutations) and low-level isoniazid resistance (*inhA* mutations);
  - 5.3.13 Strip technology, with appropriate assay procedure controls, allowing visual detection of results;
  - 5.3.14 Line probe test production under ISO 13485:2003 standards;
  - 5.3.15 Performance characteristics equal to those of conventional DST methods;
  - 5.3.16 Performance characteristics equal to those of current commercial line probe assays.
- 5.4 Adoption of line probe assays does not eliminate the need for conventional culture and DST capability; culture remains necessary for definitive diagnosis of TB in smear-negative patients, while conventional DST is required to diagnose XDR-TB. However, the demand for conventional culture and DST capacity may change, based on the local epidemiological situation and the use of line probe assays in country-specific screening algorithms;
- 5.5 As current line probe assays only detect resistance to rifampicin and/or isoniazid, countries with documented or suspected cases of XDR-TB should establish or expand conventional culture and DST capacity for quality-assured susceptibility testing of second-line drugs, based on current WHO policy guidance;
- 5.6 Adoption of line probe assays for rapid detection of MDR-TB should be phased in, starting at national/central reference laboratories or those with proven capability to conduct molecular testing. Once this has been accomplished, expansion could be considered, within the context of country laboratory strengthening plans, and considering availability of suitable personnel in peripheral centres, quality of specimen transport systems, and country capability to provide appropriate treatment and management of MDR-TB patients once diagnosed;
- 5.7 Adequate and appropriate laboratory infrastructure and equipment should be provided, ensuring that required precautions for biosafety and prevention of contamination are met:
- 5.7.1 Specimen processing for culture must be performed in biological safety cabinets (BSCs) in at least Biosafety Level (BSL) 2 facilities;
  - 5.7.2 Procedures for manipulation of cultures (conventional identification, subculture for DNA extraction and conventional DST) must be performed in BSL3 facilities;
  - 5.7.3 Laboratory facilities for line probe assays require at least three separate rooms - one each for DNA extraction, pre-amplification procedures, and amplification and post-amplification procedures. Restricted access to molecular facilities, uni-directional work flow, and stringent cleaning protocols must be established to avoid amplicon contamination leading to false-positive results;
  - 5.7.4 Successful establishment, staffing, and maintenance of BSL2, BSL3 and molecular laboratories are demanding. Upgrading of facilities and establishment of the required infrastructure for molecular assays should be carefully planned and adequately financed;
- 5.8 Appropriate laboratory staff should be trained to conduct line probe assay procedures, especially those relating to amplification and interpretation of results. Supervision of staff by a senior individual with adequate training and experience in molecular assays is strongly recommended;

- 5.9 A detailed commercial sales contract and customer support plan should be negotiated with manufacturers, guaranteeing ample and continuous supply of materials, appropriate shipment conditions, customs clearance, equipment installation, maintenance, repair and replacement, and provision of training and ongoing technical support;
- 5.10 Stringent laboratory protocols, standard operating procedures for molecular line probe assays, and internal quality control mechanisms must be implemented and enforced. A programme for external quality assessment of laboratories involved in line probe assays should be developed as a matter of priority.
- 5.11 Mechanisms for rapid reporting of line probe assays results to clinicians must be established to provide patients with the benefit of an early diagnosis;
- 5.12 WHO and partners should assist countries with operational plans to introduce line probe assays within the appropriate epidemiological and resource availability context.

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Heidi Albert, Richard O'Brien, Mark Perkins, Giorgio Roscigno.

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## **Annex 1: Expert Group Meeting Agenda and List of Participants**

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### **MOLECULAR LINE-PROBE ASSAYS FOR RAPID SCREENING OF PATIENTS AT RISK OF MULTIDRUG-RESISTANT TUBERCULOSIS**

#### **- EXPERT GROUP MEETING -**

Date and time: 31 March 2008, 09:00 – 17:00  
Venue: Crowne Plaza Hotel, Geneva

#### **BACKGROUND**

Alarming increases in multidrug-resistant tuberculosis (MDR-TB) have been documented globally, compounded by the emergence of extensively-drug resistant TB (XDR-TB) which is virtually untreatable in many settings. Lethal outbreaks of MDR-TB and XDR-TB in association with HIV infection have recently been described in South Africa and linked to institutional transmission, particularly in the absence of adequate infection control.

In response to the XDR-TB crisis, the World Health Organization (WHO) and partners constituted a Global XDR-TB Task Force in October 2006 and subsequently issued a Global XDR-TB Response Plan. Among a number of measures, the plan calls for wide-scale implementation of rapid methods to screen patients at risk of MDR-TB in order to enable prompt and appropriate treatment, decrease morbidity and mortality, and interrupt transmission. However, lack of appropriate diagnostics and inadequate laboratory capacity is a key barrier preventing an effective response to MDR- and XDR-TB.

Novel technologies for rapid detection of TB drug resistance are in development phase, undergoing laboratory validation, or in early stages of large-scale field studies to assess their feasibility, cost-effectiveness and cost-benefit. Molecular line probe assays focused on rapid detection of rifampicin resistance as a proxy for MDR are most advanced. Apart from the impact on morbidity, mortality and transmission of MDR-TB, introduction of these assays in screening and diagnostic algorithms could significantly reduce the need for sophisticated and costly laboratory infrastructure, still vastly inadequate in most high-burden countries.

An Expert Committee has been convened by WHO to assess available data on rapid line probe assays for rifampicin resistance as a proxy for MDR-TB, with the aim towards development of a policy recommendation on their use to the WHO Strategic and Technical Advisory Group for TB (STAG-TB). The Expert Committee constitutes a mix of laboratory experts with technical experience in TB molecular assays, scientists/researchers, epidemiologists and national TB programme representatives.

#### **MEETING OBJECTIVES**

- To review and evaluate data from published literature and laboratory validation studies on the performance characteristics of rapid line probe assays for MDR-TB screening;
- To evaluate preliminary data from field demonstration projects on rapid line probe assays for MDR-TB screening, coordinated by the Foundation for Innovative New Technologies (FIND);

- To define the laboratory infrastructure, human resource requirements, and further operational research data needed for programmatic implementation of rapid line probe assays.

### EXPECTED OUTCOMES

- Recommendations on the use of line probe assays for rapid screening of patients at risk of MDR-TB;
- Consensus on laboratory infrastructure, human resource requirements and further operational research data needed for programmatic implementation of rapid line probe assays.

## AGENDA

Chair: C Gilpin

<b>09:00 – 09:10</b>	Welcome	P Nunn
<b>09:10 – 09:20</b>	Introductions Meeting scope and objectives	Chair
<b>09:20 – 09:50</b>	Systematic review of data from published literature and laboratory validation studies on the performance characteristics of rapid line probe assays for MDR-TB screening	K Weyer
<b>09:50 – 10:30</b>	Discussion	All
<b>BREAK 10:30 – 10:45</b>		
<b>10:45 – 11:30</b>	Preliminary data from field demonstration projects on rapid line probe assays for MDR-TB screening	R O'Brien, FIND
<b>11:30 – 13:00</b>	Discussion	All
<b>LUNCH 13:00 – 14:00</b>		
<b>14:00 – 14:20</b>	Laboratory infrastructure and human resource requirements needed for implementation of rapid line probe assays	F Drobniewski
<b>14:20 – 14:40</b>	Further operational research data needed for implementation of rapid line probe assays	F Cobelens
<b>14:40 – 15:00</b>	Programmatic perspective on implementation of rapid line probe assays	S Egwaga
<b>15:00 – 16:00</b>	Discussion	
<b>BREAK 16:00 – 16:15</b>		
<b>16:15 – 16:45</b>	Meeting summary and consensus	Chair
<b>16:45 – 17:00</b>	Next steps and closing	Chair

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## **Annex 2:**

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**Annex 3:**

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