Molecular Detection of Drug-Resistant Tuberculosis By Line Probe Assay

Laboratory Manual for Resource-Limited Settings

Marinus Barnard[1 2]
Linda Parsons[3]
Paolo Miotto[4]
Daniella Cirillo[4]
Knut Feldmann[5]
Cristina Gutierrez[5]
Akos Somoskovi[5]

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FIND Avenue de Budé 16, 1202 Geneva tel: +41 22 710 05 90 fax: +41 22 710 05 99 www.finddiagnostics.org

[1]National Health Laboratory Services, Cape Town, South Africa
[2]DST/NRF Centre of Excellence in Biomedical Tuberculosis Research, US/MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences, Faculty of Health Sciences, Stellenbosch University, Tygerberg, South Africa
[3]Clemson University, South Carolina, USA
[4]San Raffaele Hospital, Milano, Italy
[5]FIND, Geneva, Switzerland

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Chapter 1: BACKGROUND

Drug resistance is a major threat to worldwide control of tuberculosis (TB). Drug-resistant and multidrug-resistant (MDR-TB; resistant to at least isoniazid [INH] and rifampin [RMP]) strains of *Mycobacterium tuberculosis* are man-made problems mainly related to poor case management and lack of quality drugs. Treating patients with MDR-TB requires expensive and toxic chemotherapy and often results in extremely low cure rates and increased mortality. Accurate and rapid detection of resistant strains is critical to provide appropriate treatment and to intercept the transmission of drug-resistant TB. However, lack of access to quality laboratory diagnostics continues to jeopardize efforts to control the worldwide transmission of TB. This situation has been further complicated by the recent emergence of extensively drug-resistant (XDR) TB. XDR-TB is caused by MDR strains of *M. tuberculosis* that have developed resistance to any fluoroquinolone and at least one of the following three injectable drugs: capreomycin, kanamycin and amikacin. The global threat of XDR-TB is a greater public health concern than MDR-TB since it requires more complex treatment and prolonged hospitalisation, particularly in subjects with disseminated TB. Rapid laboratory detection of MDR- and XDR-TB is critical, as delays in treatment can result in increased transmission in the community. HIV-positive individuals are especially susceptible to co-infection with TB. Poor diagnostic capacity and lack of infection control for both drug-susceptible and drug-resistant TB play a significant role in enhancing disease transmission to severely immuno-compromised individuals, especially those with HIV.

CONVENTIONAL DRUG SUSCEPTIBILITY TESTING

Shortly after the first anti-tuberculosis drugs were introduced into clinical practice in the late 1940's, resistance to these drugs was observed. In 1961, the Pasteur Institute developed the proportion method, using critical concentrations of drugs for laboratory

detection and confirmation of TB drug resistance. This assay became the gold standard for TB drug susceptibility testing (DST). Conventional DST is carried out on solid or, more recently, in liquid media after detection and identification of the pathogen previously grown from clinical specimens. Initial growth, detection and identification may take 2 - 8 weeks and susceptibility testing for first line drugs on solid medium may take an additional 3 - 6 weeks. When using automated liquid culture systems, these turnaround times can be reduced to 3 - 21 days and 4 - 7 days for isolation and DST respectively (an average reduction of 15 days in total when using automated liquid systems). When resistance to first-line drugs is detected, DST for second-line drugs may result in further, even more significant delays in treatment. Unfortunately, current methods for DST are time consuming, labour intensive, and costly. Problems with standardization of the tests and the stability of the drugs in different culture media further limit the ability of these assays to yield accurate results. Furthermore, correct execution of the assay and interpretation of results require extensive experience and demonstrated proficiency; these skills are essential, as the quality of results greatly impact both the survival and contagiousness of patients. Finally, performance of conventional DST requires a biosafety level 3 (BSL-3) laboratory, which is costly to establish and maintain especially in resource-poor settings.

Late diagnosis of drug resistance is potentially a major contributor to the transmission of TB and may compromise the efficacy of therapy. Therefore, more advanced, robust, rapid and affordable technologies are needed to strengthen laboratory capacity for rapid and accurate diagnosis of both drug-susceptible and drug-resistant strains of *M. tuberculosis*. Rapid diagnosis of drug-resistant TB will have several benefits. These include earlier initiation of treatment of patients, which will in turn save more lives, reducing the time spent on inadequate treatment when infected by drug-resistant strains (therefore promoting development of additional drug resistance), and reducing the spread of MDR-TB in the community. Moreover, early identification of MDR-TB will lead to faster initiation of laboratory testing for XDR-TB.

MOLECULAR DRUG SUSCEPTIBILITY TESTING

Recently, an improved understanding of the molecular basis of drug resistance and methods for extracting DNA directly from bacteria in clinical samples has resulted in the ability to determine susceptibility or resistance to anti-TB drugs in less than 24 hours. In today's laboratory, molecular techniques have become important adjuncts to traditional culture-based procedures, as they provide more rapid detection of drug resistance-associated mutations in the slow-growing *M. tuberculosis*. In addition, molecular detection of drug-resistance may be applied directly to clinical specimens (without the need for isolating the strain first on solid or liquid culture). Molecular methods can also be used on specimens that are not suitable for growth–based assays, such as specimens containing non-viable bacteria (killed by heat or chemical inactivation during drug therapy), specimens highly contaminated with non-mycobacterial flora, or specimens with mixed TB and non-tuberculous mycobacteria (NTM). Additional advantages of the use of non-viable specimens include the simplification and reduction of biosafety requirements for both laboratory testing and specimen transport.

Molecular methods allow the processing of a large number of specimens at the same time, and automation of the methodology provides standardization and quality assurance of testing that is independent of the proficiency of the user. Molecular methods are also able to detect mutations associated with lower levels of phenotypic resistance (Minimal Inhibitory Concentration; MIC). Such low-level resistance-associated mutations may represent an early stage in the development of high-level resistance. The ability to identify these mutations may therefore be clinical useful in optimizing treatment by switching the more potent drug regimens: an advantage over conventional DST that reports only the results from the critical concentration tested.

Finally, for some classes of antibiotics (e.g. aminoglycosides), the detection of specific mutations allows one to predict drug resistance to an entire family of drugs.

The major advantage of molecular methods is that they are fast, robust, may be adapted for high throughput use, and can require significantly fewer manipulations and technical skills compared to growth detection and conventional DST. The assays

require relatively simple-to-use equipment that can also serve as a platform for testing specimens for other diseases or purposes. Also, as previously mentioned, molecular assays do not require viable bacteria, therefore specimen transport conditions do not affect test outcomes, unlike culture, and specimens can be shipped by regular mail.

REQUIREMENTS FOR MOLECULAR TESTING

When using molecular assays to test clinical specimens (especially those suspected of containing TB), a BSL-2 laboratory is required, however when testing viable mycobacterial isolates, preparation of the isolates for DNA isolation should be performed in a BSL-3 laboratory, along with use of appropriate personal protective equipment. Only after heat-killing of the organism and DNA isolation can the sample be considered non- infectious and moved to the molecular laboratory. It is important to bear these prerequisites in mind when integrating molecular drug-resistance testing for TB into existing molecular facilities that have been established for other diseases.

If organized on a one-to-one basis, the training required for establishing laboratory staff proficiency in a particular molecular method can be accomplished within a few weeks. Interestingly, it appears to be more challenging for trainees to establish confidence and accuracy on the proper interpretation of molecular results than on actually performing the tests. These issues are described in more detail below.

MOLECULAR MECHANISMS OF DRUG RESISTANCE TO INH AND RMP

The number of drugs currently available for effective anti-TB treatment is limited due to the intrinsic or natural resistance of *Mycobacterium tuberculosis* to the majority of common antibiotics. This is due to the fact that *M. tuberculosis* and other members of

the *M. tuberculosis* complex use several strategies to resist the action of antimicrobial agents. First, the mycobacterial cell is surrounded by a specialized, highly hydrophobic cell wall that results in decreased permeability to many compounds. Active drug efflux systems, degrading or inactivating enzymes, and the genes associated with these functions, have been identified in *M. tuberculosis*. However, genetic studies indicate that resistance to anti-mycobacterial drugs is the consequence of spontaneous mutations in genes encoding either the target of the drug, or enzymes involved in drug activation. Resistance-associated point mutations, deletions, or insertions have been described for all first-line drugs (isoniazid [INH], rifampin [RMP], pyrazinamide [PZA], ethambutol and streptomycin) and for several second-line and newer drugs (ethionamide, fluoroquinolones, macrolides and nitroimidazopyrans). However, no single genetic alteration has yet been found that results in the MDR phenotype (resistance to at least INH and RMP). Rather, MDR develops by sequential acquisition of mutations at different loci, usually because of inappropriate patient treatment. Because MDR strains are the result of accumulative mutations, growth of *M. tuberculosis* can be successfully controlled in the host by concomitant treatment with more than one drug. Thus, treatment regimens consisting of three to four drugs are used routinely to treat TB patients. **Table 1** shows the principal *M. tuberculosis* genes implicated in resistance to key anti-TB drugs.

The frequency of mutations that cause the onset of resistance is nearly 10^{-6} for INH and streptomycin, 10^{-8} for RMP and 10^{-5} for ethambutol. It follows that the frequency of concurrent mutations, conferring resistance to both INH and RMP, is 10^{-14}, making the combined resistance to these two drugs a rare occurrence in naive patients. It appears that patients with large populations of *M. tuberculosis* could host “a priori” resistant organisms.

Drug	Gene	Gene product	Mutations
RIFAMPIN	*rpoB*	RNA pol (β subunit)	hot-spot region (98%) cod. 508 to 535; N-term region
RIFABUTIN	*rpoB*	RNA pol (β subunit)	cod. 144, 146, 148, 505, 512, 526, 531
INH	*katG*	Catalase-peroxidase	cod. 315 (60-80%) Polymorphism Leu463Arg
	inhA	NADH-dep enoyl-ACP red	promoter region (ribosome binding site) pos. -8; -15 (15%); coding region (example: Ser94Ala)
	ndh	NADH dehydrogenase	coding region
	ahpC	small subunit of alkylhydroperoxide reductase	promoter region (mutations relatively rare)
	OxyR	regulon (controls expression of katG and several other genes including ahpC)	(mutations relatively rare)
ETHIONAMID	*inhA*	NADH-dep enoyl-ACP red	Ribosome binding site pos. -8; -15 coding region: cod. 16, 21, 47, 78, 94, 95
	ethA	monooxygenase	coding region
	ethR	putative monooxygenase repressor	coding region
	ndh	NADH DH	coding region
PZA	*pncA*	Pyrazinamidase	coding region (72-98%)
ETHAMBUTOL	*embB*	Arabinosyl transferase	ERDR (70%) cod. 306; 239, 240, 247, 282, 285, 299, 311, 330, 368, 397, 466, 469, 471, 630
	embC	Arabinosyl transferase	cod. 251, 254, 270
	embR	(regulator) - ?	
FLUOROQUINOLONES	*gyrA*	DNA gyrase (sub. A)	QRDR (nt. 220 to 339) cod. 74, 88, 90, 91, 94 (70%) Polymorphism at cod. 95
	gyrB	DNA gyrase (sub. B)	QRDR (nt. 1414 to 1530) cod. 495, 516, 533
	mfpA	protein that mimic DNA	?
STREPTOMYCIN	*rpsL*	12S ribiosomal protein	cod. Lys43Arg, Lys88Gln/Arg, Arg9His, Val93Met (60%)
	Rrs	16S rRNA	reg. 530: C to T 491, 512, 516; A to C/T 513 (8%); reg. 915: C to A/G 903; A to G 904; C to T 798; G to A 877; A to C 906
CAPREOMYCIN	*rrs*	16S rRNA	A to G 1401
	tlyA	rRNA methyltransferase	coding region
VIOMYCIN	*rrs*	16S rRNA	coding region
KANAMYCIN	*rrs*	16S rRNA	C to T 1402; 1401, 1483, (>) 1400 (67%)
AMIKACIN	*rrs*	16S rRNA	1400

Table 1: Principal *M. tuberculosis* genes implicated in resistance to key anti-TB drugs

INH inhibits the synthesis of mycolic acids of actively growing mycobacteria. A lack of mycolic acid synthesis eventually results in the loss of cellular integrity and viability. INH is a pro-drug that is activated in the bacterial cell by the mycobacterial enzyme catalase. The emergence of INH resistance is multi-factorial and involves mutations in several genes such as *katG*, which encodes the activating enzyme, catalase, or regulatory genes such as *inhA*, *ahpC-oxyR*, *ndh* and *furA*.

More than 60% of INH-resistant strains of *M. tuberculosis* isolated from patients have missense mutations or small deletions/insertions in *katG*. Most *katG* mutations are found between codons 138 and 328 with the most commonly observed gene alteration at codon 315 (60 - 80% of cases). The most frequent mutation at codon 315 is a Ser→Thr substitution that is estimated to occur in 30 – 60% of all INH-resistant isolates. Interestingly, the *katG* 463 (CGG-CTG) (Arg-Leu) amino acid substitution is the most common polymorphism found in *katG* and is not associated with isoniazid resistance.

In the mid-1950's, Middlebrook et al. demonstrated that loss of catalase-peroxidase activity can result in INH resistance. The peroxidase activity of this enzyme is essential

to convert INH into its active form, capable of killing TB. The enzymatic activity of catalase in INH-resistant mutants is significantly lower or absent, compared to INH susceptible wild-type strains.

Several studies have shown that certain strains with low-level INH resistance frequently develop co-resistance to ethionamide, suggesting that the two drugs share at least one molecular target. Although the two drugs have a different activation mechanism, other studies revealed that both of them are targeting the *inhA* gene. *inhA* encodes a mycobacterial enoyl-ACP reductase enzyme that is probably involved in the biosynthesis of mycobacterial cell wall fatty acids. INH and ethionamide act to neutralize this enzyme, which in turn inhibits cell wall synthesis of the bacteria. The most common mutations of *inhA* occur in the promoter region of the gene and are most frequently seen at positions -24 (g→t), -16 (a→g), or -8 (t→g/a) and -15 (c→t). Changes in this region result in increased expression and production of the enoyl-ACP reductase enzyme, which consequentially increases the level of the drug target and counterbalances the interruption in cell wall synthesis due to drug titration. Strains bearing mutations in the coding region of *inhA* show low-level resistance. Six point mutations associated with INH resistance within the structural *inhA* gene have been identified: Ile16Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro. These mutations in the structural *inhA* gene are rarely found in clinical isolates of *M. tuberculosis*. Overall approximately 70–80% of INH resistance in clinical isolates of *M. tuberculosis* can be attributed to mutations in the *katG* and *inhA* genes.

It is interesting to note that mono-resistance to INH is relatively common while mono-resistance to RMP is quite rare. In fact, nearly 90% of RMP-resistant strains are also INH-resistant. Therefore, resistance to RMP may be used as a surrogate marker for MDR-TB. However, some evidence suggests that this may not be true in every setting, emphasizing the importance of collect information on the local prevalence of drug resistance patterns before implementing a molecular assay.

RELEVANCE OF SPECIFIC MUTATIONS

A low-level resistant organism is an organism with a 'MIC higher than is common for the susceptible population, devoid of any acquired resistance mechanism'. As defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), low-level resistance starts at the upper limit of the cut-off values. It affects the relationship between the bacteria and the antimicrobial agent to a degree where treatment success cannot be guaranteed.

Studies determining MICs have shown that high-level RMP resistance is associated with mutations in codon 526 and 531, whereas alterations in codon 511, 514, 515, 516, 518, 521, 522 and 533 result in low-level RMP resistance. Some mutations affecting codon 526 such as His→Leu and His→Asn represent an exception and do not confer high-level resistance. It has been shown that certain mutations in *rpoB* (Leu511Pro; Asp516Tyr; Leu533Pro) showed a gradual increase in growth indices in the presence of 2 mg/ml RMP in the BACTEC 460TB system. RMP-susceptible strains do not grow in the presence of this concentration of drug, while high-level resistant strains grow much more rapidly. In addition, in contrast to drug susceptible controls, these strains were shown to be resistant to RMP at 0.5 mg/ml indicating elevated MICs. A recent report on highly discordant RMP susceptibility results during proficiency testing highlights the importance of determining the frequency of strains with mutations such as these, and the shortcomings of conventional methods. In a recent systematic sampling from Hong Kong, these *rpoB* mutations were found in 22% of all RMP-resistant strains tested.

Formation of reactive-oxygen-species, due to treatment with low levels of bactericidal antibiotics, can lead to mutagenesis and the emergence of a range of mutations, which may result in varying MICs and resistance to a wide range of antibiotics. Treatment with one drug class can lead to heterogeneous increases in MIC against other classes of antibiotics.

Since drug resistance may not always be uniform throughout a population, some cells within a population may remain susceptible to the antibiotic, whereas other cells display varying degrees of drug resistance, a phenomenon referred to as hetero-resistance.

Hetero-resistance has been reported for several anti-TB drugs (INH, RMP and ethambutol) based on the simultaneous detection of wild type and mutant molecular susceptibility. Hetero-resistance likely represents a natural variation in the population of cells of *M. tuberculosis* and could play a significant role in the emergence of resistance.

CONSIDERATIONS WHEN IMPLEMENTING MOLECULAR TESTING

A critically important question is the clinical significance of predicting drug resistance by molecular methods. There are at least two important issues that require attention in this regard.

The first is the detection limit of molecular tests when testing a hetero-resistant population of cells. As mentioned previously, in the early stages of the development of drug resistance in TB, only a relatively small proportion of resistant bacteria are present in the total population. Drug resistance will influence the treatment outcome in the patient only if the number of these strains reaches a clinically significant proportion, previously described as 1% of the population when performing the agar proportion method. However, the ability of molecular tests to detect a clinically significant proportion of resistant cells in the patient specimen has not been properly addressed. Evaluations of these tests have primarily focused on the analytical sensitivity (total amount of DNA detected). Recently, the detection limit of the Inno-LiPa Rif line probe assay (LPA) was assessed with mixtures of DNA from RMP-susceptible and resistant strains and compared to the detection limit with an in-house multiplex PCR-based macroarray. The Inno-LiPa Rif LPA was able to detect 20% RMP-resistant DNA in the presence of 80% wild type DNA, while the macroarray was able to detect 2% resistant DNA. In a similar experiment, where heat-killed mixtures of RMP-susceptible H37Rv and H37Rv *rpoB* mutants were mixed at different ratios (from 0 to 100% resistant cells), the GenoType® MTBDR*plus* LPA was able to detect as low as 5% resistant cells.

The second issue relates to the ability of molecular methods to provide information on the level of resistance, particularly to INH. As described above, there is general

agreement that mutations in *katG* tend to confer high-level resistance to INH, while mutations in *inhA* or its promoter region generally confer low-level INH resistance. Thus, identifying and reporting whether the specific mutation was detected in *katG* or *inhA* would help the clinician determine whether the patient would benefit from continuing treatment with a higher dose of INH. It is essential not to discontinue INH unnecessarily, especially since INH has potent bactericidal activity that rapidly reduces the infectivity of newly diagnosed cases.

LINE PROBE ASSAYS

Reverse hybridization-based assays, referred to as line probe assays (LPAs), are available commercially and are being tested under field conditions in endemic areas for rapid detection of mutations resulting in resistance to INH and RMP. These assays are a useful tool for cost-effective detection of drug resistance in selected patients. In brief, LPAs are based on the hybridization of PCR products from patient specimens to specific probes for wild-type and mutant alleles of genes involved in drug resistance and have shown high specificity and sensitivity.

The Genotype MTBDR (Hain Lifescience, Nehren, Germany) is a commercial LPA developed for the detection of RMP and/or INH resistance in *M. tuberculosis*. The assay is based on reverse hybridization between amplicons derived from a multiplex PCR and nitrocellulose-bound probes covering wild-type sequences and, in this first version of the assay, includes the most frequent mutations in the 81 base-pair region of *rpoB* and mutations at codon 315 in *katG*.

In order to increase the capacity for detection of drug resistance, a new version of the assay, called GenoType® MTBDR*plus*, was developed. This version has the ability to detect a broader variety of *rpoB* gene mutations and has added probes for wild type and mutations in *inhA*. By covering mutations in the regulatory region of *inhA*, additional INH-resistant strains have been detected. In fact, the identification of mutations in the promoter region of *inhA* has increased sensitivity for molecular detection of INH

resistance up to 31.4% as compared to detecting only mutations at codon 315 in *katG*. In particular, Brossier and colleagues demonstrated that the identification of mutations in the *inhA* promoter region allows an increase of over 50% in the detection of low-level INH-resistant strains.

In spite of the ease of performance of the assay, correct interpretation of LPA patterns appears to be a problem, even for technically skilled laboratorians in the field. As described above, LPA results are based on banding patterns detected on a strip following hybridization with PCR products amplified from target DNA in a patient specimen. Often, confusion arises if a wild-type band (found in drug-susceptible strains) is missing, but a corresponding mutation band (found in drug-resistant strains) is not present. It is likely that this banding pattern is the result of a drug resistance-associated mutation different from the common ones identified by the specific probes on the strip. However, there is a slight possibility that the pattern represents a silent mutation, one that does not result in an amino acid change. This is one of the reasons that mutations observed with molecular methods should be confirmed with a phenotypic assay. Even if the known molecular mechanism is showing a high level of association with the respective phenotypic drug resistance (*rpoB* and RMP resistance, *pncA* and PZA resistance, *katG* and INH resistance), the molecular test may not include probes for all possible mutations. Most molecular tests (including the LPAs, molecular beacons and real time PCR) only interrogate the 81 base-pair hot spot of *rpoB* and the 315 codon of *katG,* while resistance-associated mutations may occur in other parts of these genes. Furthermore, molecular mechanisms are not fully identified and understood for all drugs, and therefore mutations in known targets may not be associated with phenotypic drug resistance in all cases (e.g. INH, aminoglycosides, fluoroquionolones etc.).

Systematic reviews and meta-analyses of the performance of LPAs compared to conventional DST methods showed that LPAs are highly sensitive (>=97%) and specific (>=99%) for the detection of RMP resistance, alone or in combination with INH (sensitivity >=90%; specificity >=99%), on isolates of *M. tuberculosis* and on smear-positive sputum specimens. When RMP resistance alone was used as a marker for MDR, the overall accuracy for detection of MDR was equally high, at 99%. These

results were confirmed by laboratory validation and field demonstration data in several countries, most notably in the large-scale demonstration project in South Africa, executed by FIND (Foundation for Innovative New Diagnostics), the South African Medical Research Council (SAMRC) and the South African National Health Laboratory Service (NHLS). Data from the studies in South Africa also indicated the feasibility of introducing LPAs in high-volume public health laboratories. Detailed costing data from South Africa showed that by using the LPAs in routine diagnostic algorithms, the reduction in cost amounted to between 30% and 50% when compared to conventional DST methods. As expected, the cost was lowest when the LPA was applied directly to smear-positive specimens and highest when the assay was used on isolates from liquid primary culture. Introducing these assays into screening and diagnostic algorithms could significantly reduce the need for sophisticated and costly conventional laboratory infrastructure, still vastly inadequate in most high-burden countries. Based on these studies, the use of LPAs has been recommended by WHO according to the following guidelines:

- Adoption of LPAs for rapid detection of MDR-TB should be decided by Ministries of Health (MOH) 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-TB drugs

- The performance characteristics of the LPA have been adequately validated in direct testing of sputum smear-positive specimens, and on isolates of the MTBC grown from smear-negative and smear-positive specimens. Direct use of LPAs on smear-negative clinical specimens is not recommended.

- The use of commercial LPAs, 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 LPAs should be subject to adequate validation when first introduced into the field, that is, published 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 LPAs

- Adoption of LPAs does not eliminate the need for conventional culture and drug-susceptibility testing (DST) capability; culture remains necessary for definitive diagnosis of TB in smear-negative patients, and 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 LPAs in country-specific screening algorithms

- As current LPAs only detect resistance to RMP and/or INH, 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.

SUGGESTED BACKGROUND READING

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Chapter 2:
INFRASTRUCTURE REQUIREMENTS

Implementing Nucleic Acid Amplification Testing (NAAT) using the line probe assay (LPA) to detect *Mycobacterium tuberculosis* and mutations associated with drug resistance often necessitates laboratory scale-up and strengthening. Before the LPA can be implemented for routine use, both the design and layout of a mycobacteriology laboratory must be carefully planned with regard to biosafety (containment of *M. tuberculosis* within the facility) and bio-risk (containment of DNA and DNA-related products). Appropriate consideration of these issues is essential to avoid contamination of personnel, the environment and exogenous samples, as well as to maintain a high standard of quality-assured diagnostic output.

This section will focus on the layout requirements for a basic PCR laboratory, which consists of three to four smaller areas or "rooms", and the associated levels of biosafety and bio-risk in each. Knowledge and know-how of Good Laboratory Practice is strongly advised and must be applied throughout in order to maintain an ultra-clean and contaminant-free environment[1].

PCR FACILITY

There are two diagnostic PCR facility layouts that can be used by tuberculosis (TB) laboratories planning to implement the LPA, whether these are new or existing.

The **ideal** PCR facility consists of four small areas as follows:

1. Pre-amplification area (reagent preparation)
2. DNA extraction area (specimen preparation)
3. Amplification area (template addition & amplification)
4. Post-amplification area (hybridization/detection)

The **most basic** PCR laboratory must consist of at least three different areas, each dedicated to the different processes involved in the LPA, namely:

1. Pre-amplification
2. DNA extraction
3. Amplification and post-amplification (which normally houses the Thermal Cycler as well as the hybridization detection area)

This arrangement will aid in avoiding the risk of cross-contamination or carry-over contamination. **Figure 1** illustrates the setup of the two different facilities.

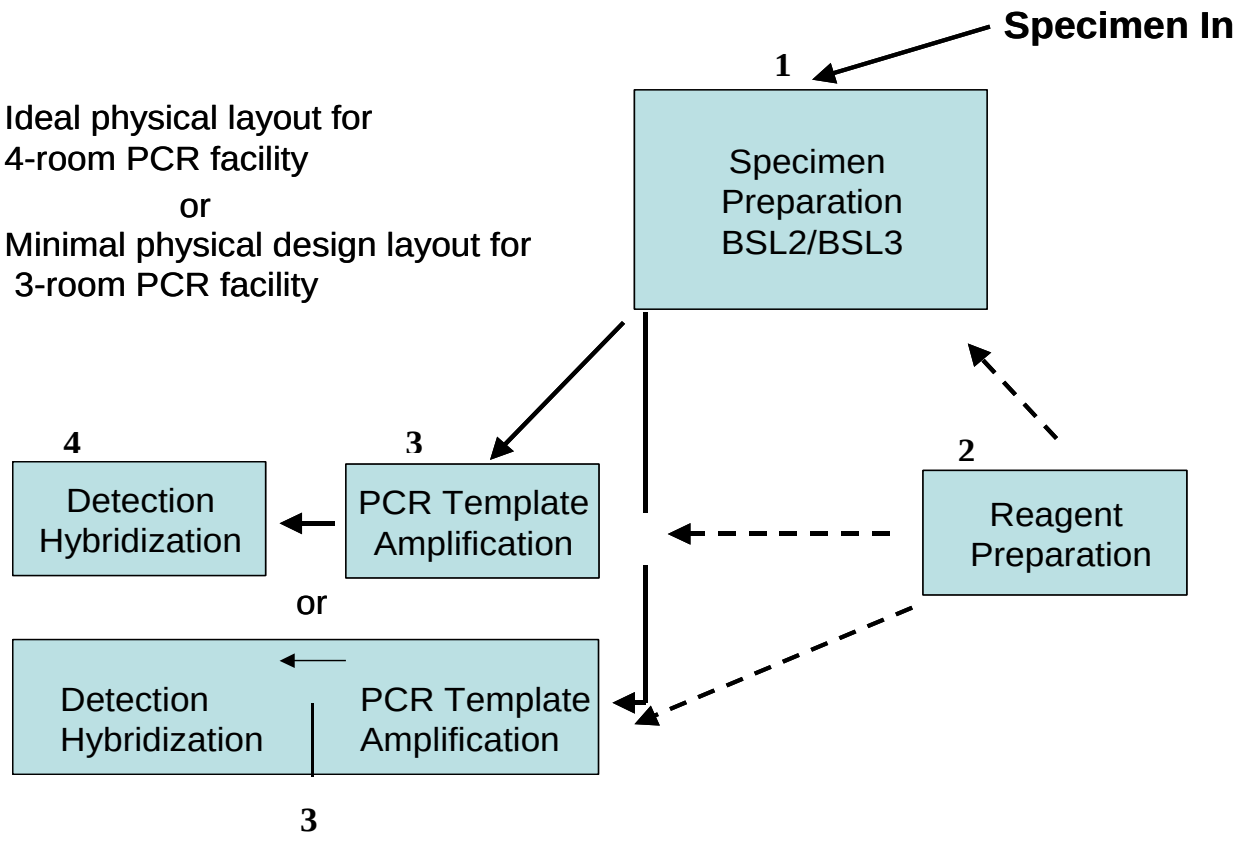


Figure 1: An illustration of the work flow for both the ideal physical design layout (4-room PCR facility) and a basic three-room PCR facility. Reagent Preparation in the pre-amplification room must always be performed before any other procedure can be done.

A Biological Safety Cabinet (BSC) must be used for both specimen decontamination and DNA extraction. If existing laboratories are to be renovated or changed to accommodate molecular testing, the BSC in the existing specimen decontamination area (where negative pressure is already in place) can be used for DNA extraction. The advantage of using an existing BSC is that it eliminates the need to purchase a new BSC, which would require a large double-door for installation and accommodation for exhaust and proper ventilation. Smaller rooms can be used for the other areas since the pre-amplification and DNA addition areas must be located in positive pressure environments. However these smaller areas must be access-controlled, with doors closed at all times and may also include an anteroom. Some laboratories go so far as to have their DNA extraction area in another building or on another floor to ensure that new specimens are not contaminated with DNA from previously amplified specimens. When converting an existing laboratory, it is essential that the entire area be thoroughly decontaminated before beginning molecular diagnostic work.

It is imperative that the separate areas have their own dedicated instruments, supplies, reagents and pipettes. In addition, it is equally essential that area 2 and area(s) 3 and 4 or combined area (3 - 4) must have separate and dedicated refrigerator/freezers.

BIOSAFETY REQUIREMENTS

When setting up a PCR facility for the LPA, it is important to distinguish between two types of “risk”. The “biohazard risk” is the potential that a laboratory worker will become infected when working with live *M. tuberculosis*, while the “bio-risk” is the potential that specimens and/or reagents will become contaminated with DNA, amplified products (amplicons) or exogenous contaminants that lead to false positive PCR results. It is crucial that only DNA extracted from the new specimen enters the PCR reaction as the template. To ensure this, DNA extraction must be performed in a laboratory with adequate and appropriate biosafety level precautions i.e. BSL-2 and BSL-3, depending on the sample. DNA extraction from clinical specimens can

be performed in either a BSL-2 or BSL-3 laboratory, while DNA extraction from TB isolates must be performed in a BSL-3 laboratory. The other two areas, for amplification and post-amplification, only require a BSL-1 laboratory. The WHO Laboratory Biosafety Manual (**Third Edition, available from WHO website**) provides practical guidance on biosafety techniques and equipment for all biosafety levels and the development of national codes of practice (**Table 2**).

Assigning an infectious agent to a specific biosafety level for laboratory work must be based on risk assessments. At present, it is widely acknowledged that decontamination, concentration and inoculation of culture media with clinical specimens suspected of containing TB can be performed in a BSL-2 laboratory with a certified and well maintained BSC. DNA preparation using concentrated sediments can also be performed in this setting. However, handling positive cultures of viable *M. tuberculosis* grown on solid or liquid media (for the purposes of identification, drug susceptibility testing or molecular methods) must occur in a BSC within a BSL-3 containment laboratory. The main requirements for the different biosafety levels are given in **Table 3**.

Table 2: Biosafety levels, laboratory type, practices and equipment

BIOSAFETY LEVEL	LABORATORY TYPE	LABORATORY SAFETY	EQUIPMENT PRACTICE
Basic - Biosafety Level 1	Basic teaching, research	GMT[1]	None, open bench work
Basic - Biosafety Level 2	Primary health service, diagnostic services, research	GMT plus protective clothing, biohazard sign	Open bench plus BSC[2] for potential aerosols
Containment - Biosafety Level 3	Special diagnostic services, research	Same as Level 2 plus special clothing, controlled access, directional airflow	BSC and/or other devices for all activities
Maximum Containment - Biosafety Level 4	Dangerous pathogens unites	Same as Level 3 plus airlock entry, shower exit, special waste disposal	Class III BSC or positive pressure suits in conjunction with Class II BSCs, double-ended autoclave (through the wall), filtered air

[1] GMT- good microbiological techniques

[2] BSC - biological safety cabinet

Table 3: Summary of main biosafety level requirements

	Biosafety Levels			
	1	**2**	**3**	**4**
Functional isolation of laboratory	No	**No**	**Yes**	Yes
Room sealable for decontamination	No	**No**	**Yes**	Yes
Ventilation:				
inward airflow	No	**Desirable**	**Yes**	Yes
controlled ventilating system	No	**Desirable**	**Yes**	Yes
HEPA-filtered air exhaust	No	**No**	**Yes**	Yes
Double-door entry	No	**No**	**Yes**	Yes
Anteroom	No	**No**	**Yes**	-
Autoclave:				
on site	No	**Desirable**	**Yes**	Yes
in laboratory room	No	**No**	**Desirable**	Yes
double-ended	No	**No**	**Desirable**	Yes
Biological safety cabinets	No	**Desirable***	**Yes**	Yes
Personnel safety monitoring capability	No	**No**	**Desirable**	Yes

*Required when infectious aerosols may be created, as in a TB culture laboratory.

In a BSL-2 laboratory, access should be limited and the entrance labeled with a biohazard sign (preferably make from fire resistant material). Access to a BSL-3 laboratory should be restricted to authorized personnel only. This can be controlled both administratively and physically, using the following biosafety measures:

- The laboratory should be physically separated from other laboratories within the same complex.

- The laboratory should have a double door entry system i.e. two inter-lockable doors. The laboratory doors should remain closed, preferably equipped with a self-locking device.
- The international biohazard warning symbol and sign for BSL-3 containment (**Figure 2**) must be displayed on laboratory access doors.
- The name and contact details of the laboratory supervisor (i.e. the person who controls access to the laboratory) should also be displayed on laboratory access doors.
- Maintenance personnel should only be allowed to enter a BSL-3 laboratory if accompanied by laboratory staff with experience in biosafety measures. Maintenance personnel should also be educated as to the risks involved.
- Additional protective gowns suitable for visitors, maintenance and emergency response personnel should be available and, if possible, distinguishable from that worn by the routine workers.

The Fourth Edition of the WHO Laboratory Biosafety Manual contains several sections that focus specifically on TB and provides basic guidance on laboratory design to ensure biosafety standards are maintained. Based on a risk assessment for each technical procedure, the manual describes the minimum requirements for facilities performing the following tests:

- Direct sputum microscopy (Basic TB)
- Concentrated sputum microscopy, culture and/or line probe assay (LPA)
- Culture manipulation, drug susceptibility testing (DST) and LPA

Note. Upgrades to any laboratory facility should always be preceded by a comprehensive risk assessment.

Biosafety in laboratories includes three components:

1. Facility layout
2. Essential equipment, purchased according to standard specifications with a serviceable maintenance plan (including [re-]certification)

3. Work practice. It is generally acknowledged that good laboratory practice and good microbiological techniques are essential to minimize the generation of infectious aerosols and the spread of DNA (genomic products and amplicons).

Also in the WHO manual, the following issues are addressed and elaborated for each laboratory type:

- Code of Practice:
    - Biosafety Management
    - General Laboratory Safety
    - Personal protection and Personal protective equipment
    - Laboratory Procedures
    - Organization of the laboratory working areas
- Laboratory Design
- Laboratory Equipment
- Health and Medical Surveillance
- Training
- Waste handling
- Chemical, fire, electrical, radiation and equipment safety.

BIOHAZARD

ADMITTANCE TO AUTHORIZED PERSONNEL ONLY

Responsible Investigator: _____

In case of emergency call: _____

Special conditions: _____

Daytime phone: _____**Home phone:** _____

Authorization for entrance must be obtained from the Responsible Investigator named above.

Figure 2: International biohazard warning symbol and sign for BSL-3 containment

LABORATORY LAYOUT AND SETUP

1. Pre-amplification area

The pre-amplification area (**Figure 3**) is the “ULTRA-CLEAN area” and is dedicated to the preparation of reagents for the “master mix”, to be used during the amplification procedure. Unlike the DNA extraction area, this area does not require negative pressure, but rather positive pressure so that, upon entering the room, all air currents brought in with the laboratorian are pushed back out. It is therefore important to have an air-conditioner in the room, optimally placed directly opposite the door, with the condenser on the outside of the building.

The room must be large enough (at least 9 m^2) to house a refrigerator/freezer combination for the storage of the PCR reagents, as well as an adequately sized bench top with space to work. There is no need for a BSC since the room is used only to prepare the reagents for the PCR master mix: no specimens, DNA or amplicons should ever be brought into this room. If there is cause for concern, a PCR “dead-box” can be used for contamination prevention, provided the bench top is large and sturdy enough. It is also advisable to equip this area with a small under-counter cupboard for storing the PCR supplies that do not need to be refrigerated or frozen.

In addition to the room itself, 3 to 6 m^2 of space is required for an anteroom, leading to the pre-amplification area. The anteroom should have space for a small table where disposable gowns and shoe covers can be kept, and coat hangers on the wall for laboratory coats that are to be used exclusively during reagent preparation.

The spaces between the door and its frames need to be sealed, in order to prevent dust and other contaminants from entering the area. Many laboratories have their pre-amplification area as far away as possible from the post-amplification area, some are even located on separate floors or in entirely separate buildings.

Figure 3: Pre-amplification area.

2. DNA extraction area (specimen processing area)

This area is used exclusively for **sample** preparation. Under no circumstances should any previously amplified PCR products (amplicons) be handled anywhere in this room or area. Similarly, reagent preparation must not be carried out in this room, but in the pre-amplification room described above.

In order to perform the LPA, the sputum specimens containing live bacilli and normal flora must first be decontaminated using the N-acetyl-L-cysteine (NALC)-sodium hydroxide (NaOH) method, which includes concentration by centrifugation and proper buffering with a phosphate solution (pH 6.8). DNA for the LPA is extracted from the concentrated sediment of the specimen after solid and/or liquid cultures have been inoculated and a smear has been prepared. At present, the test is validated for smear-positive specimens that have been decontaminated using this method. These manipulations, and subsequent steps of the DNA extraction for the LPA, may create aerosols that pose a major health risk to the operator and may also cross-contaminate specimens if appropriate containment measures are not followed. Thus, all manipulations must be done in a BSC in order to filter the aerosols produced, thereby protecting the operator and minimizing sample-to-sample contamination. It is also

crucial that the centrifuges used during the manipulations have aerosol-tight rotors. Both precautionary measures are designed to increase personal safety and to comply with the standards for a BSL-2 and BSL-3 laboratory.

As stated previously, if patient material (clinical specimens) is to be tested directly in the LPA, the NALC-NaOH decontamination method and subsequent DNA extractions should be executed in a BSC under BSL-2 conditions. However, if the LPA is going to be performed on cultured bacterial isolates (from liquid or solid media), the laboratory is required to adhere to BSL-3 standards.

A dedicated air supply with no recirculation of air is desirable for a BSL-2 laboratory and required for a BSL-3 laboratory. It is also essential for negative pressure to be established in this laboratory so that all incoming air is drawn into the BSC and filtered through HEPA filters before release into the outside environment. The laboratory should be free of draughts, since any disturbances in the airflow of the BSC may cause infectious aerosols to escape and possibly infect the operator and other staff as well.

The DNA extraction equipment (**Figure 4**), i.e. the microfuge, heating block, ultrasonic water bath, and positive-displacement pipettes[1] or any other consumables (such as filter tips, 1.5 ml O-ring screw-top tubes, plastic loops, 3 ml Pasteur pipettes, etc.), should be dedicated to this room and never used in any other room.

Due to the limited availability of BSCs and dedicated space in some laboratories, the BSC that is used to extract the DNA, can also be used for the addition of the DNA to the PCR master mix.

Note: This can only be done if the BSC has been decontaminated after specimen processing and DNA extraction with 1% sodium hypochlorite followed by 70% ethanol. It is also important to ensure that the person who prepares and aliquots the PCR master mix in the ultra-clean pre-amplification area does so before working on either the original specimen, the extracted DNA or the amplified products.

[1] The positive-displacement pipettes prevent aerosol carry-over created during pipetting, thus reducing the possibility of sample-to-sample contamination.

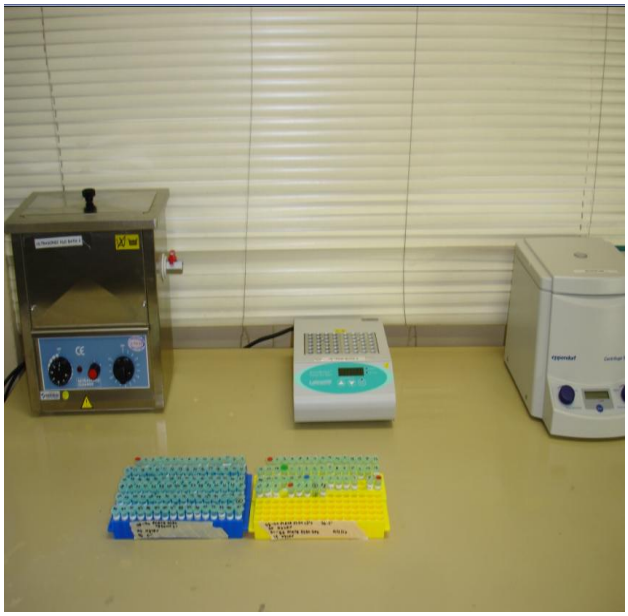


Figure 4: Equipment used in the DNA extraction area

3. Amplification & post-amplification area(s)

The final stages of the LPA procedure take place in this area (**Figure 5**), which is a BSL-1 laboratory. It can be considered a “dirty laboratory”, since we are working with the amplified products, or **amplicons**.

If there is an area dedicated to template addition, it must also have positive pressure inside. As with the pre-amplification room, any air currents are pushed back into the anteroom upon entering. Again, the air-conditioner must be placed directly opposite the door, with the condenser on the outside of the building.

The post-amplification stage of the LPA procedure is made up of a number of smaller steps. It is strongly advised that dedicated areas be set aside for these individual procedures (**Figure 6**), including:

A. A strip preparation bench.

B. A bench for the addition of the amplicons to the denaturing solution.

C. A bench for the hybridisation machine(s).

D. A bench for the “pasting” the hybridised strips to the datasheet and interpretation of the strips.

E. A bench next to the sink for the drying of the re-usable plastics such as the PCR racks, the 1.5 ml eppendorf tube racks, the trays (should the GT-Blot 48 be in use), the discard container, and the measuring cylinders.

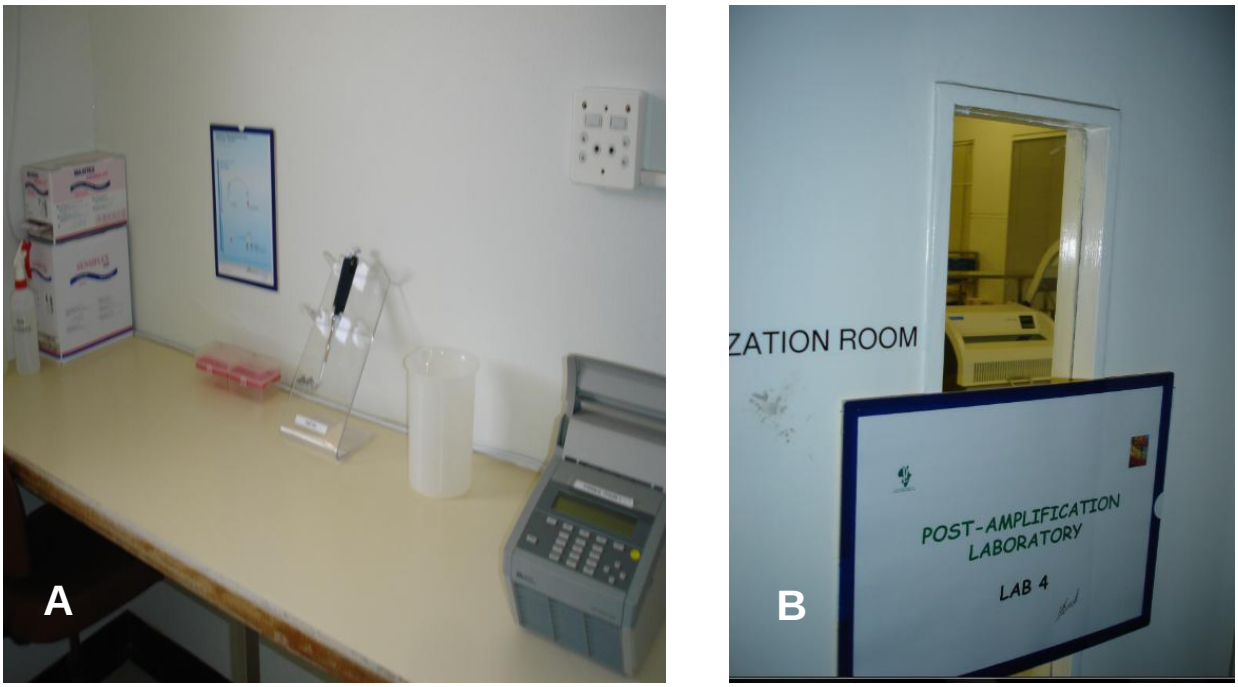


Figure 5: (**A**) Amplification and (**B**) Post-amplification areas. As mentioned in the text, the ideal facility would have these two areas in separate rooms.

Note: There is no BSC/PCR cabinet in Area (A), since this room in this particular facility is dedicated to the addition of the template DNA. As mentioned previously, template addition can also be carried out in the DNA extraction area, provided there is a suitable BSC.

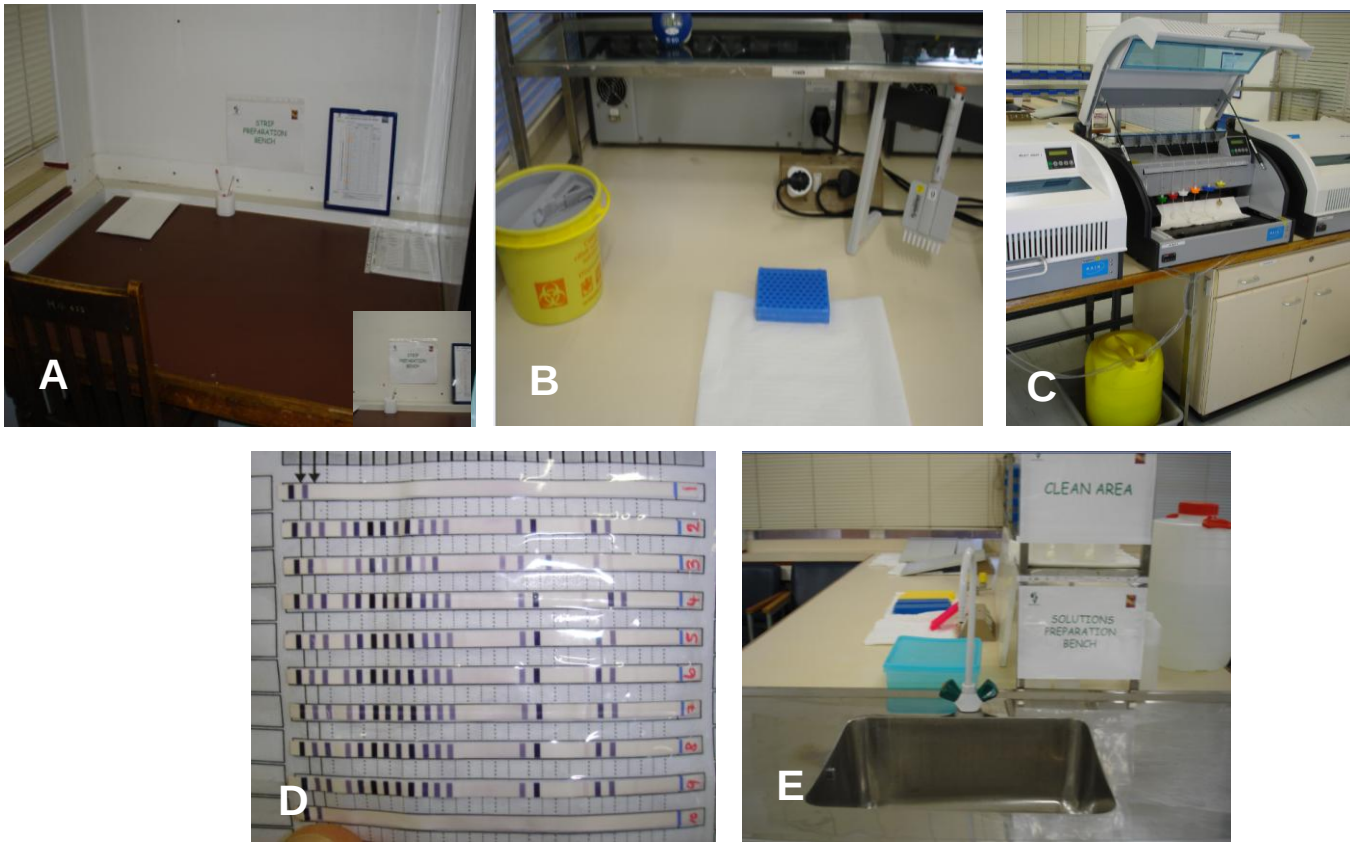


Figure 6: Dedicated bench spaces in the post-amplification area. (**A**) Strip preparation bench, (**B**) Amplicon denaturation bench, (**C**) Hybridization machine bench, (**D**) Post hybridization bench for pasting of the strips and interpretation thereof, and (**E**) The "Clean Area" bench. Note the sealable blue plastic container, which contains sodium hypochlorite. The eppendorf and PCR racks are dipped in this solution in order to decontaminate them before they are used again.

The need for this "separation" of work areas within the laboratory will aid in contamination prevention and helps with the natural workflow. It is also recommended that the sink be cleaned daily with 5% sodium hypochlorite and 70% alcohol. In addition, a container filled with sodium hypochlorite should be used to decontaminate all re-usable consumables, especially the racks that are used for 1.5 ml and PCR tubes. The containers for the freshly prepared 1% sodium hypochlorite and 70% alcohol, to be used in each of the four areas, must also be immersed in these two decontamination solutions before they are filled and placed in their respective areas. Also, pipettes and benches should be wiped with these solutions both before and after the day's procedure.

Each laboratory should have a small under-counter cupboard, to act as an “immediate” stock cupboard, where all consumables required for detection and interpretation will be kept.

The amplification and post-amplification rooms must be locked when not in use to ensure controlled access. It is also recommended that self-closing door devices are installed to help control air movement. General cleaning staff should not enter these rooms, move equipment around or clean the floor with a mop that has been used to clean other laboratories. Many laboratories have found that these actions introduce contaminants that interfere with the PCR reaction. Thorough decontamination of the laboratory is then required before testing can resume, which significantly delays the testing turn-around time, with a subsequent negative impact on patient care and treatment.

Useful tips

The “backbones” of the PCR facility are the 3 or 4 dedicated areas that will be used for the different steps of the procedure. It is recommended that all equipment, pipettes etc., be labeled with a different colour of masking tape, based on the area in which they are used, e.g., red for the pre-amplification area, green for the DNA extraction area, blue for the amplification area and yellow for the post-amplification area. This ensures that the equipment will stay dedicated to each area. Also, violations can be clearly seen by visual inspection, e.g. if a “blue” labeled pipette is found in a “green” area, where it should not be.

GENERAL INFRASTRUCTURE REQUIREMENTS

A detailed list of all laboratory equipment and necessities required to optimally perform the LPA, within the prescribed biosafety requirements, is presented below.

Working Environment

Cleanliness is of utmost importance in the PCR facility. All walls, floors, doors, door handles, ceilings, light-switches, sinks and bench-tops must be smooth and easy to clean. Surfaces must be impermeable to liquids and resistant to harsh disinfectants, since everything must be cleaned thoroughly and often.

If existing “rooms” are to be converted into PCR “rooms”, it is essential that the walls be repainted white with high-gloss enamel or other gloss-based paint. This will ensure easy cleaning of the walls. The floors must be covered with vinyl, linoleum or ceramic tiles (carpets should not be used anywhere). The use of melamine bench-tops is suggested because of their impermeability. Wooden bench tops should not be used, as tiny crevices in the top might harbour contaminants that are nearly impossible to eliminate.

Electricity

A reliable and adequate supply of electricity is a necessity. Some of the LPA associated reagents need to be refrigerated or frozen, and the various machines used in the procedure require electricity. Circuit breakers must be able to accommodate the electrical demand needed to operate the various laboratory equipment, and all electrical boxes and wiring should be certified to avoid surges that might render the equipment inoperable. An Uninterrupted Power Supply (UPS) device, or a back-up generator is recommended, especially if the supply of electricity is sporadic. If information is available on planned power outages, laboratory work should be scheduled around the outages so that procedures are not interrupted.

Air Conditioning

The laboratory may be located in a geographical region of the world that routinely experiences high temperatures. With additional heat generated by refrigerators, freezers, autoclaves and some equipment used in the LPA, there must be sufficient air conditioning to offset both the heat from the environment and from the equipment, especially in laboratories with low-ceilings where heat has a tendency to build up rapidly.

Air conditioner units must be placed in an outside wall with a condensate drain line, but should not be placed in close proximity to ducts and vents to the outside, since this will allow potentially infectious air to be re-circulated into the laboratory. The opening around the air conditioner must be properly sealed to avoid dust or other exogenous contaminants from entering the laboratory. Care should also be taken to ensure that the vents of the air conditioners are aimed away from BSCs, so as to avoid disturbances that would direct fumes and potential aerosols toward laboratory workers.

Biological Safety Cabinets

BSCs must be placed in contained or low traffic areas and orientated in such a way that draughts and air disturbances across the cabinet are limited. If a new BSC is to be purchased, entry doors must be wide enough to accommodate the cabinet. Professional staff should install the BSCs and perform bi-annual servicing and annual certification.

Extractor fans

Extractor fans are placed in the laboratory to create negative air pressure. Air pressure should be the most negative in the areas where the most potentially infectious work is performed. An extractor fan should also be placed near the autoclave to aid in exhausting excess heat. Extractor fans should always be placed on an outside wall so that they will not vent into a corridor. The area where the exhaust is vented should not be a high traffic area or an area with nearby air intakes, as this might pull the exhaust back into the building.

There should be no extractor fan in the reagent preparation room since infectious material is not processed here and the negative pressure generated may bring in contaminants when the door is opened.

PLACEMENT OF LABORATORY EQUIPMENT

A detailed reasoning behind the design of the laboratory is provided below.

Biological safety cabinet

The BSC should be placed in the contained laboratory area that will be considered the BSL-2 or BSL-3 suite. This is the area where all infectious specimen manipulation will take place and which is separate from all other laboratory areas. If a spill should occur while manipulating infectious material, staff exposure will be limited since the negative air pressure generated by the BSC and the extractor fans will rapidly remove any suspended droplet nuclei.

Air conditioners

Most air conditioners are single units and not part of a centralized system. When deciding where to place these units, special attention should be paid to the direction of the airflow, especially if there is no choice but to place the units in close proximity to the BSC. As stated previously, BSCs are very sensitive to disruptions in airflow, so these must be minimized for the BSC to work properly. The vents or louvers should be angled toward the ceiling to disperse the flow of air. The air conditioner unit may need to be turned off for a short time when working in the pre-amplification room, in order to avoid excessive air movements that may lead to contamination of reagents.

Autoclave

The autoclave is needed to disinfect laboratory waste generated in the different areas of the laboratory. Because autoclaves generate a great deal of heat, they should be located far away from the refrigerator and freezer. If the freezer is located near the autoclave, the excess heat produced by the autoclave will unnecessarily tax the freezer motor and decrease its life. A floor model autoclave should be placed under an extractor fan, in order to exhaust the heat and steam produced by the autoclave. It is essential to have two autoclaves in the facility: one to sterilize “clean” things that will be used again, and the other to disinfect the “dirty” laboratory infectious and contaminated waste that is to be discarded.

Carts

Carts should be conveniently located to transport specimens, cultures, or waste within the laboratory. When transporting infectious materials it is always much safer to use a cart rather than carrying items by hand. It is important to note that carts used in the specimen preparation room or the amplification and post-amplification rooms should never be taken into the reagent preparation room.

Laboratory coat racks

Coat racks should be placed at the entrance to the laboratory, near the hand sink so that laboratory coats can be donned before entering the laboratory and removed when preparing to exit the laboratory, before hand washing. There should be a separate coat rack near the entrance to the BSL-3 laboratory for staff member to leave the BSL-1 or BSL-2 laboratory coat and put on a separate laboratory gown and other appropriate personal protective equipment (PPE) for BSL-3 work. It is advisable to wear over-shoes both in the BSL-3 and in the reagent preparation area to avoid exiting the BSL-3 area with contaminated shoes, or contaminating the clean reagent preparation area.

Biohazard waste containers

Waste containers should be placed conveniently throughout the laboratory, near work stations where biohazard waste is generated and near doors where gloves can be disposed of before exiting an area. Pipettes, glass slides, and other sharps need to be placed in special “sharps” containers that are puncture resistant. All biohazard waste should be autoclaved, chemically disinfected or incinerated to ash.

Hand sinks

Hand sinks must be placed in convenient areas of the laboratory, especially near laboratory exits, for hand washing: following removal of PPE, but before exiting the laboratory; when moving from one laboratory area to another; and after work has been completed. The sinks should always be kept clean, connected to a constant supply of clean water, and stocked with an ample supply of tuberculocidal hand soap.

Eye wash

An eye wash station should be located near a sink that is connected to a consistent clean water source, so that in the event of use, the sink can be flushed with clean water and drained. If the eye wash station is mounted onto the wall, the station must contain a sealed bottle of sterile saline that is refilled with a freshly prepared solution on a month basis.

Fire extinguishers

Fire extinguishers should be placed in easily accessible locations in the laboratory. All laboratory staff should be trained on what to do in case of a fire, including being aware of the location and operation of the extinguishers.

Biohazard spill kit

It is good to have two spill kits; one should be placed in the BSL-2 or BSL-3 area to clean spills that occur inside the BSC or other contained area. The other should be located outside the containment laboratory. This second spill kit should be used when a spill occurs outside the BSC and personnel have been alerted to exit the laboratory. After the lab has been vacated for a previously specified amount of time, the PPE, disinfecting equipment, and instructions located in the spill kit can be used to safely return to the laboratory and clean up the spill.

Emergency shower

The emergency shower should be available for use if caustic chemicals come into contact with a large area of a worker's body and requires flushing. It is not necessary for the shower to be in the laboratory, but it is recommended it be in close proximity to the laboratory, in the event of an emergency.

Safety Centrifuge (with aerosol sealed rotor)

The safety centrifuge is used to concentrate specimens during processing. If the centrifuge has an aerosol-sealed rotor it should be located within the BSL-2 or BSL-3 suite, optimally near the BSC, in order to limit staff exposure to potential aerosols. **If it does not have a sealed rotor it must be placed under a BSC or Extractor Fan.**

Note: All equipment involved in specimen processing should be kept in the same room or area.

Sonicator

The sonicator used in the DNA extraction procedure should be placed in the specimen preparation area. The sonicator's water tank should be emptied and the insides of the tank wiped out with 70% alcohol after each use.

Heating Block

The heat block used in the DNA extraction procedure should be placed in the specimen preparation area. This instrument can be placed inside the BSC or on a bench top in the BSL-2 or BSL-3 laboratory. At this point in the procedure, the specimens are enclosed in 1.5 ml tubes with screw caps, and thus will not flip open during the heating process, when pressure builds up inside the tubes.

PCR Hood in reagent preparation area

A PCR hood can be placed in the reagent preparation area and used to prepare the master mix and aliquot appropriate portions into the PCR tubes. Using the hood will decrease the possibility of contamination of the reagents or combined master mix. However, the use of such a hood is optional, and “on-the-bench” preparation can be done cleanly without the introduction of contaminants, provided the area is sufficiently separated from other parts of the laboratory.

PCR Hood for addition of DNA to master mix

As in the reagent preparation area, the operator may use a PCR hood for the addition of the extracted DNA to the master mix contained in the PCR tubes, thereby decreasing the risk of contamination during this step in the procedure. The PCR hood should be placed either in the specimen preparation area or in a separate room.

It is important to note that, by this stage of the process, the DNA preparations are non-infectious. Original specimens or isolates containing live TB should always be handled in a BSC and never under a PCR hood. The main advantage of using a PCR hood is to separate the work areas used for DNA extraction (in the BSC) and addition of the DNA to the master mix. This lowers the chances for contamination of the PCR reaction with previously processed specimens. In conjunction with appropriate cleaning, the UV light in the PCR Hood will destroy any possible contaminating DNA.

PCR clean freezer (-20º)

This freezer should be located in the **reagent preparation area** and used to store the PCR reagents (components of the master mix). It should not be placed near sources of intense heat, such as autoclaves. Because refrigerators and freezers produce large amounts of heat themselves, sufficient air conditioning is required to offset the heat production. A special effort should be made to keep the filters clean in both refrigerators and freezers in order to increase their life span.

DNA extract freezer (-20 or -80°C)

This freezer should be located in the **specimen preparation (BSL-2 or BSL-3) area** to store the DNA samples that have been extracted from clinical specimens.

Specimen refrigerator (4-8°C) and/or freezer (-20 or -80°C)

This refrigerator should be located in the **specimen preparation (BSL-2 or BSL-3) area** to store clinical specimens.

Refrigerator for post-amplification reagent (4-8°C)

This refrigerator should be located in the **amplification/post-amplification area** to store specific reagents used for strip hybridization and detection of bands.

Thermocycler

This instrument should be placed in the **amplification/post-amplification area**.

TwinCubator®

This instrument should also be placed in the **amplification/post-amplification area.**

SUMMARY STATEMENT

The main drawback of PCR technology is the “false-positive” results that occur when reagents are contaminated with PCR products (amplicons) from previously-positive samples. This can be a considerable problem, even in the most fastidious laboratories, and has led to the requirement for dedicated rooms or areas for the different steps in the procedure. All TB laboratories that currently use the LPA, or are planning to do so, must have the appropriate laboratory infrastructure in place in order to avoid sample contamination and produce reliable results. **Figures 7 to 11** are suggested laboratory lay-outs to ensure that all infrastructural needs are appropriately met.

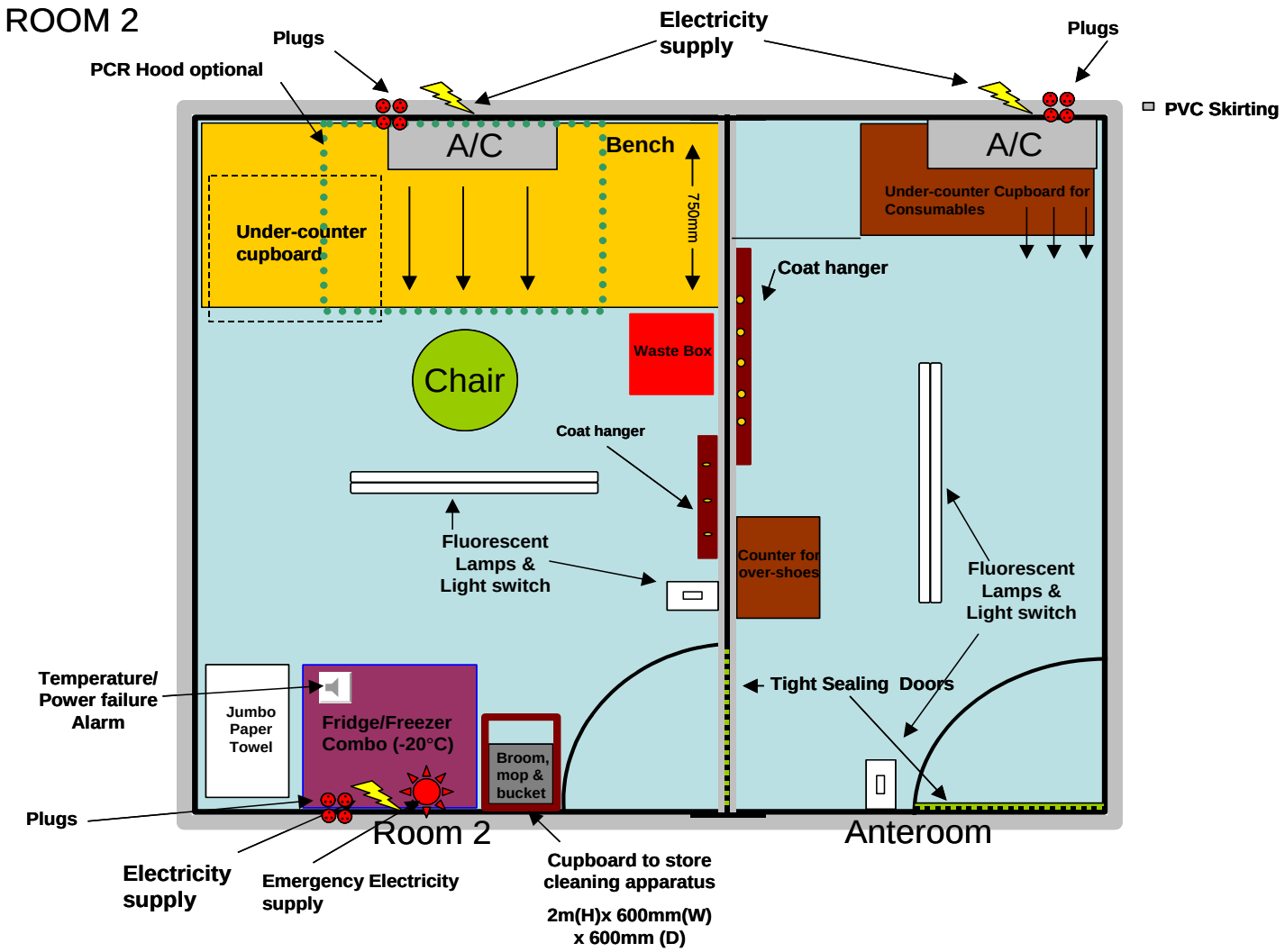


Figure 7: Suggested lay-out for the pre-amplification area, with adjacent anteroom.

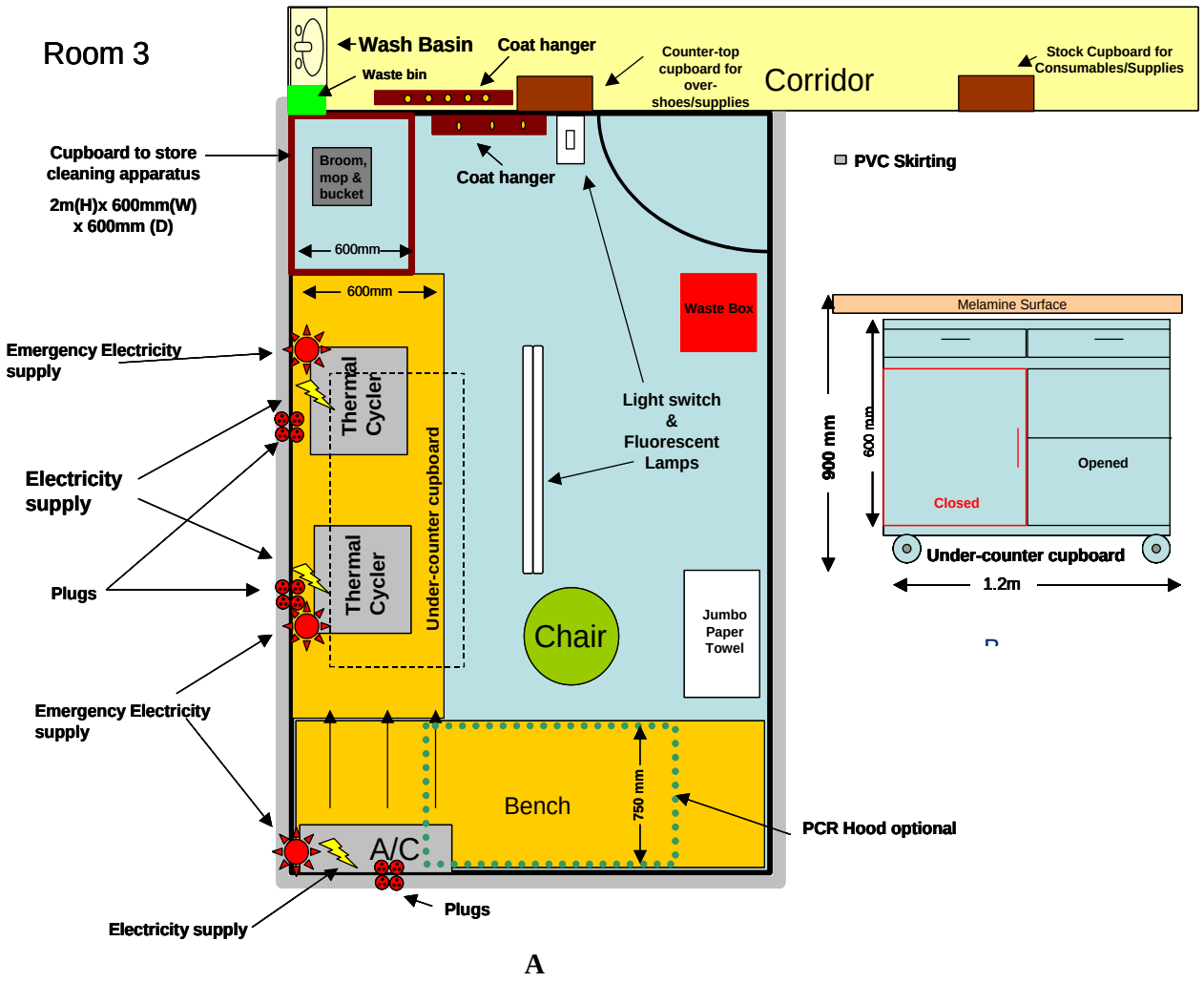


Figure 8: (**A**) Suggested lay-out for the amplification area. (**B**) Bench dimensions with move-able under-counter cupboard.

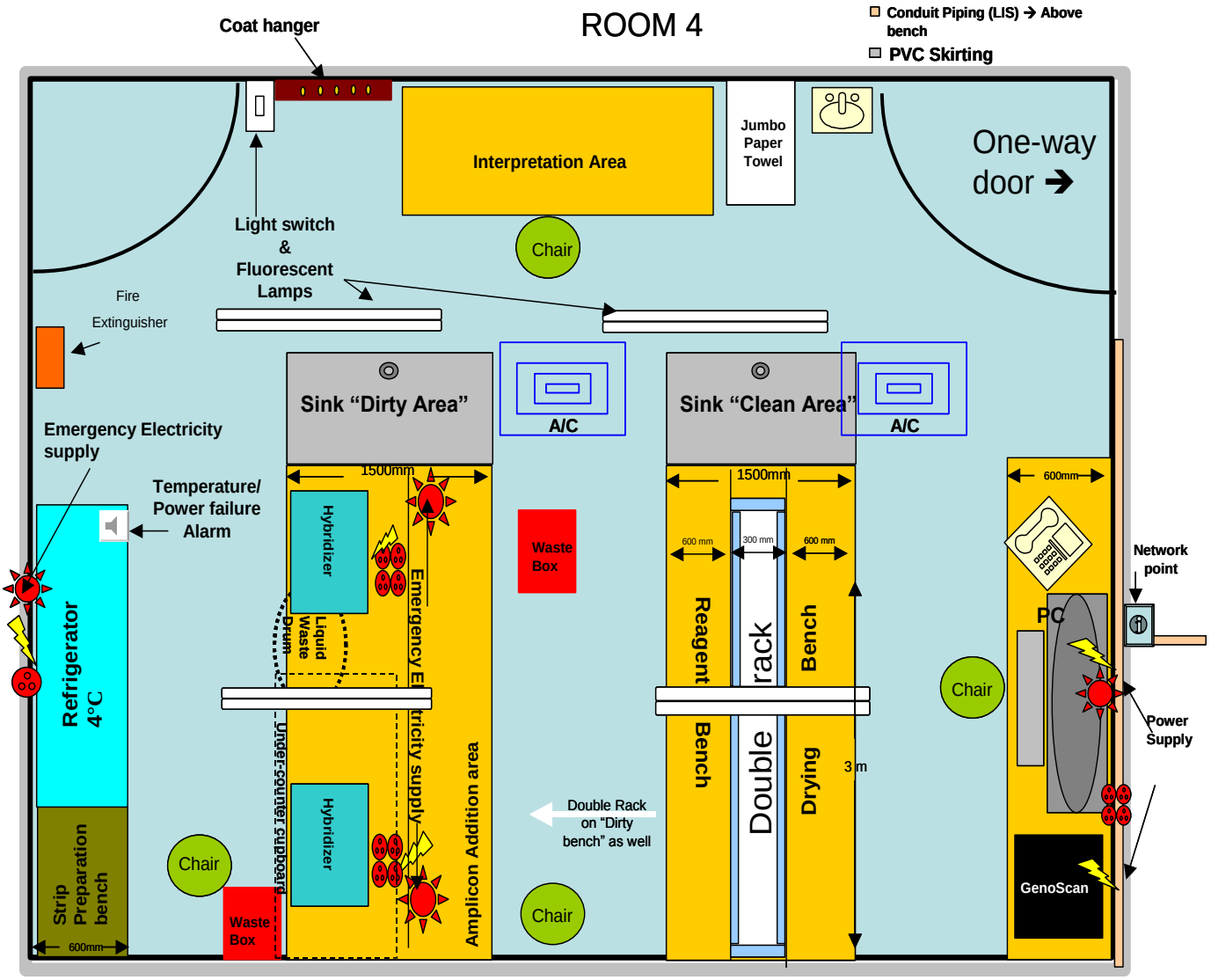


Figure 9: Suggested lay-out for the post-amplification area.

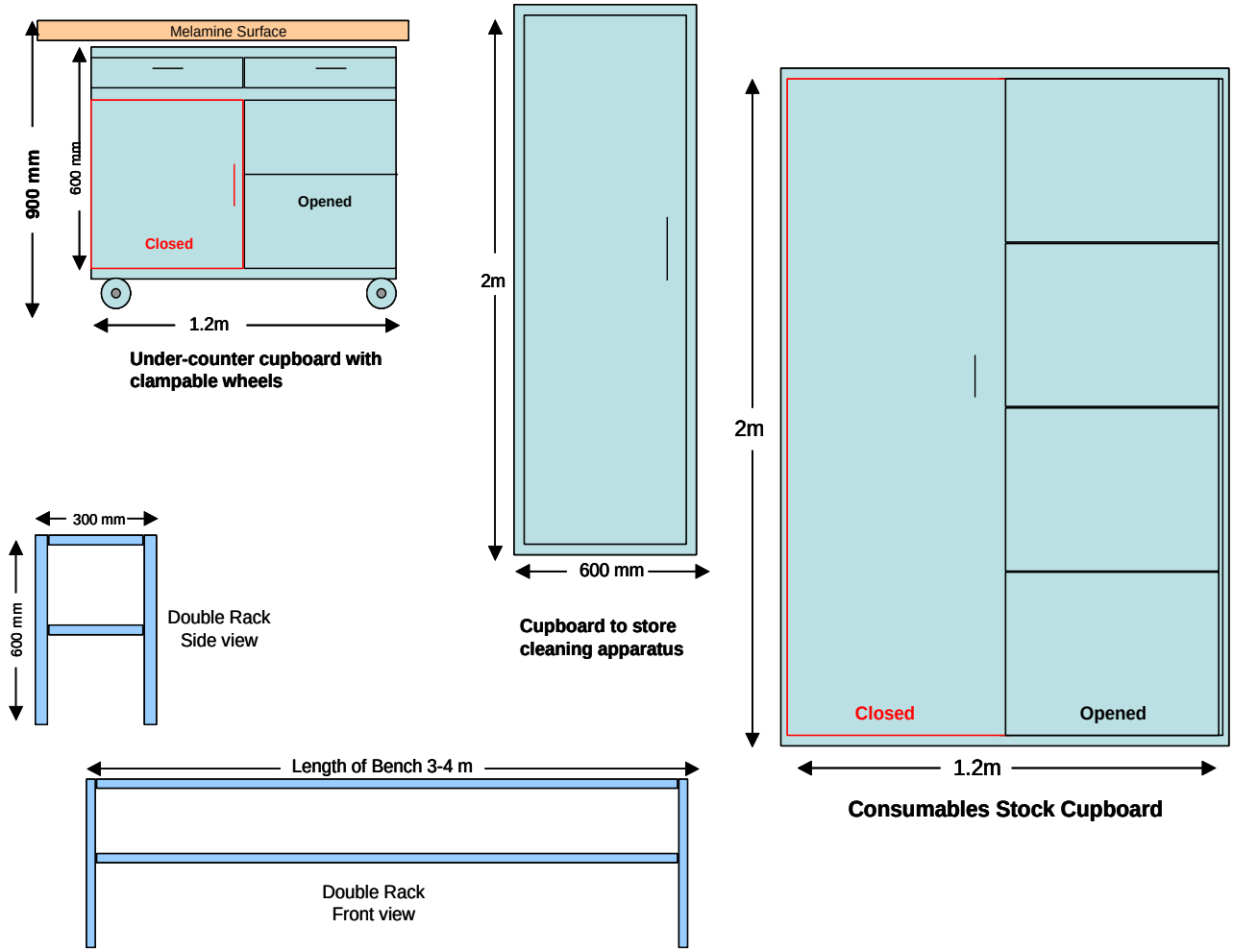


Figure 10: Dimensions of racks and storage cupboards.

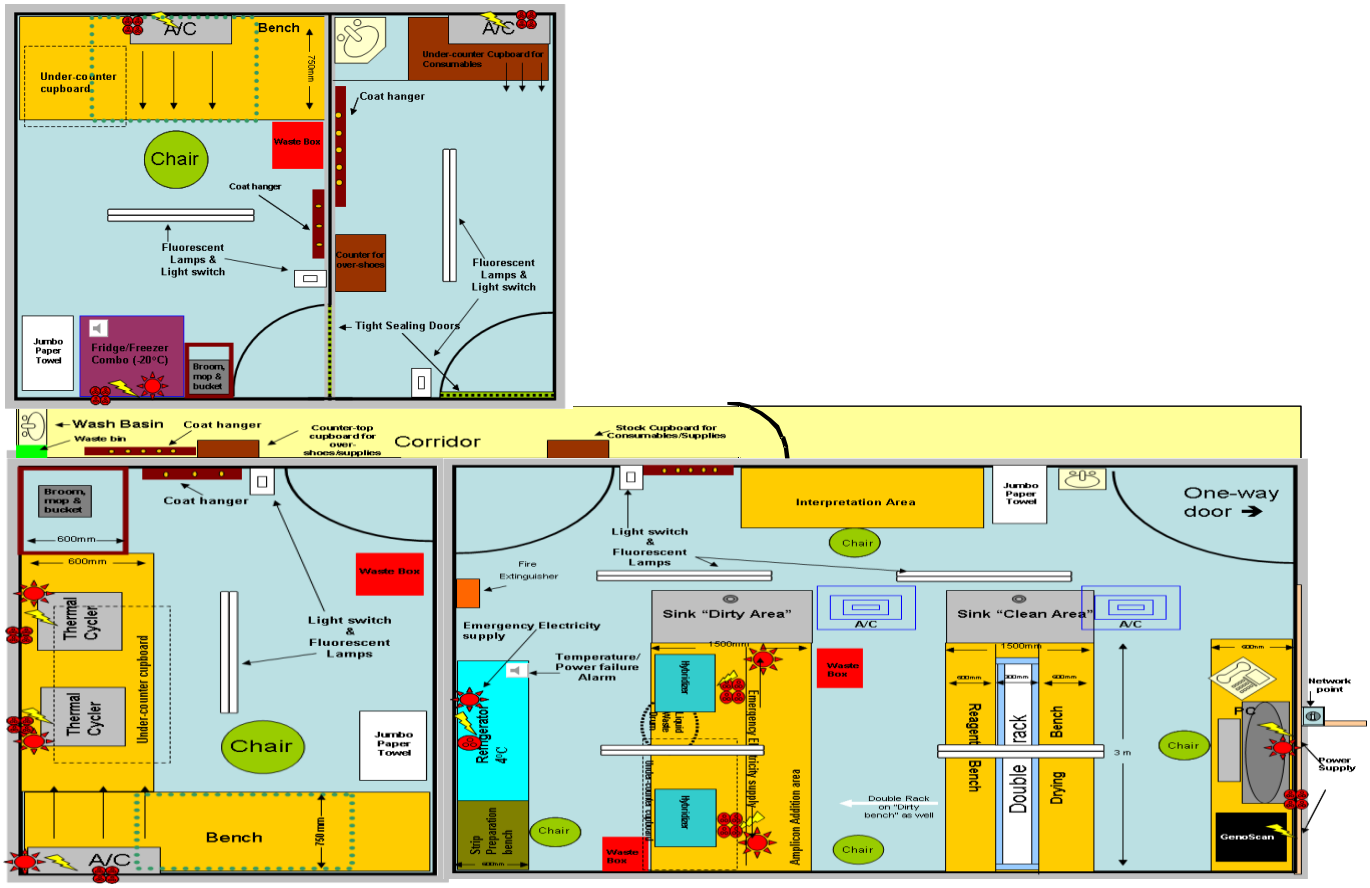


Figure 11: Amalgamated lay-out of the three PCR areas. Note that this fits into the "skeleton" observed in Figure 1. The door in the corridor creates an additional anteroom for the molecular diagnosis of *M. tuberculosis*.

Chapter 3:
PRINCIPLE OF THE PROCEDURE

The **Geno**Type® **MTBDR*plus*** test is based on **DNA•STRIP**® technology and allows for the molecular identification of the *Mycobacterium tuberculosis* complex (which includes *M. tuberculosis*, *M. canettii*, *M. africanum*, *M. microti*, *M. bovis* and *M. bovis* BCG) and its associated genotypic susceptibilities to Rifampicin (RMP) and Isoniazid (INH). This assay can test smear-positive patient material, cultured isolates from various media sources and NaOH-NALC decontaminated specimens.

In wild-type *M. tuberculosis* strains (susceptible strains), RMP and INH inhibit the activity of enzymes involved in RNA and cell wall synthesis respectively, thereby killing the bacilli (**Figure 12**). However, resistant strains have mutations in genes encoding these critical enzymes, thereby altering the structure of the proteins so that a complex cannot be formed between the altered enzymes and the drugs. The bacilli with these specific mutations (resistant bacilli) can therefore still replicate and multiply in the presence of the drug.

The molecular identification of RMP resistance is accomplished by detecting the most significant mutations in the 81-bp (base pair) region of the *rpoB* gene (which encodes the β-subunit of RNA polymerase, the essential enzyme that is inactivated by RMP). High-level resistance to INH is detected by screening for the most common mutations in the *katG* gene (which encodes catalase, the enzyme that activates INH). Low-level resistance to INH is detected by screening for mutations in the promoter region of the *inhA* gene (which encodes the NADH enoyl ACP reductase, involved in cell wall biosynthesis).

As stated above, when testing uncultured specimens, only NaOH-NALC pre-treated patient material that is acid-fast bacilli (AFB) smear-positive (based on the smear grading of 1+ or more) can be used. If the smear results are scanty or negative, the specimen must first be cultured and subsequent positive culture isolates used for the line probe assay (LPA). Routine NaOH-NALC decontamination practices should be followed as per the publication by the U.S. Center for Disease Control and

Prevention (CDC) entitled “Public Health Mycobacteriology: A Guide for the Level III Laboratory”.

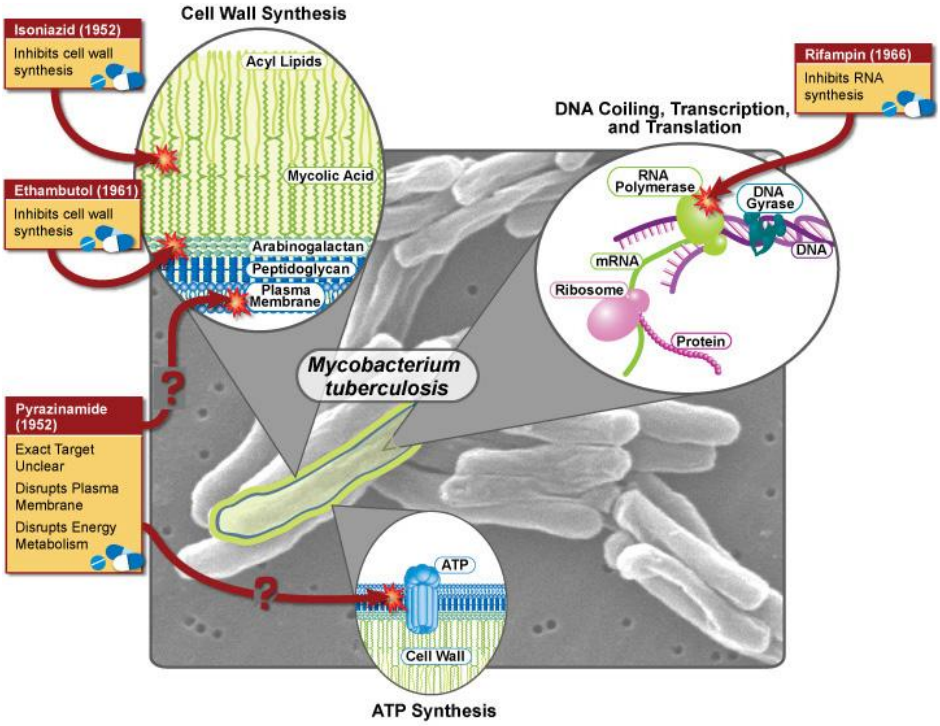


Figure 12*: First Line treatment of tuberculosis (TB) for drug susceptible strains.

*Obtained from the National Institute of Allergy and Infectious Diseases (NIAID) www.niaid.nih.gov.

Use of the LPA eliminates the diagnostic delay associated with phenotypic conventional drug susceptibility testing since it provides a “snapshot” of the genotype of a particular strain of the *M. tuberculosis* complex within a few hours. The genotypic pattern is then used to interpret the drug susceptibility pattern for a particular strain of *M. tuberculosis*. When using the LPA, the bacilli’s DNA is first extracted, and then regions of genes associated with INH and RMP resistance are amplified from the DNA using the **P**olymerase **C**hain **R**eaction (PCR). Following the PCR, reverse hybridization of the amplified DNA is performed on membrane strips: the banding pattern on the strips provides a visual interpretation of the genotype of the bacilli being tested.

The molecular LPA can therefore be divided into three procedures (**Figure 13**):

1. DNA extraction from NaOH-NALC decontaminated smear positive specimens, or from cultured isolates (solid or liquid media);
2. A multiplex PCR with conditions that are specific for the type of specimen that was extracted and;
3. Reverse hybridization, where probes (reaction zones or bands) on the strips are used to interrogate the *M. tuberculosis* target DNA associated with RMP and INH resistance by detecting sequences complementary to the probes on the strip.

The principle of each procedure will be discussed individually below.

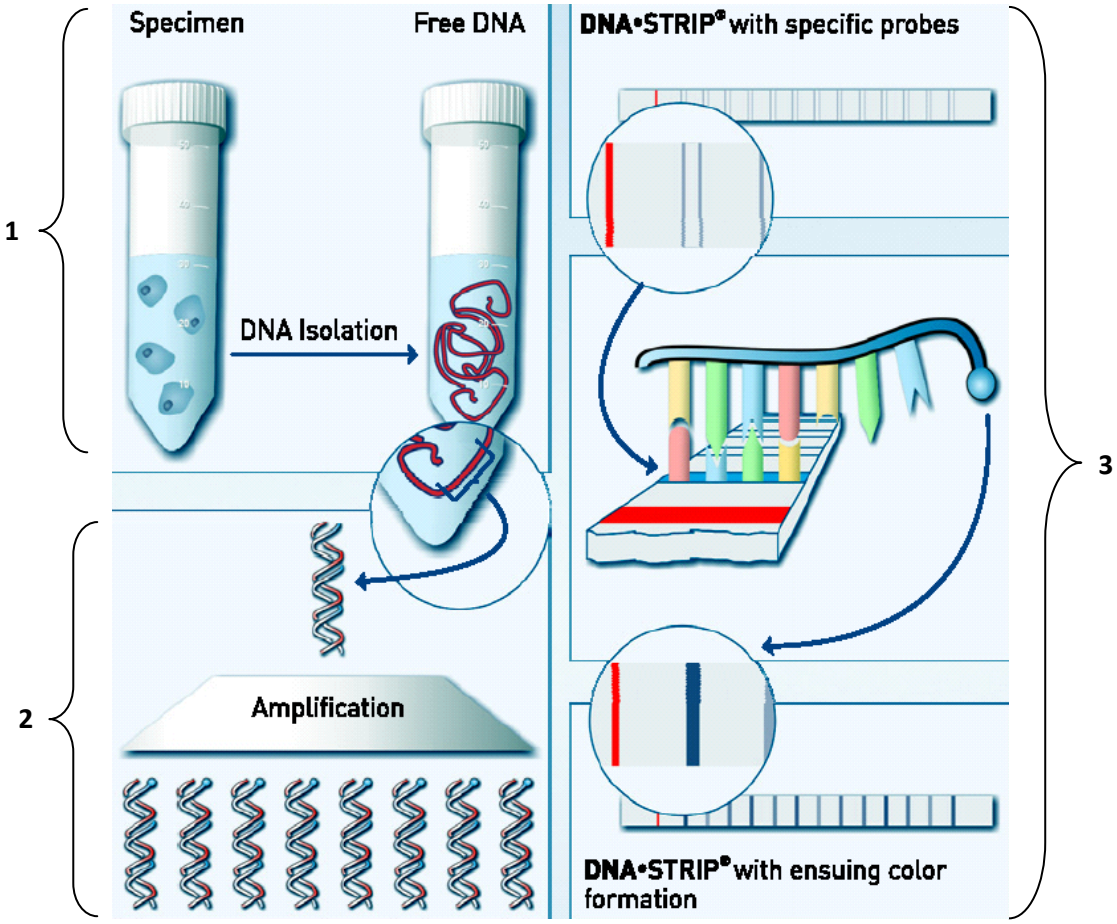


Figure 13: Schematic representation of the three LPA procedures. The first procedure is DNA extraction, followed by amplification of genes with biotinylated primers. Then, the amplified DNA is hybridized to probes and the bands visually detected by means of a colour-forming enzymatic reaction involving streptavidin adhering to biotinylated primers.

DNA EXTRACTION

The LPA diagnostic technique is based on the PCR amplification of specific regions of DNA. DNA must therefore be extracted from the specimen under investigation in order to make a genotypic diagnosis of the drug susceptibility pattern. The extraction of the DNA from the AFB in the specimen is done by sonification after partial cell lysis during a heat-killing step. **Figure 14** illustrates the principle of obtaining the DNA from the tubercule bacilli.

An initial centrifugation step concentrates the bacilli at the bottom of the microcentrifuge tube to form the bacterial containing “pellet”. The supernatant, which may consist of either the neutralising buffer from the NaOH-NALC decontamination procedure, Tween® 80 saline, MGIT growth liquid, or molecular grade water (if colonies were picked from solid media), should be discarded. The pellet should be resuspended in molecular grade water, to ensure that the suspension of extracted DNA is free of impurities that might inhibit the PCR reaction. The tubercle bacilli tend to clump so the resuspended solution should be mixed well so that it appears slightly opaque (milky). This will ensure that the dispersed bacilli will be killed during the heat-inactivation step and a higher yield of DNA obtained.

The heat generated by the heating-block, hot air oven or water-bath is meant to kill the bacilli and partially lyse the cells, thereby rendering the solution non-infectious. The solution can be now be transferred out of the BSL-3 laboratory for performance of the rest of the assay. The powerful ultrasonic shockwaves created by the sonicator disrupt the cell walls of the tubercule bacilli, causing further cell lysis, and releasing the DNA and other cell debris into the molecular grade water. The final centrifugation step, at maximum speed, is needed to separate the impure cell debris (containing the cell wall, proteins and other macromolecules) from the DNA. The heavier debris will form the pellet and the lighter DNA (free from impurities) will be suspended in the supernatant (molecular grade water). The extracted DNA is now pure and should be stored at 4ºC until the next day, when it will be added to the master mix and the procedure continued.

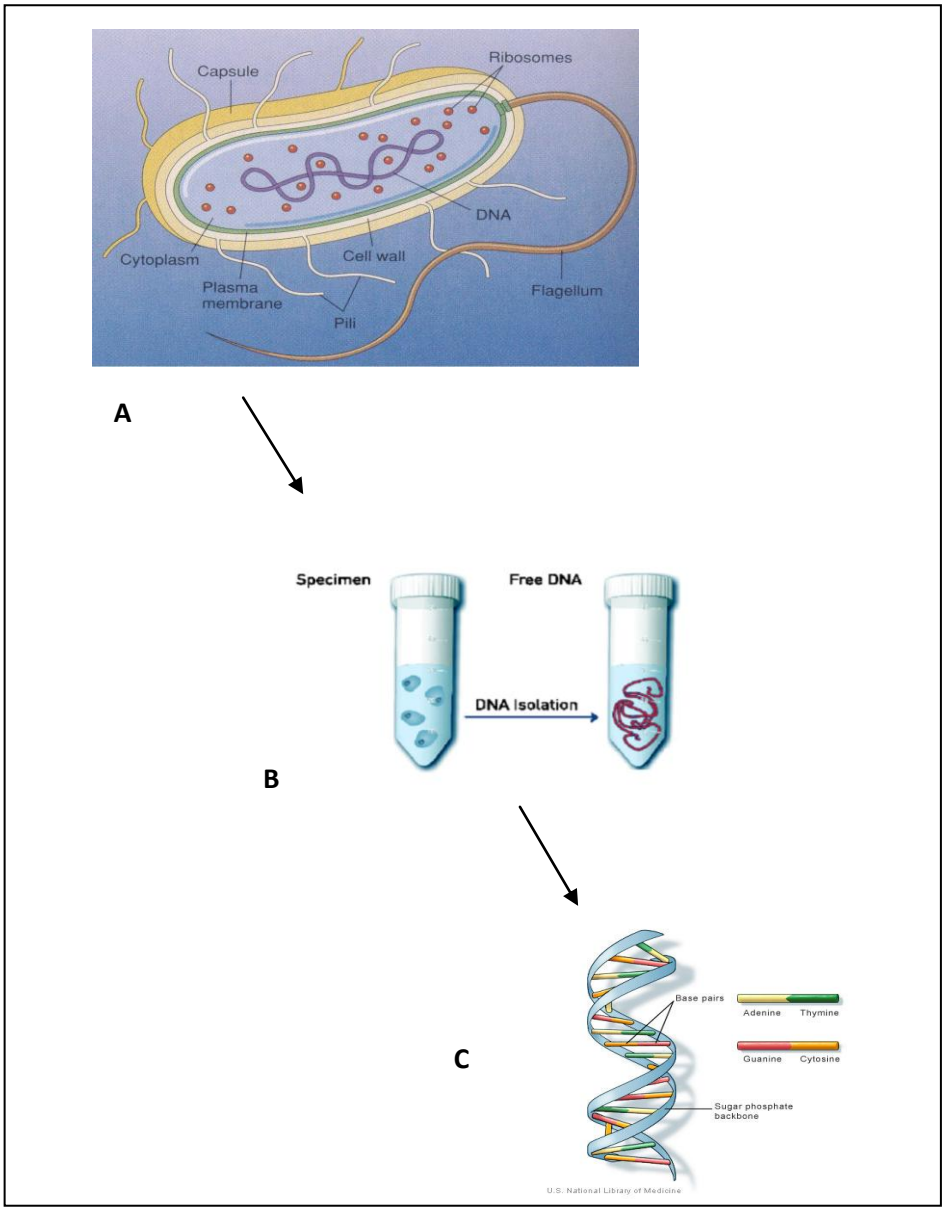


Figure 14: Illustration of the principle of the DNA extraction procedure. (**A**) Bacterial DNA is found in the chromosome. (**B**) The cells in the specimen will be disrupted by heat and sonication to lyse the cells thereby releasing the DNA into the supernatant. (**C**) The DNA consists of corresponding base pair nucleotides (A=T and G≡C) which is structured around a sugar phosphate backbone.

PCR PRINCIPLES

Polymerase Chain Reaction (PCR) is an *in vitro* method for amplifying target DNA sequences. It is referred to as a “chain reaction” because newly synthesised DNA strands serve as templates for further synthesis of DNA strands in subsequent cycles. This technique allows us to generate millions of copies of specific DNA fragments (containing the genes of interest) from a small amount of DNA (**Figure 15**).

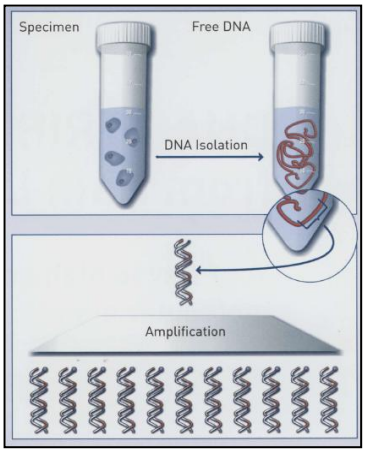


Figure 15: Representation of the DNA Extraction from a specimen and its subsequent amplification. The gene(s) of interest such as *rpoB*, *katG* and *inhA*, associated with RMP and INH resistance, respectively, will be amplified due to the specificity of the primers.

Within a heterogeneous mixture of DNA sequences, only specific DNA sequences are amplified due to the use of DNA primers that are complementary to DNA regions of interest and bind these preferentially. For successful amplification, the PCR requires the presence of:

1. Target DNA that will serve as the template to be amplified;
2. Primers (short oligonucleotides of between 18 and 26 base pairs) that will recognise their corresponding sequence on the single stranded DNA and initiate the reaction;

3. DNA precursors (the four deoxynucleoside triphosphates, dATP, dCTP, dGTP and dTTP) which will be used as building blocks during the elongation of the single stranded DNA and;
4. A thermostable *Thermis aquaticus* DNA polymerase (Hot Start *Taq*) which will elongate the DNA molecule by facilitating the incorporation of the free nucleotides onto the end of the primer, according to the complementary base on the single stranded target DNA.

These reactions are fundamental in ensuring that sufficient DNA is amplified and will be easily detected in the hybridization process. **Figure 16** illustrates the PCR cycle where biotinylated primers are incorporated into the newly synthesized DNA amplicons.

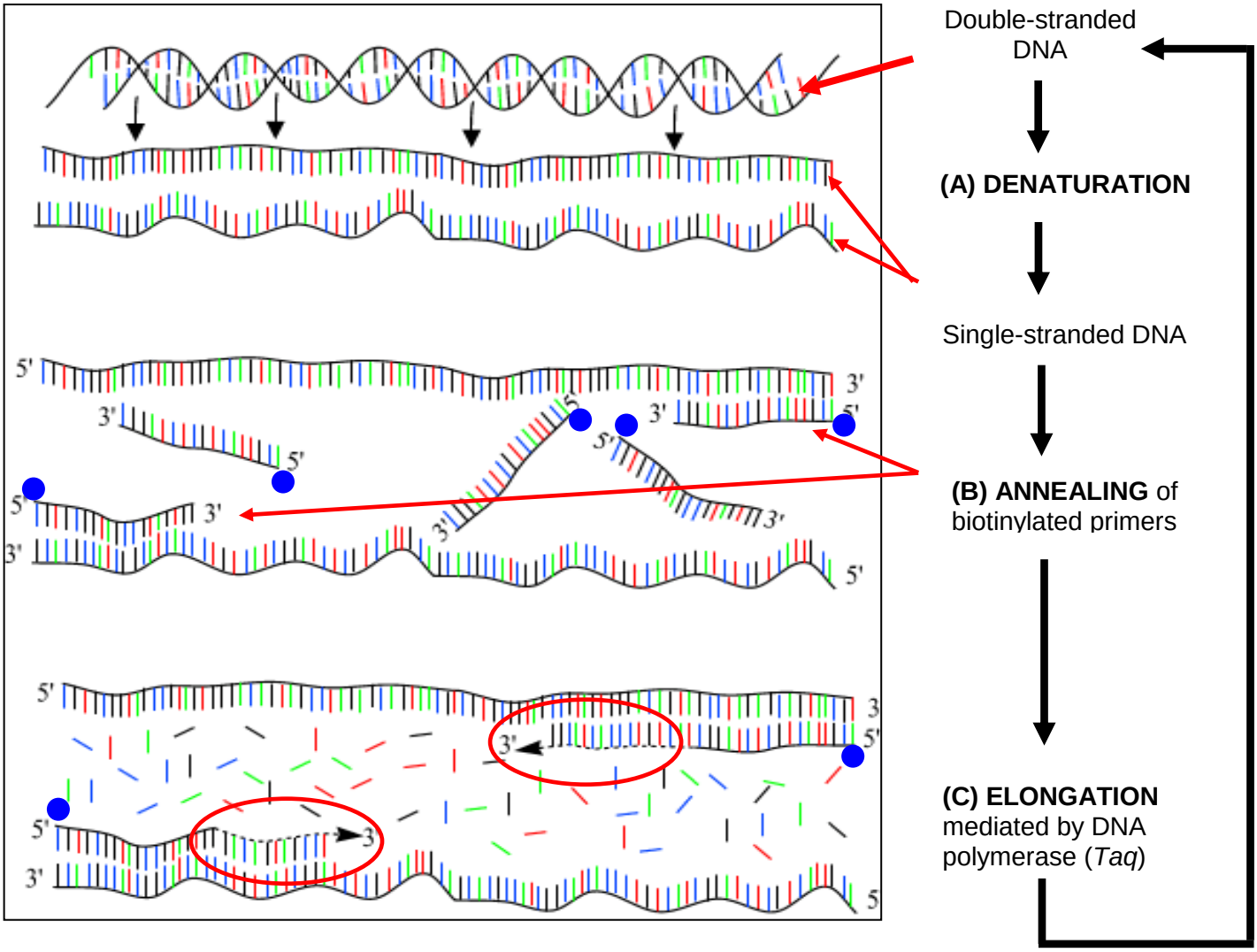


Figure 16: Diagrammatic representation of the Polymerase Chain Reaction (PCR) where 30 to 40 cycles of three steps are repeated. (**A**) **Denaturation** of the double-stranded DNA into single-stranded DNA at 95ºC for 25 seconds, (**B**) **Annealing** of the forward and reverse primers at 53ºC for 40 seconds, and (**C**) DNA polymerase mediated **Elongation/Extension** of the DNA by incorporation of the dNTPs (nucleotides) at 70ºC for 40 seconds.

After 30 cycles of amplification, the DNA products will be in the order of 2^{30} (two to the thirtieth power), with sufficient product to be easily visualized as a distinct band when bound to probes on the LPA strip. **Figure 17** illustrates the exponential amplification of the DNA during the chain reaction event.

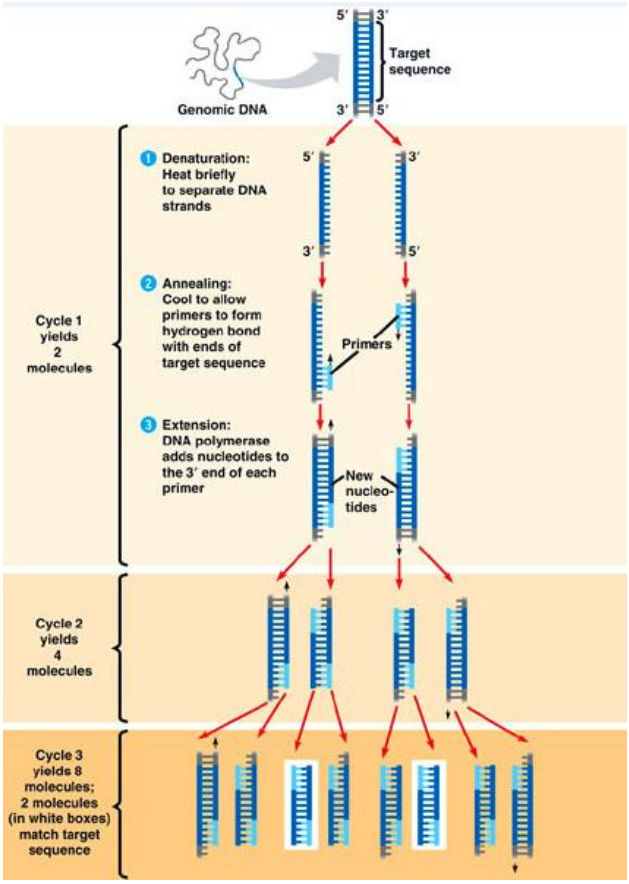


Figure 17: Principle of the PCR. Genomic DNA is denatured, the primers anneal to specific regions within the gene, and DNA polymerase enables the extension of the primers to form 2 copies of the gene. The procedure is exponential, and two copies will become 4, 4 will become 8, etc. After the 30^{th} cycle, the amount will be in the order of 2^{30}.

Thus, the PCR procedure relies on three sequential steps:

1. Denaturation
2. Annealing
3. Extension / Elongation

This allows the primers to gain access to ssDNA templates. During the Annealing process (step 2), the reaction mixture is cooled to a lower temperature (50 - 65°C), thereby allowing for the primers to select and anneal (or “attach”) to their complementary positions on the ssDNA. During Extension/Elongation (step 3), the ssDNA/primer couplings are heated to 70ºC and the heat stable *Thermis aquaticus* (*Taq*) DNA polymerase will transcribe the complementary strands, in conjunction

with the PCR buffer, dNTP's and magnesium (Mg^{2+}). This results in dsDNA copies of the regions of interest. By continuously repeating these three steps, in a "chain reaction", exponential amplification of the original target DNA (or template) will occur (**Figure 18**).

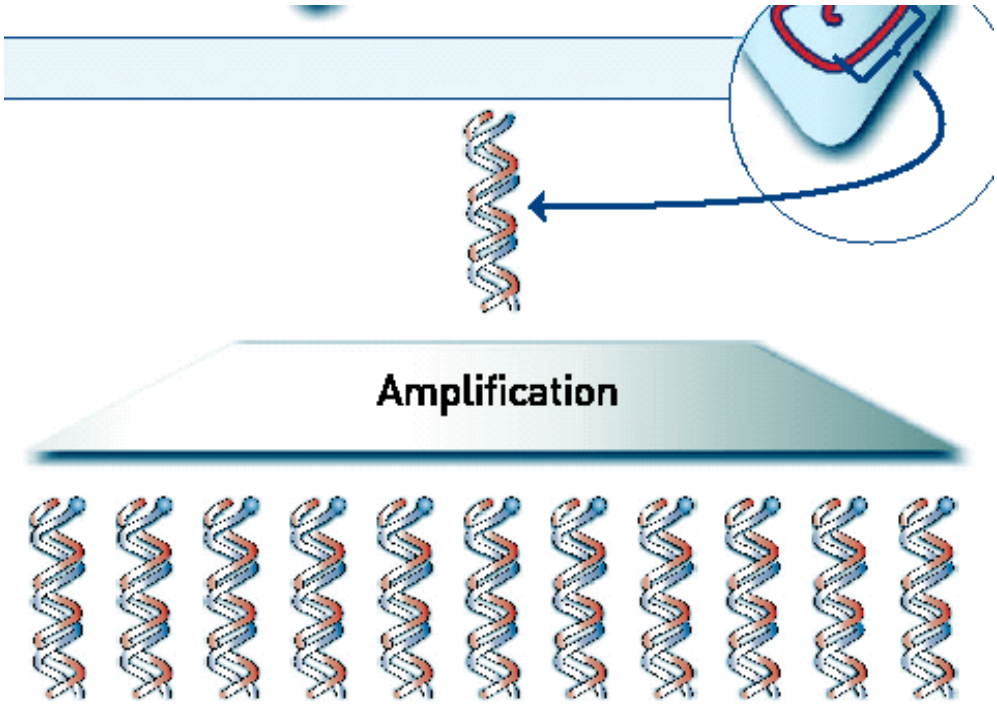


Figure 18: Simplified diagram of the PCR, where a single specific fragment (gene of interest) in the DNA is amplified to produce multiple copies of the same specific fragment.

If hot start PCR is to be used, an initial extended heating phase (95ºC) is needed to ensure the removal of an antibody that inhibits the Hot Start DNA polymerase activity before the start of the PCR cycle. This method also ensures that there is no non-specific binding of the primers to the template DNA, before the denaturation cycle starts at 95ºC.

It is essential that the correct cycle setting be used for the different specimen types: sputum specimens should undergo 40 cycles, but only 30 cycles should be used for cultured isolates.

OPTIMIZATION OF THE PCR REACTION

There is no need to optimise the GenoType® MTBDR*plus* LPA since the manufacturer has already determined the various factors involved in the PCR reaction. However, if a home-brew PCR reaction is to be developed and validated, the following issues need to be considered:

- The Choice of primers

  The GenoType® MTBDR*plus* LPA is a multiplex PCR and thus there are several primer sets in the PNM mix. Every PCR assay has at least one set of two (2) primers: a Forward primer and Reverse primer located at either end of the region to be amplified. When designing or choosing primers for a home-brew PCR, the following factors must be considered: the size of the DNA fragment; the GC content, melting temperature, and specificity of the primers; and the possibility of primer-dimer formation (complementary regions within the primers that would result in folding back on itself or annealing with the other primer).

- The Annealing temperature

  The annealing temperature is the most critical determinant of the specificity of the PCR and is chosen according to the melting temperatures of the primers. When optimising a PCR assay with a new primer set, it is advisable to test different annealing temperatures (mostly between 50°C and 65°C) so that a temperature that will result in optimal specificity can be chosen.

- The $MgCl_2$ concentration

  The concentration of $MgCl_2$ has a marked influence on the specificity of the binding of the primers and hence the specificity of the PCR. The Mg^{2+} forms soluble complexes with the free nucleotides allowing for the DNA polymerase to recognise them as substrates during the amplification procedure. The $MgCl_2$ must be thoroughly thawed and mixed so that the solution is homogenous and the final concentration in the master mix is exact. Standard buffers are commonly used with a final concentration of 1.5mM $MgCl_2$.

Since the GenoType® MTBDR*plus* LPA is a commercial product that has been optimised, it is very important to keep all components as constant as possible in order to reduce the chances of failure and to ensure that there are no variations in the PCR yield.

DNA POLYMERASE

During the reagent preparation procedure, all the PCR reagents are mixed together before the initial heat denaturation step. This combination of reaction components and DNA should be kept on ice before loading into the thermal cycler, since exposure to ambient temperatures will usually increase the chances of non-specific binding of the primers to the DNA. For this reason, it is recommended that HotStarTaq® from Qiagen be used when preparing the master mix as it reduces non-specific product amplification during the heating of the thermal cycler at the beginning of the PCR. This product ensures that the amplification cannot start until the reaction temperature is above that where non-specific annealing of primers to template DNA may occur (**Figure 19**). Thus, the DNA polymerase will only be activated when the thermal cycler reaches 95ºC. The optimum working temperature of the *Taq* enzyme is 80ºC so the annealing temperature of 70ºC ensures that the primers will bind specifically to the ssDNA.

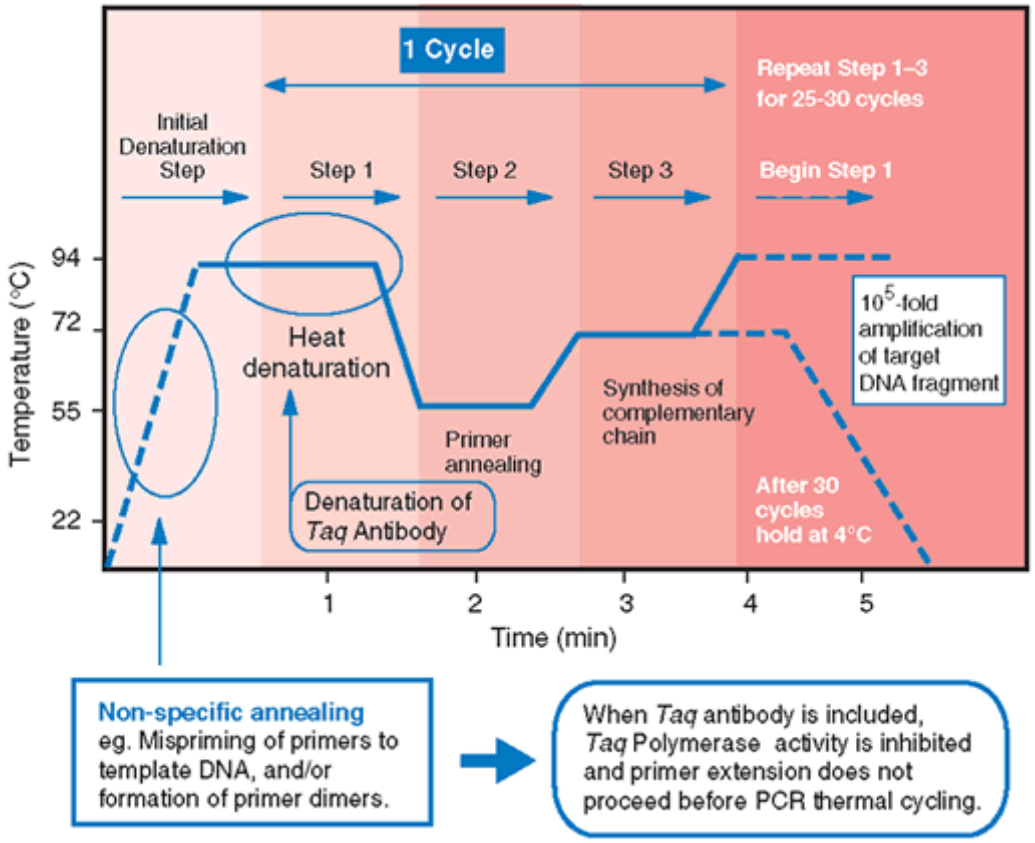


Figure 19: Illustration of a Hot Start PCR Reaction.

HYBRIDIZATION PRINCIPLES

DNA hybridization is one of the oldest molecular techniques and is based on the natural event of complementary strands of ssDNA hybridising or binding to each other to form dsDNA. As the first step in the hybridization procedure, the dsDNA in a sample is rendered single-stranded by chemical denaturation. This allows it to bind to a complementary single-stranded probe. The reaction is accomplished by two essential nucleic acid base parings: cytosine (C) forms a base pair with guanine (G), and adenine (A) forms a base pair with thymidine (T). For the hybridization to take place there must be a very high degree of complementary base pairing between the two single strands of DNA.

In “normal” hybridization reactions, the unlabelled target DNA is fixed onto a nitrocellulose membrane and exposed, most often, to only a single radio- or biotin-

labelled probe that is in the fluidic state. The LPA reverses this approach, and makes use of Reverse Hybridization. This expands the scope of the assay by allowing interrogation and differentiation of a large number of wild-type and mutant sites within the *rpoB*, *katG* and *inhA* regions. In this assay, the biotin-labelled amplicons (amplified DNA of the genes of interest generated during amplification of the target DNA) are in the fluidic state and the wild-type and/or mutated probes (reaction zones) are unlabelled and immobilized as bands onto the positively charged nitrocellulose membrane strips (**Figure 20**).

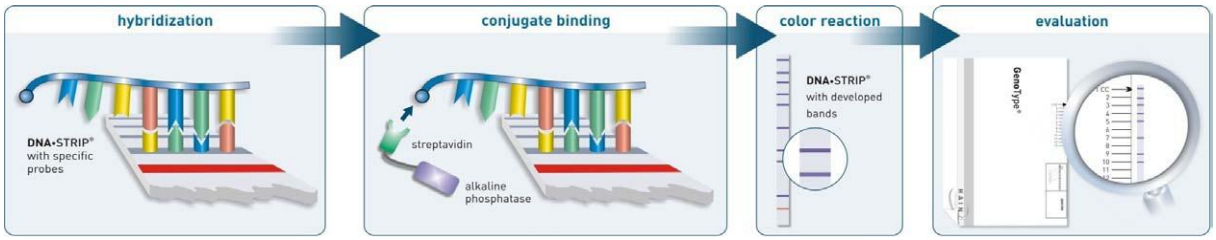


Figure 20: Overview of the LPA hybridization procedure.

Following hybridization of the biotin-labeled amplicons to the reaction zones, the strips are exposed to a streptavidin-alkaline phosphatise conjugate. The ligands biotin (a natural occurring vitamin) and streptavidin (a bacterial protein) have a high affinity for one another and binding takes place at band sites on the strip where hybridization has occurred. Next, the enzyme alkaline phosphatase conjugated to the streptavidin reacts with hydrogen peroxide, allowing the colorimetric detection of the bound biotin-streptavidin complex. The specific regions of genes (wild-type or mutant) present in the heterogeneous mixture of the target ssDNA can be thus be detected.

The following incubation, washing, and rinsing steps are carried out sequentially during the hybridization procedure and are described in detail below:

1. Chemical denaturation of the amplified products;
2. Hybridization of the single-stranded-biotin-labelled amplicons to membrane-bound probes;
3. Stringent washing of the non-specifically bound amplicons;

4. Addition of a streptavidin /alkaline phosphatase (AP) conjugate and;
5. An AP-mediated staining reaction.

Chemical Denaturation

In order for the hybridization process to take place, the biotinylated dsDNA must be separated into ssDNA that can recognize and bind to complementary probes on the strip (**Figure 21**). The LPA uses sodium hydroxide (NaOH) to denature the dsDNA by breaking the hydrogen bonds between the paired nucleotides. The resulting ssDNA can then be used for the hybridization step.

Hybridization

In order to hybridize, the biotinylated-single-stranded amplicons in solution must have a specific sequence that is perfectly complementary to specific probes (wild-type or mutant) on the strip (**Figure 22**). The temperature at which the amplicon and the probe hybridize is determined by the strand length, the chemical environment and the nucleotide base pairs. It is important to understand that the hybridization conditions are critical in ensuring formation of stable hybrids. The LPA method uses an optimal temperature that will allow precise hybridization of the amplicons to the probes. If the temperature of a specific test is higher than recommended, there may be no binding at all, and if the temperature is too low, there may be non-specific binding to various reaction zones on the strip. Furthermore, monovalent cations in the hybridization buffer stabilize exact nucleotide matches between the probe and the ssDNA. After the hybridization step, the buffer solution containing the unhybridized ssDNA is removed, and the strip is then exposed to a stringent washing step that disrupts any unstable hybrids that are not a perfect match (see below).

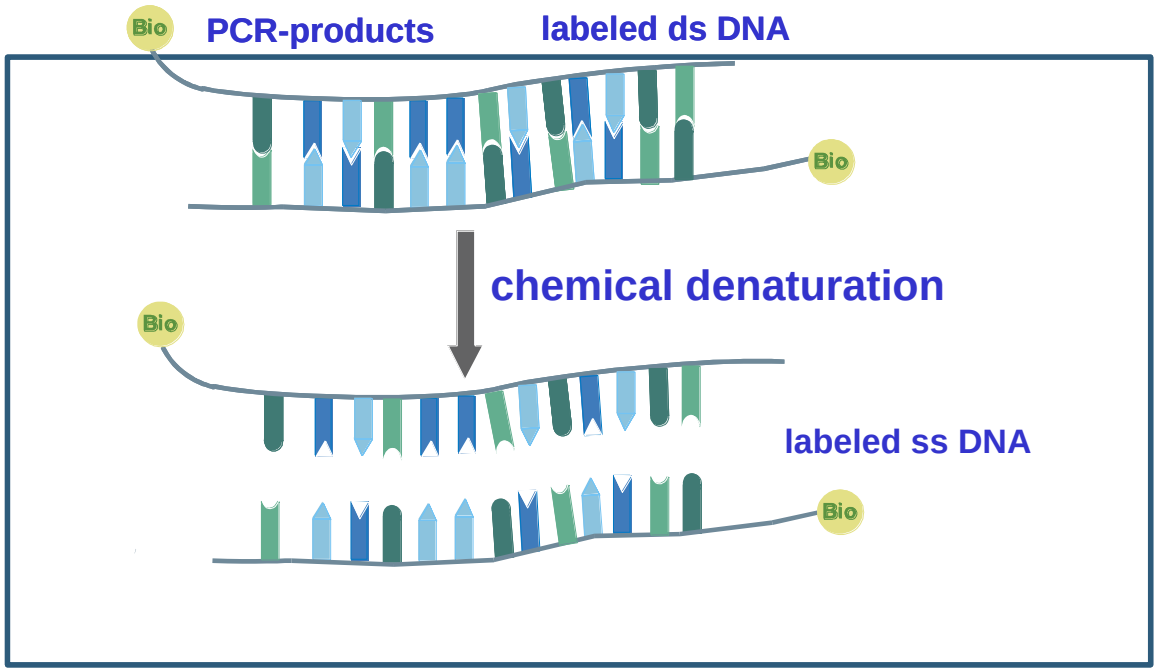


Figure 21: Chemical denaturation with sodium hydroxide (DEN buffer) of the biotinylated double-stranded DNA (dsDNA) into biotinylated single stranded DNA (ssDNA). This allows the ssDNA to bind to a specific single-stranded probe (reaction zone) on the strip.

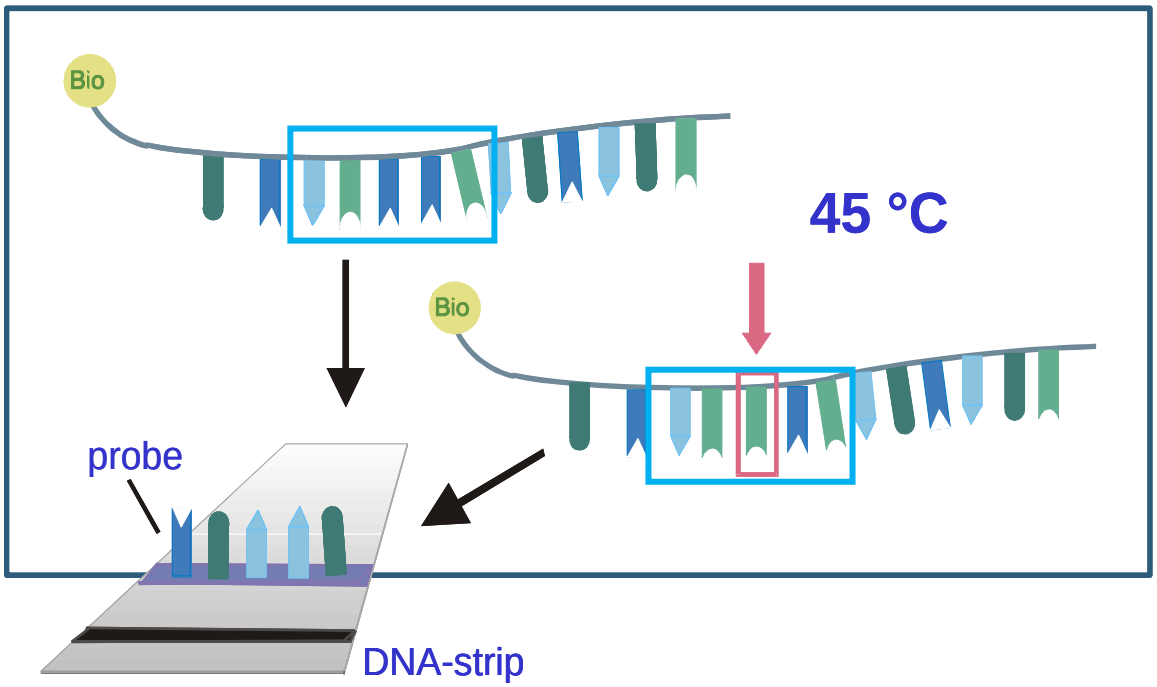


Figure 22: Hybridization of the PCR-amplicons to the probe in the green hybridization buffer (HYB). Note the top ssDNA strand (within the blue box) perfectly matches the probe on the strip, while the strand on the lower right has a single nucleotide mis-match (red arrow).

Stringent Wash

Single nucleotide mismatches (e.g. A-G) are unstable at high stringency washes. After the hybridization step has been completed, a high stringency wash at 45ºC (in red stringent wash buffer, STR) will ensure that any potential mismatches that may have formed between the hybridized amplicon and the probe are removed (**Figure 23**). This wash is essential in order to eliminate all non-specific bands on the strip that might influence the read-out of the final result.

Conjugation

The conjugation buffer consists of a streptavidin-alkaline phosphatase, which recognises the biotinylated primers (**Figure 24**) that are hybridized to probes on the membrane. If this step is accidentally skipped, the strips will have no banding patterns at all.

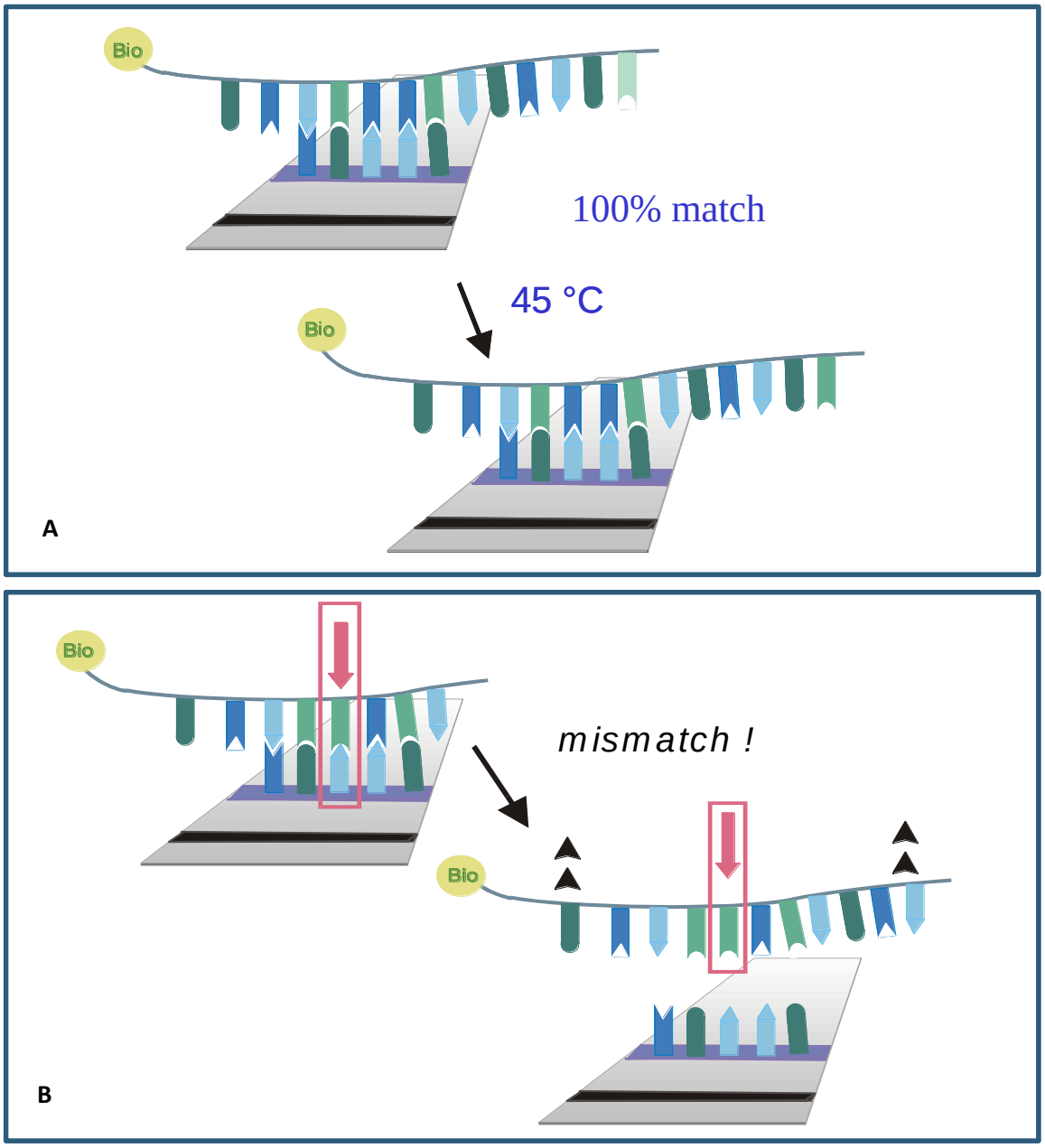


Figure 23: Stringent wash removal of non-specifically bound amplicons. (**A**) 100% nucleotide recognition (A=T & C≡G) resulting in a perfect match between the amplicon and the probe. (**B**) A mismatch at a single nucleotide position. The stringent wash removes this amplicon from the probe in order to eliminate non-specific hybridization bands.

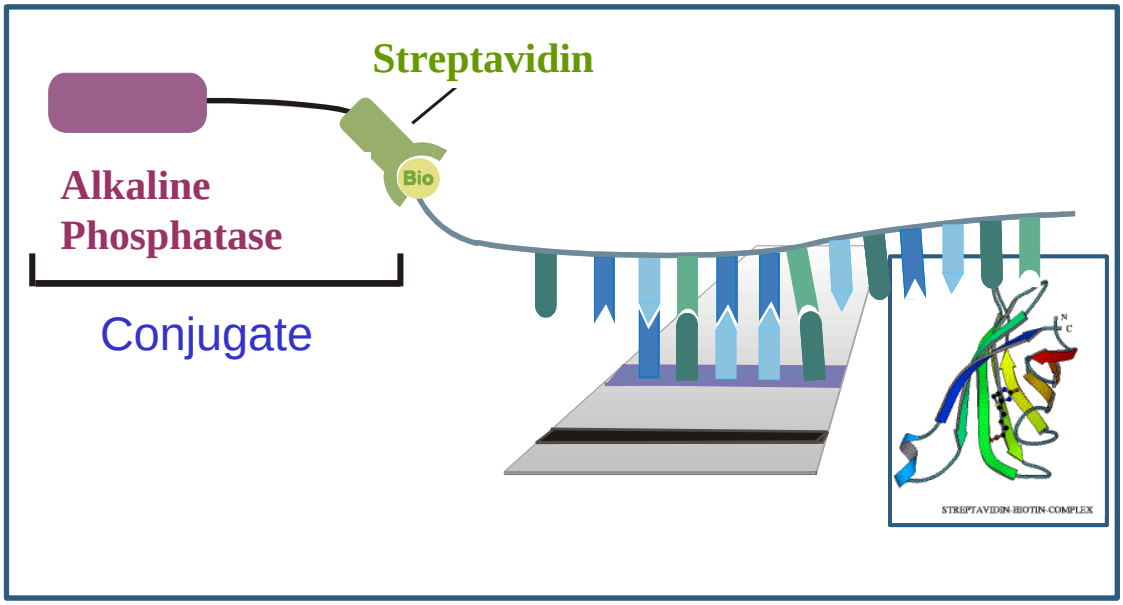


Figure 24: Conjugation of the Streptavidin-Alkaline Phosphatase to the biotinylated primer end of the amplified product.

Substrate addition

The visualization of the banding patterns is produced when the substrate (hydrogen peroxide) reacts with the alkaline phosphatase and undergoes a calorimetric change (**Figure 25**), that turns the bound amplicons purplish-brown. The intensity of colour in the bands is directly related to the number of amplicons that hybridise to the probes, and indirectly to the numbers of copies that were produced during the amplification.

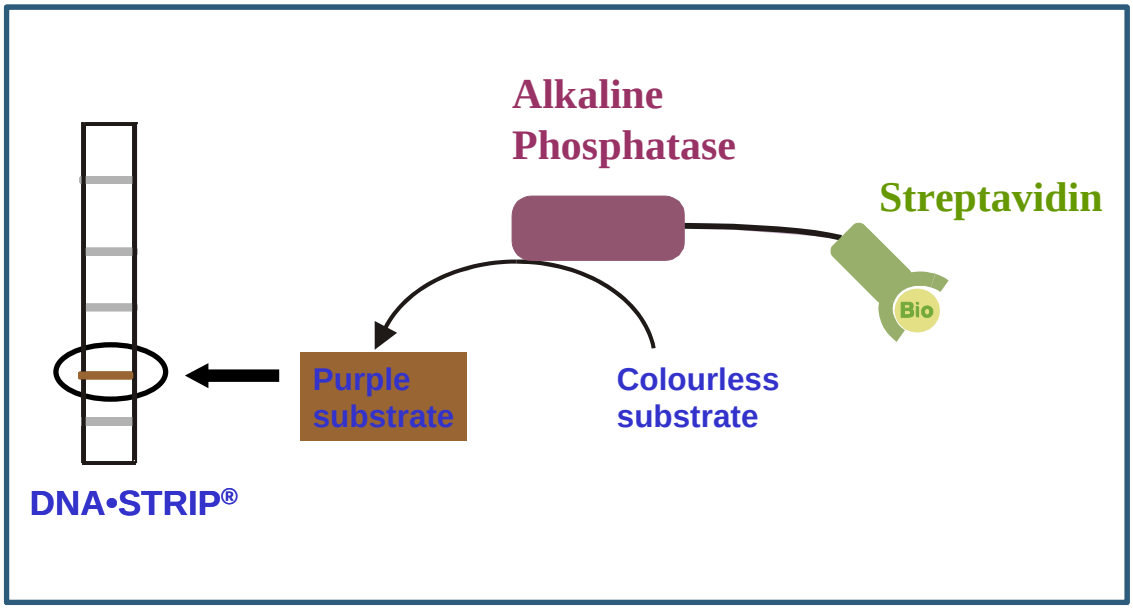


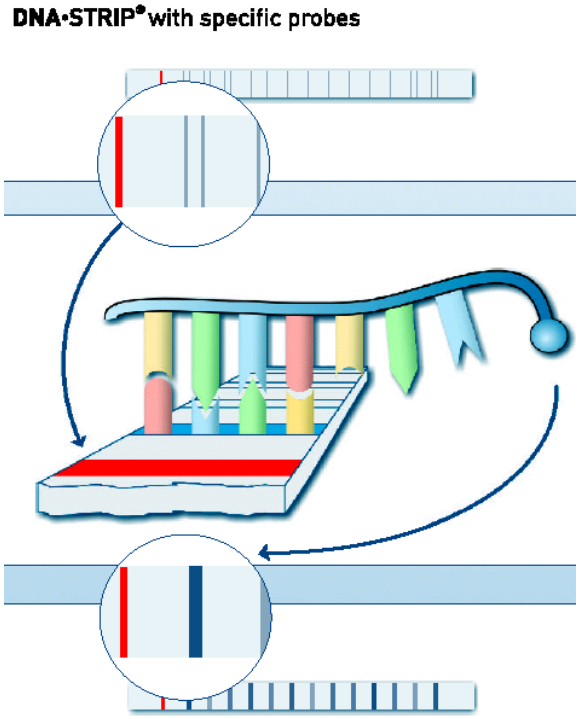
Figure 25: Colorimetric detection of the banding patterns on the strip by means of enzymatic reaction of the substrate with the alkaline phosphates. The banding on the strips is produced by the conversion of the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium Chloride into a purple/brown precipitate.

RINSE BUFFER & DEIONISED/DISTILLED WATER

The rinse buffer washes away the salts and "soapiness" of the two pre-heated hybridization buffers from the test strips. The rinse buffer also removes the excess conjugate buffer, thereby ensuring that the strip will not have high level of background staining. After the second post-conjugate rinse, water is added to remove the salts in the rinse buffer. The two final washing steps post-substrate development also require the use of water to stop the colorimetric reaction and to remove excess substrate buffer that may also result in increased background noise.

Figure 26 illustrates the hybridization of the amplicons onto the clean strip and what the strips will look like after the colorimetric reaction that allows the visualisation of the banding patterns. The interpretation score card (template) should then be aligned with the banding patterns to give a final diagnostic result based on the interpretative guidelines.

A



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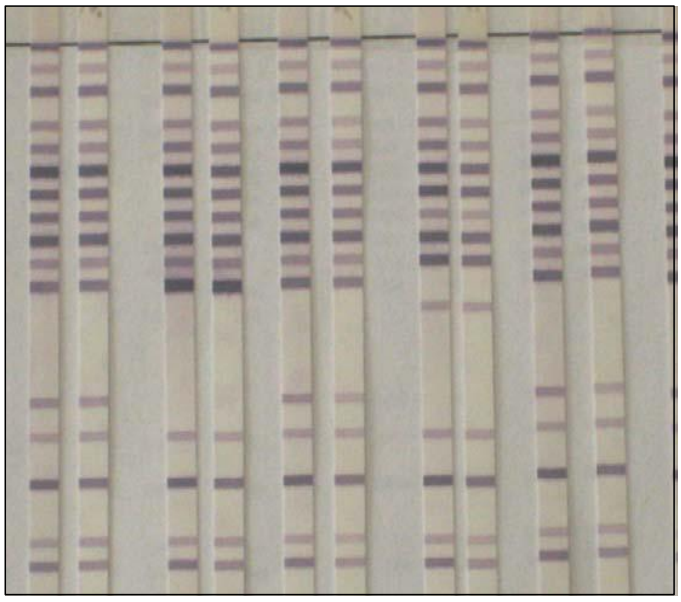

Figure 26: From (**A**) concept and principle to (**B**) real-life visualization of the banding patterns on the MTBDRplus® LPA.

TERMINOLOGY

Annealing: The joining of two complementary DNA strands (primer and the single stranded DNA) to form a double strand.

Complementary DNA: The bases of DNA forms specific pairings. Two strands whose nucleotides form a sequence of perfect pairings that enable them to form a stable double strand are referred to as complementary strands.

DNA: deoxyribo**n**ucleic **a**cid, the chemical substance of genes.

Nucleic acids: Molecules that consist of long chains of paired nucleotides.

Nucleotides: Basic building blocks of DNA; they compromise the four base pairs adenine, thymine, cytosine and guanine (A,T,C and G), a sugar and at least one phosphate group.

Primer: A short DNA fragment (oligonucleotides) with a defined sequence that serves as an extension point for polymerases.

Polymerases: Enzymes that link individual nucleotides together to form long DNA chains.

Sequence: The order of the nucleotides in DNA.

Chapter 4: PROCEDURE FOR THE LINE PROBE ASSAY

In order to perform the GenoType® MTBDR*plus* line probe assay (LPA), it is essential that the diagnostic testing is founded on a strong Quality Assurance program, that the laboratory infrastructure adheres to guidelines developed by the WHO, and that the required equipment is functioning properly. Each run of the LPA must be performed using the exact same procedure under identical conditions so that the banding patterns on the strips are consistent and interpretable. Contamination can have a devastating impact on the patient, the laboratory, and the budget, so efforts to prevent it must be instituted in each of the different procedural areas.

The complete LPA procedure for specimen extraction, amplification, hybridization and test interpretation is summarized in **Figure 27.** In addition to carefully following the steps outlined in the procedure, it is essential to guard against cross- or carry-over contamination during the following essential steps: the NaOH-NALC decontamination procedure; the extraction procedure; the template addition procedure; and the hybridization procedure. Ideally, each of the four procedures must be done in dedicated areas, as outlined in the Infrastructure chapter. Even if this is so, the different procedures must be carried out with the utmost care in order to avoid contamination.

Good laboratory practice requires that the specimen preparation be done on a separate day (or early in the day). Once the extraction procedure is completed, the DNA should be kept at 4ºC until the next day. If the DNA is going to be used later in the week, the DNA can be frozen at - 20ºC. The day after the DNA extraction (day two), the reagent preparation, template addition (amplification) and hybridization (detection) can be sequentially done, thereby ensuring a unidirectional workflow.

This chapter will describe the individual steps of the LPA in detail. The required contamination control at each step is also stressed, but described in more detail in the chapter on contamination control.

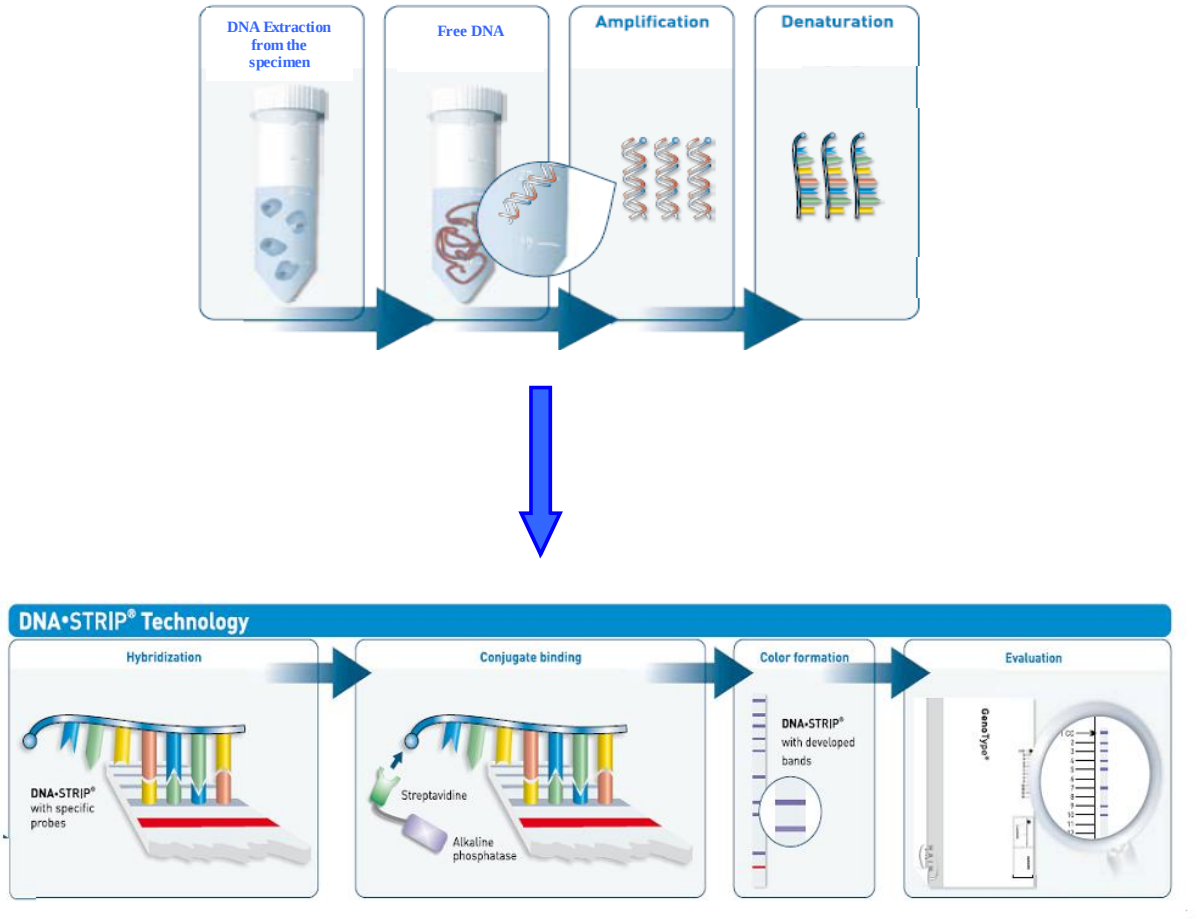

Figure 27: Illustration of the DNA-strip technology by Hain LifeScience. The GenoType® MTBDR*plus* molecular LPA is divided into three procedures: (1) DNA extraction from NaOH-NALC decontaminated smear positive specimens or from cultured isolates (solid or liquid media), (2) a multiplex **P**olymerase **C**hain **R**eaction amplification (PCR) and (3) the reverse hybridization (including detection and evaluation).

OVERALL CONTAMINATION CONTROL

DNA and amplicon contamination must be prevented at all times. It is especially important that no supplies or equipment from the amplification and hybridization ("dirty") areas are ever taken into the areas dedicated for reagent preparation, specimen processing or template addition. Likewise, items used in any of the other areas should not be taken into the reagent preparation ("cleanest") area. This

ensures the desired unidirectional workflow between the different areas. **Figure 28** gives a diagrammatic representation of DNA contamination control.

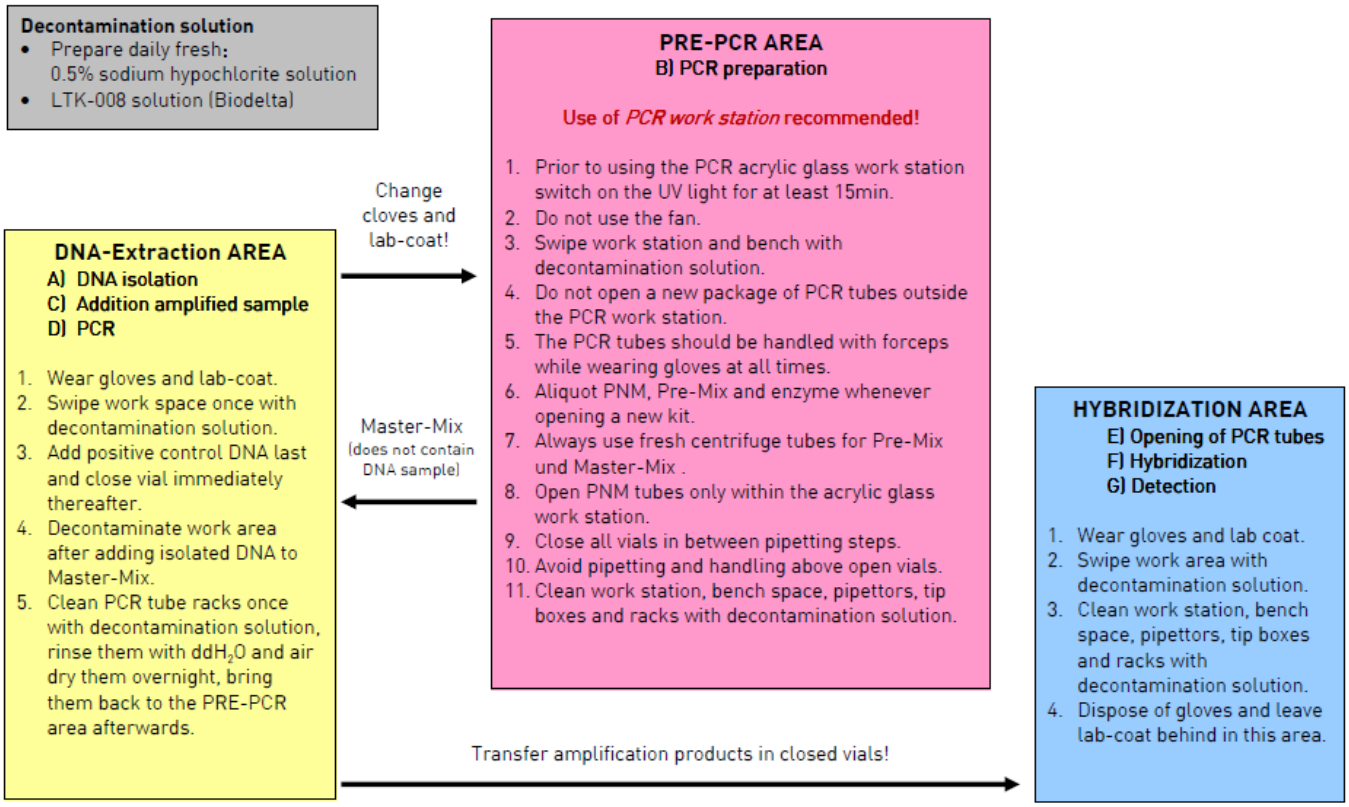

Figure 28: DNA Contamination safeguard procedure.

ORGANIZING SPECIMENS TO BE TESTED

Following staining and reading of the smears prepared from the NaOH-NALC decontaminated sputum specimens, the tubes that contain the AFB smear-positive specimens should be selected from all those tested and placed into a rack. Similarly, all the AFB smear-positive cultured isolates must also be selected and placed into an appropriately sized rack. An empty space should be left in the first hole of each of the racks so that, once an aliquot has been transferred from the first specimen tube into the tube for DNA extraction, the specimen tube can be moved across to the empty space. This process should be repeated as each specimen is transferred, so that there is always a space between the specimens that have been transferred and those specimens that have yet to be transferred. Although the extraction procedure

is similar for processed specimens and isolates, it is necessary to keep these two groups separated and well labeled since the PCR thermal cycler conditions are different for the different specimen types.

Before starting the DNA extraction procedure, worksheets listing the specimens must be created. First, the initial accession numbers for each of the specimens should be listed in numerical order (lowest number to highest number) on a worksheet. Each specimen can be then be allocated a simpler worksheet number that will be used for labeling the tubes and strips during the procedure. One or more worksheets should be created for the processed specimens that are to be tested, with other worksheets created for the isolates to be tested. The thermal cycler program to be used for each batch to be amplified should also be noted on the worksheet, e.g. processed specimens require 40 amplification cycles, while isolates only require 30 cycles (**Table 4**).

Once the worksheet(s) have been created, the worksheets and specimens must be double-checked to ensure that the specimen numbers on the worksheet correspond to the numbers of the specimens in the rack. This is an important step in preventing laboratory mix-ups.

When the worksheet and specimens are in order, a duplicate set of 1.5 ml screw-cap conical microcentrifuge tubes should be placed into an appropriately sized rack and labelled. It is recommended that the first set of tubes have the corresponding specimen worksheet number on their lids, and the second set of tubes have both the specimen worksheet number on their lids and the unique patient identifier number on their sides. These numbers must correspond to the original accession patient numbers, and to the worksheet number of that particular specimen in the batch.

In addition, the first set of tubes must be marked on one side with a small line or dot. This mark must face towards the center when loading the tubes into the microfuge. As the microfuge has a fixed-angle rotor of 45º, the pellet will be found on the opposite side of the mark following centrifugation. This enables the easy visualization of the pellet when removing the supernatant.

Table 4: Grouping of specimens when drawing up a worksheet.

Specimen Type	Specimen Source	40X cycles	30X cycles
Direct patient material from a clinical specimen	NaOH-NALC decontaminated smear-positive sputum.	✓	
Liquid media	Ziehl-Neelsen (ZN) positive MGIT cultures.		✓
	ZN positive MGIT cultures that are contaminated.		✓
	ZN positive tuberculosis (TB) nutrient broth.		✓
	ZN positive blood culture bottles (Myco-F-Lytic) that is inoculated onto 6.6 Middlebrook control slopes. These specimens will then be harvested from solid media, which does not contain heme-compounds that will inhibit the PCR.		✓
Solid Media	ZN positive Lowenstein-Jensen slopes.		✓
	ZN positive 6.6 Middlebrook control slopes inoculated from blood culture bottles (the colonies closest to the lid must be picked).		✓

It is recommended that specimens are grouped according to the capacity of the microfuge used. For example, if there are 93 specimens to be extracted and the microfuge can accommodate a maximum of 24 specimens, then the batch of 93 should be sub-divided into 3 groups of 24 and one of 21. Please note that when the centrifuge is run with an odd number of tubes it must always be balanced with water blanks.

PREPARATION OF SUPPLIES AND EQUIPMENT

Before starting the DNA extraction procedure, the specimen preparation checklist should be consulted to ensure that all the tools, materials and consumables for the procedure are to hand, and there is enough stock to complete the entire procedure. All material containing live bacilli (infectious material) should be manipulated inside the BSC during the specimen preparation procedure. The items required for this step should therefore be placed near the BSC, but not in the BSC until it is disinfected (as described below). The pipettes required for extraction are a P1000 and a P200, which should be colour-coded and numbered as pipettes 1 and 2 respectively. The pipettes or consumables that are used for DNA extraction should never be taken **from** other areas used in later stages of the LPA procedure. Similarly, supplies should never be taken **to** the Reagent Preparation Area from this area or any other area used in later stages of the LPA.

ISO-15189 requires that all molecular diagnostic laboratories use commercially prepared filtered pipette tips. It is also required that screw cap tubes are used when transferring suspensions of live bacilli. However, care must be taken when using screw cap tubes for both the live bacilli and the extracted DNA. If the tubes become mixed up, the operator may become exposed to live bacilli. It is therefore advisable to use two different coloured screw cap tubes, e.g. “colourless” for live bacilli and “green” for extracted DNA.

Before use, the BSC must be disinfected using appropriate decontamination agents. Appropriate personal protective equipment (PPE), as described in the Biosafety section of the Infrastructure chapter, must be worn when working in the BSL3 laboratory. The PPE required in the BSL3 laboratory is suitable for all TB diagnostic

purposes, including: specimen processing and decontamination; centrifugation; inoculation of specimens into MGIT growth media; preparation of smears from processed specimens and cultures; manipulation of cultured isolates; inoculation of cultured isolates onto solid or into liquid media for drug susceptibility testing (DST); biochemical testing to identify TB (Capilia); and DNA extraction of smear-positive samples (before heat-kill) for LPA testing. Double gloving during the extraction procedure is advised since it ensures that the operator will always have gloves on if the outer gloves become contaminated. In addition, the second pair of gloves must be removed before moving out of the safety cabinet and replaced before moving back into the BSC, between each step of the purification procedure.

After donning the PPE, the magnehelic pressure gauge in the exhaust duct must be checked for any drop in pressure across the filters. If the pressure has dropped below the optimal levels, the work must be moved to a functional BSC. If the BSC is functioning properly, the cabinet must then be decontaminated with freshly prepared hypochlorite (1% working solution from a 5% stock solution), aired for 20 minutes, and then wiped with 70% alcohol. It is important to ensure that all the surfaces on the inside are properly decontaminated before placing the required equipment and consumables into the cabinet.

Note: The label on the commercial hypochlorite bottle should always be checked to ensure that the concentration is between 4.5 and 5%. Never purchase commercial undiluted bleach if the concentration of the product is below 2%. The initial concentration of the undiluted hypochlorite is required to calculate the dilution needed to prepare a fresh solution of 1% hypochlorite (usually 200 ml of 5% bleach in 800 ml of water).

Items should be placed inside the cabinet according to the handedness of the extractor so that the process is unidirectional, i.e. working from a “clean side” to a “dirty side”. It is advised that the specimens be kept on one side of the BSC, with the vortex placed directly next to them. A tray lined with a hypochlorite-soaked paper towel should be placed in the middle of the BSC and used as the immediate working surface (details below). The filter tips and pipettes should be placed towards the back of the BSC, behind the tray. The waste containers should be placed on the opposite side of the BSC from the specimens, and the pre-labeled screw-cap tubes

should be placed on the immediate working surface tray (**Figure 29**). All items should be decontaminated by working from one side to the other (as described above), first with the 1% hypochlorite, then, working in the same direction, with the 70% alcohol. This will give all the items an equal amount of exposure to the hypochlorite. When placing items inside the BSC, care must be taken so that the grill is not blocked. All subsequent manipulations must be carried out at least 12 cm away from the front grill, on the working surface tray. Equipment that may generate an aerosol, such as the vortex, must be placed towards the back of the BSC.

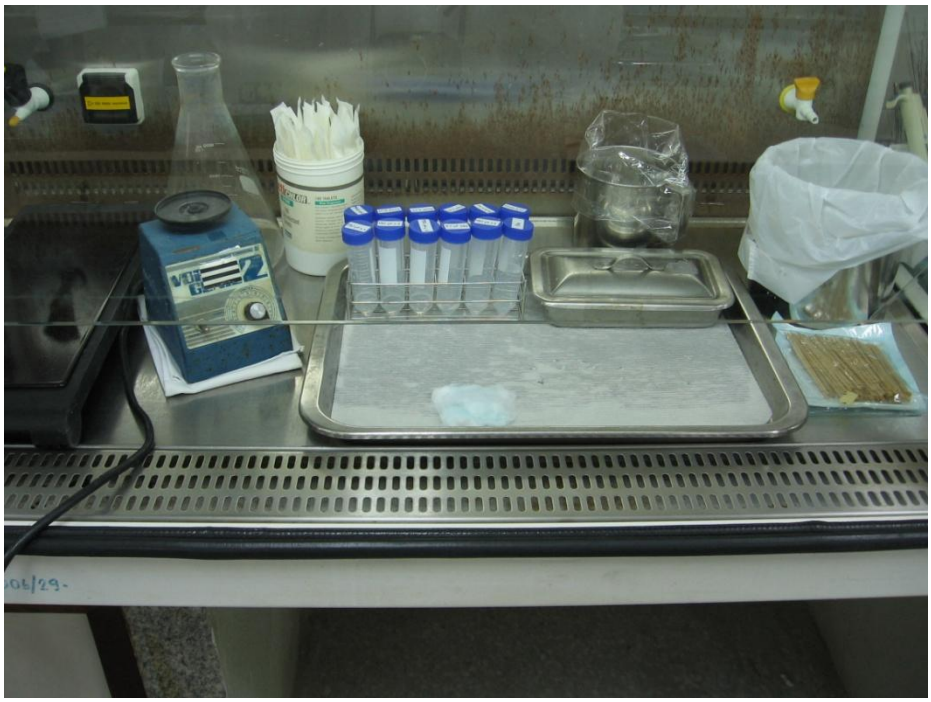

Figure 29: Placement of tools and consumables around the hypochlorite soaked towel on the immediate working surface tray in the BSC.

To prepare the immediate working surface, an area in the centre of the BSC should be covered with an absorbent paper towel and sprayed with 1% hypochlorite. If there happens to be a spill, this will prevent contamination of the BSC and the outside of specimen tubes. Ideally, the hypochlorite-soaked towel should be placed in a stainless steel tray, as shown in Figure 3, so that any spillage is contained within the tray. A discard container, lined with a plastic bag and sprayed with 1% hypochlorite, should be available for discarding the used tips (solid waste). The plastic bag should not be over-loaded with used tips, since a large number of tips may puncture the

bag. Liquid waste should be discarded into a 50 ml disposable tube that contains approximately 15 ml of 5% hypochlorite. By separating the solid and liquid waste, spillage of liquid waste is avoided, should the plastic bag be accidentally punctured.

EXTRACTION PROCEDURE

As mentioned above, it is advisable to leave a space (**Figure 30**) in the racks between the specimens that have been transferred into the 1.5 ml conical screw-cap tubes and those that still need to be transferred. It is very important that a different pipette tip is used for each specimen, and that the tip is discarded into the solid waste container immediately after. Liquid specimens should be well mixed (by vortexing) before the desired volume is transferred into a labelled conical 1.5 ml screw-top tube. The transfer volumes for different specimen types required for the extraction procedure are shown in **Table 5**.

It is essential that only one specimen tube is opened at a time during this part of the LPA, and several others. The lids on the other specimens must remain securely closed. Containers or centrifuge tubes should never be left open in the BSC.

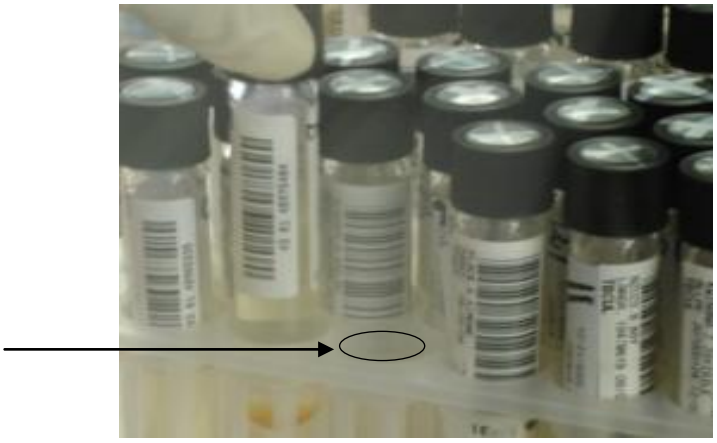

Figure 30: Example of the “space” that needs to be left to distinguish between specimens that already have been transferred into the 1.5 ml screw-cap conical tubes from those that still need to be transferred.

Table 5: Specific volumes required according to the different specimen types.

Specimen Type	Transfer Volume
Direct patient material	500 µl
Isolates from liquid media*	1,000 µl
Isolates from solid media**	300 µl

* Vortex for 1 minute to break up the clumped bacilli inside the MGIT growth medium. Vortex again once the isolates are transferred to ensure the complete break-up of flakes and clumps.

** Colonies picked from solid media and resuspended in molecular grade water should be vortexed for 1 minute to completely break up the colonies and clumps.

If clinical specimens are frozen after the NaOH-NALC decontamination stage, they must be thoroughly thawed before starting the extraction procedure. If, after the preparation of the smear and culture, the residual volume is not enough to perform the LPA, Tween® 80 saline can be added to the sample to make up the required 500 µl. The detergent Tween® 80 also helps eliminate clumping of the bacilli, thereby making the specimen more homogeneous.

Tubercle bacilli tend to clump together during growth, therefore suspensions of liquid media must be vortexed before transferring the 1000 µl of specimen into the 1.5 ml conical tube. Vortexing ensures that the liquid is homogeneous and that sufficient bacilli are transferred for DNA extraction. For solid media, representative colonies should be picked using disposable plastic loops and then re-suspended in 300 µl of molecular grade water and vortexed. Care must be taken to ensure that portions of the egg-based LJ medium are not scraped off along with the bacterial colonies, as some components can inhibit the PCR reaction.

Once the specimens have been organized in batches according to specimen type and aliquoted into the 1.5 ml conical tubes, the specimen-containing tubes (50 ml disposable tubes, MGIT cultures and solid media slopes) can be wiped with hypochlorite and removed from the BSC. The aliquoted 1.5 ml conical tubes should

also be wiped with hypochlorite before being placed in the aerosol-tight rotor of the 24-well capacity microfuge (with the dots on the tubes facing the centre). Always ensure that the rotor lid is securely in place before closing the machine and starting centrifugation. The bacterial suspensions must be centrifuged for 15 minutes at 10,000 x g in order to form a decent pellet. Although the pellet may not be visible after centrifugation, the marking on the tube will indicate where the pellet should be and the laboratorian must work carefully that area when removing the supernatant.

When the centrifuge has stopped, the lid should not be opened for 2 - 5 minutes to allow any aerosols created during centrifugation to settle. Ideally, the tubes should be removed from the aerosol-tight rotor inside the BSC, in case a tube broke during centrifugation. After opening the rotor, the tubes should be removed very carefully and placed in a rack on the working surface inside the BSC. The tubes should stand in the rack for 1 - 2 minutes before opening to allow aerosols to settle. During this time the next 24 specimens can be placed in the microfuge and centrifuged for 15 minutes. After the last batch of live specimens has been centrifuged, the rotor and its lid must be wiped with a bactericidal disinfectant, followed by 70% alcohol. This decontamination will ensure that no live bacilli could come into contact with the tubes during the final spin in the extraction procedure.

Following centrifugation, the supernatant fluid must be removed from the pelleted specimens. The cap should be removed from the first tube and the pellet or pellet mark located. Once identified, a P1000 pipette (1 ml pipette) fitted with a filter tip must be used to gently aspirate the supernatant from the tube without disturbing the pellet. The supernatant fluid must be discarded into the liquid waste container and the tip discarded into the solid waste bag. The supernatants of subsequent specimens are to be removed and discarded in the same fashion, with a new filter tip for each sample. Only one specimen tube should be opened at a time. If the pellet is disrupted, the tube needs to be centrifuged again.

Once the supernatants of the 24 specimens have been removed and discarded, each pellet should be resuspended in 100 μl of molecular grade water, using a P200 pipette and filter tip (**Figure 31**). The molecular grade water can be aliquoted from larger containers into McCartney bottles, and the smaller aliquots (working stock) made into 1.5 ml tubes, using sterile technique. Using the 1.5 ml tubes of molecular

grade water for the resuspension step will prevent the stock water from becoming contaminated from continual opening and closing of the container. The addition of 100 µl of molecular grade water to each sample should be followed by vigorous pipetting. To ensure that the pellet is thoroughly resuspended, the tube should also be vortexed for 1 - 2 minutes.

To ensure efficient heat-kill, the bacteria must be thoroughly suspended. The suspension may have a slightly milky appearance, however if clumps are visible these should be crushed using a filter tip and followed by repeated vortexing.

Figure 31: Resuspension of the bacterial pellet with 100 µl molecular grade water using a P200 pipette (200 µl pipette).

After the pellet has been resuspended, the specimens must be heat-killed at 95ºC for 20 minutes. The outside of the tubes should be wiped with a damp hypochlorite-soaked paper towel before being placed in the hot air oven (or heat block or water bath) for the heat-kill inactivation step. Provided the inactivation is carried out at the correct temperature, the heat both kills the bacilli and aids in the disruption of the cell wall, rendering the specimen non-infectious. The heat-kill instrument must always be pre-warmed and the temperature checked immediately before placing the specimens in it.

It is recommended that a small hot air oven be used for the heat inactivation step since the small condensation droplets that form in the caps of the screw-cap tubes may contain viable *Mycobacterium tuberculosis* bacilli. When using a heat block or water bath, these caps might not reach 95ºC during the 20 minute incubation. If

either of these instruments is used, the lids of the screw-cap tubes should be tapped to merge the droplets and then be quickly swung down by hand, forcing the droplet to fall back into the suspension. If a water bath was used for the heat kill step, the lids of the tubes must be tightly closed in order to prevent contamination of the water. The same floating rack can be used in the ultrasonic waterbath.

While the first batch of 24 specimens is heat inactivated, the second batch can be removed from the rotor, placed inside the BSC and the pellets resuspended, as described above.

An ultrasonic waterbath is used to further disrupt the bacilli. This must be filled with distilled water to the level recommended by the manufacturer and the water must then be degassed by sonication for 15 minutes before any specimens are placed into the filled tank. By exposing the distilled water to sonication, tiny bubbles of gas present in the water implode, ensuring that the specimens will be optimally targeted by the high pulse frequencies during sonication.

Following heat-kill, the first batch of 24 specimens should be placed in floating racks that will keep the tubes up-right in the ultrasonic waterbath. The solutions in the tubes should be below the surface of the water, thereby exposing the heat-killed bacilli to the high-pulse frequencies generated during sonication. The sonication must be carried out at 100% power for 15 minutes at room temperature. Afterwards, the floating rack containing the tubes should be removed from the ultrasonic waterbath and placed on paper towel to remove the water on the outside of the tubes. After all batches of tubes have been sonicated, the water must be discarded and the instrument cleaned with 1% hypochlorite followed by 70% alcohol.

Once the first batch of 24 specimens have been placed in the ultrasonic waterbath, the second batch that has been resuspended in 100 μl of molecular grade water can be placed into the hot air oven (heat block or water bath) for the heat inactivation stage.

Following sonication, the first batch of 24 specimens must be placed in the microfuge rotor. If possible, two centrifuges should be used: one for the first centrifugation of suspensions of live bacilli, and the second for separating the extracted DNA from the cell debris. However, if using the same microfuge rotor, this should have been wiped

with a disinfectant after the final centrifugation of the specimens containing live bacilli. As before, the marked lids of the tubes must be facing towards the centre of the microfuge. Even though the specimens are no longer viable, it is good laboratory practice to transfer the tubes from the floating rack to the rotor inside the BSC. After placing the closed rotor into the centrifuge, the sonicated specimens must be spun down at 13,000 x g for 5 minutes. If the pellet has not formed properly, or if it dislodges after the centrifugation, the specimen(s) can be centrifuged for up to 15 minutes.

After this centrifugation step, the supernatant (approximate 100 µl) must be carefully aspirated with a filter tip and transferred into the duplicate tube that was labelled prior to the extraction procedure. The supernatant now contains the DNA (to be used for the amplification procedure) and the pellet contains the cell debris created during the heat-kill and sonication steps. The pellet must not be disturbed when aspirating the supernatant because there may be inhibitors in the debris that could cause the subsequent amplification to fail. Screw-cap tubes are recommended for storing the extracted DNA, but either screw-cap or flip-top tubes can be used. The use of flip-top tubes can result in aerosol contamination if the tubes are snapped open quickly.

Once extracted, the DNA must be placed into a 4ºC refrigerator and used for amplification within 1 - 7 days. If the DNA is kept for too long at 4ºC it may degrade enough to prevent clear results. In addition, LPA is a rapid test that can provide results within 1 - 2 days: it makes very little sense to wait a week before completing the test. The leftover processed sputum specimens must be kept at - 20ºC, and the liquid and solid media isolates can be kept indefinitely at room temperature. If the amplification is not successful, these stored specimens can be used to re-extract the DNA.

Figure 32 is a diagrammatic representation of the entire DNA extraction procedure. Please note that, according to the figure, suspensions of cells from solid media can be heat-killed directly in the 300 µl of water, without the initial centrifugation step. However, it may be best for these suspensions to undergo the same initial centrifugation and resuspension procedure as the specimens from other sources. This will standardize and simplify the extraction procedure especially if several batches of different specimen types are being performed during the same day.

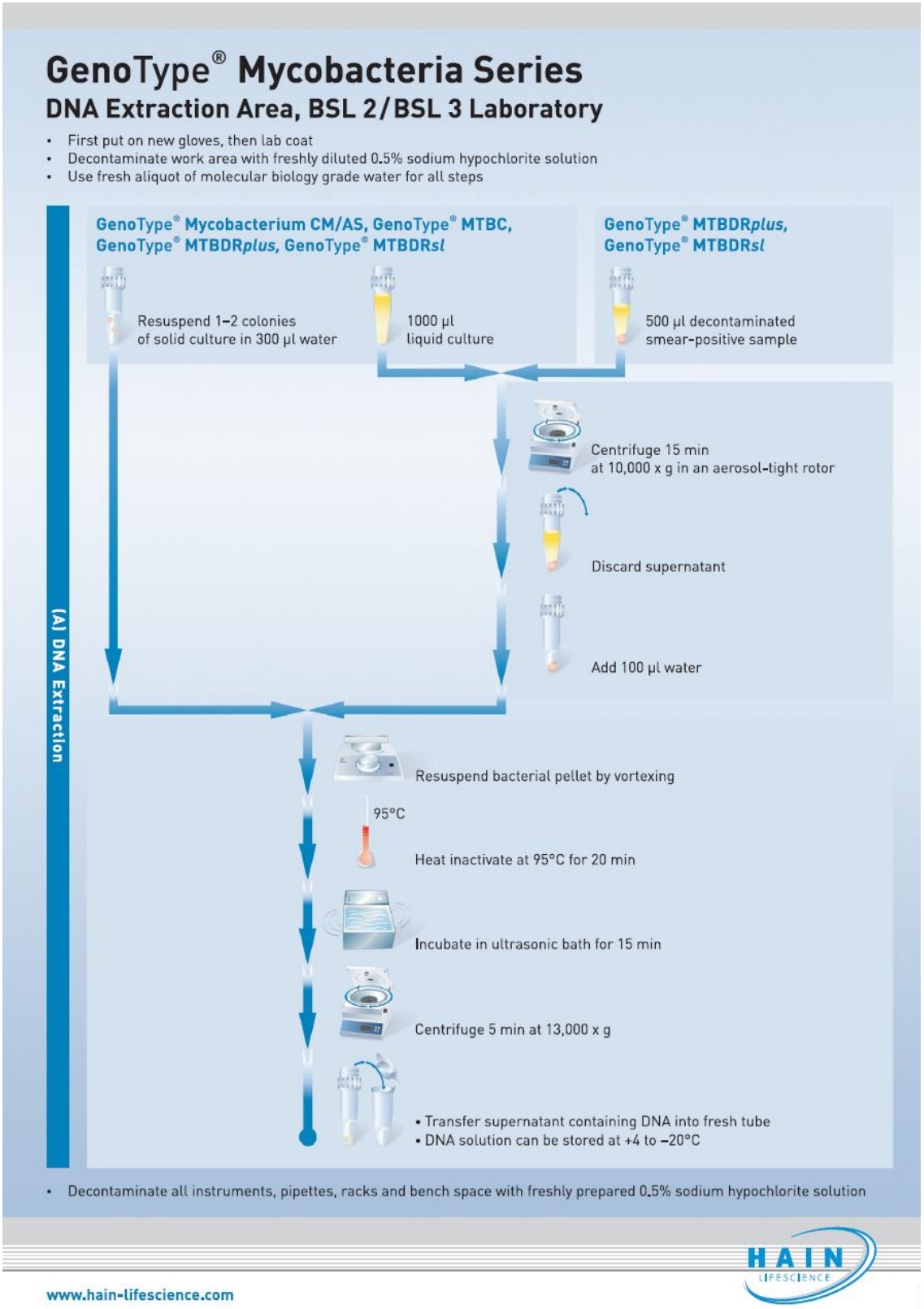

Figure 32: Work-flow diagram illustrating the different steps of the DNA extraction procedure.

REAGENT PREPARATION

It is critical that the area used for reagent preparation is kept clear of contamination. Clean paper towels (not a piece of cloth) must be used to decontaminate the surfaces and equipment. When performing the weekly area-specific decontamination procedures, this area must be cleaned first, and should not be entered after cleaning any of the other areas.

As described in the chapter on contamination control, shoe covers must be put on before entering the reagent preparation area. The following briefly describes the recommended method for putting on shoe covers:

- Stand in front of the door leading to the reagent preparation room
- Lift one leg and cover the shoe on that foot
- Keep the foot with the covered shoe in the air
- Open the door and place the foot with the covered shoe on the floor inside the room (The foot with the uncovered shoe will still be on the floor outside the room)
- Lift the leg outside the room and cover the shoe on that foot
- Keep the foot with the second covered shoe in the air
- Step inside the reagent preparation area with the second covered shoe and close the door.

Once inside the room, the powder-free gloves and dedicated laboratory coat should be worn, and the work area decontaminated with freshly prepared 1% hypochlorite (20 minutes) followed by 70% alcohol. The tools and equipment should also be wiped with 1% hypochlorite followed by 70% alcohol as described above for the supplies in the BSC. Kits containing the reagents should be removed from the refrigerator and freezer. In order to avoid degradation of the reagents, the master mix reagents must be kept in a tabletop cooler, especially the Hot Start *Thermis aquaticus* (*Taq*) DNA polymerase. Sterile molecular grade water should be aliquoted into smaller 1.5 ml tubes to avoid contamination of the stock bottles.

Before preparing each day's master mix, it is necessary to know how many specimens need to be tested. The number must include the specimens and the required controls plus one or two more to account for loss of volume on pipet tips. The master mix is made up of 5 components with a total volume of 45 µl for each PCR reaction (35 µl of the PNM mix, 5 µl of buffer, 2 µl of $MgCl_2$, 3 µl of H_2O and 0.2 µl of *Taq* polymerase). These volumes should be used in the calculations. It is recommended that a table showing master mix volumes (see **Table 6**) should be permanently placed on the bench top to assist in the calculations of the volumes needed for each day's master mix.

Table 6: PCR master mix reaction volume table

	POSSIBLE REACTION VOLUMES				
PRODUCT	**1X**	**2X**	**8X**	**24X**	**24X + 2X**
PNM MIX	35 µl	70 µl	280 µl	840 µl	910 µl
BUFFER	5 µl	10 µl	40 µl	120 µl	130 µl
$MgCl_2$	2 µl	4 µl	16 µl	48 µl	52 µl
H_2O	3 µl	6 µl	24 µl	72 µl	78 µl
TAQ	0.2 µl	0.4 µl	1.6 µl	4.8 µl	5.2 µl
TOTAL	**45 µl**	**90 µl**	**360 µl**	**1080 µl**	**1170 µl**

The molecular grade water and other reagent preparation solutions must be stored in the reagent preparation (DNA free) area. Three pipettes must be dedicated to this area and labeled number 3 (P1000), number 4 (P200) and number 5 (P10) (**Figure 33**).

Before starting the procedure, all reagents must be completely thawed. Once thawed, the calculated amounts of the different components can be aliquoted into a 1.5 ml screw cap with O-ring (the same type used for the initial specimen spin down). The master mix should be prepared in the order listed in Table 6: first the PNM Mix, then 10X buffer, $MgCl_2$, molecular grade water and Hot Start *Taq* polymerase. Visual inspection of the addition of the DNA polymerase to the master mix should be done to ensure that it has not been left out. Only one reagent vial should be opened at a time: when the desired volume has been removed, it should be closed immediately

and placed back in the tabletop mini cooler. After all components have been added to the vial and the lid screwed back on, the master mix can be gently vortexed and spun down in a mini-centrifuge for 10 - 15 seconds. The master mix must not be vortexed too vigorously, since this action may disrupt the integrity of the primers in the PNM. In the absence of a vortex and/or a mini-centrifuge, the tube can be gently inverted to homogenize the various components. Any residual fluid in the cap can be swung down by hand or pipetted out and transferred back into the master mix.

Figure 33: Area specific numbering of the pipettes. Shown here are the pipettes that are used in the reagent preparation area.

If more than one vial of master mix is being prepared, the same tip can be used when adding the same components into one or more vials. However, care must be taken not to immerse the tip in the master mix in one vial if it will be placed back into the reagent stock (e.g. to retrieve reagent for another vial). Most importantly, a new filter tip must be used when transferring each different reagent. A new tube of molecular grade water must be used each day, and discarded after the master mix preparation. After the master mix has been well mixed, the 45 µl required for each specimen should be transferred into the PCR tubes. **Figure 34** illustrates the reagent preparation procedure.

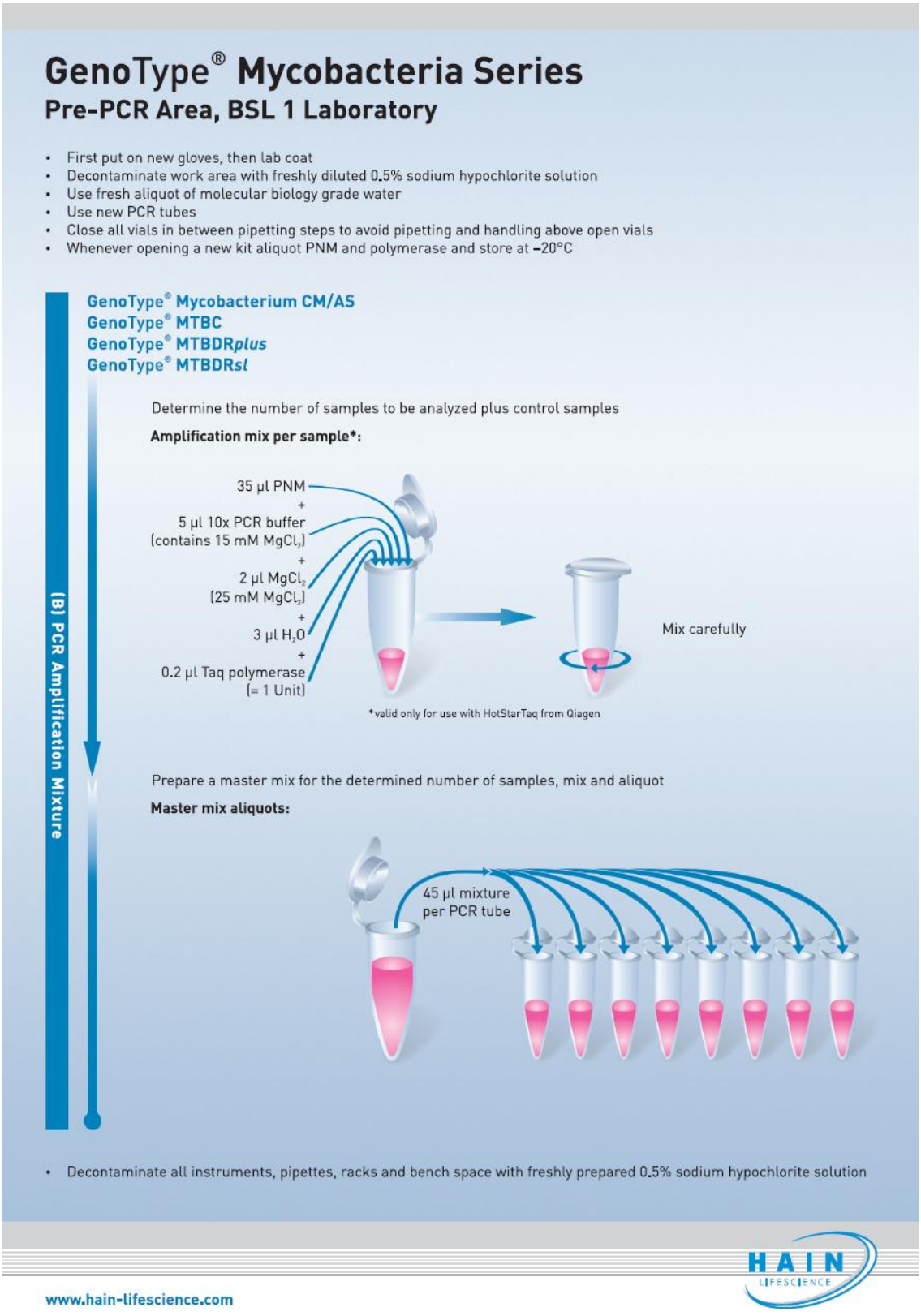

Figure 34: Work-flow diagram illustrating the different steps of the reagent preparation procedure.

The PCR tubes should be removed from the plastic bag by letting them drop out naturally, and not by hand, since reaching into the bag may contaminate other tubes. Only the sides of the tubes should be labeled since ink on the lid will come off when the heated lid of the thermal cycler presses down on it. The PCR tubes should be labeled according to the number of specimens plus controls, as determined by the worksheets that were created during the sample preparation. However, the worksheets cannot be brought into this area so the laboratorian must remember how many tubes need to be labeled. The numbered PCR tubes should be placed in numerical order in the PCR rack, opened, filled with 45 μl of the master mix and immediately closed. In order to test for contamination in the master mix, 5 μl of the molecular grade water used to prepare the mixture should be added to the 45 μl of master mix in one of the negative control tubes.

Once all tubes have been closed, the PCR tubes can be covered with the lid of the PCR rack and moved to the side of the bench or taken out of the reagent preparation area for template addition. If a glass beaker is used (ideally containing ice), aluminum foil must be placed over the beaker and the PCR tubes placed in small holes in the foil. The tube-pierced foil should be covered with another piece of foil and moved to the side of the bench or taken out of the reagent preparation area. If the procedure will not be completed until the next day, the PCR tubes should be stored at - 20^{o}C.

Once the PCR tubes containing the 45 μl of master mix are removed from the reagent preparation areas, the working surface, pipettes, tip racks and hood must be cleaned with the freshly prepared 1% hypochlorite (for 20 minutes), followed by 70% alcohol. If the work has been performed inside a PCR hood, the working area and equipment should also be treated with UV light. While the UV light is on in the PCR hood, a notice may be posted stating: “UV-irradiation in process – do not enter”. Before leaving this area, use a checklist to make a quick assessment of the stock of reagents and consumables that will be required when preparing the next batch of master mix. When leaving, the laboratory coat dedicated to this area must remain in the reagent preparation area and cover-shoes must be removed once outside the reagent preparation area.

Contamination can be prevented by labeling the tools, instruments and decontamination bottles and ensuring that these items are not used interchangeably between the different areas. As mentioned above, the pipettes required for the reagent preparation procedure are numbered as pipettes 3, 4 and 5 respectively (**Figure 33**). The labels demonstrate that the pipettes are dedicated to this specific area and re-enforces the uni-directional approach to the work. The pipettes must be decontaminated before calibration, and it is recommended that the pipettes are autoclaved on a regular basis. The use of a PCR hood with a fitted UV-lamp is recommended as another approach to control contamination. High throughput laboratories may consider using the 8-strip 0.2 ml tubes for PCR, however this requires very strict contamination prevention procedures. Single PCR tubes (0.2 ml) are therefore preferred.

TEMPLATE (DNA) ADDITION

If the facility has four areas dedicated to the LPA, the addition of the DNA can be done directly on a decontaminated bench in the template addition room (the use of a PCR hood is optional). If the facility has only three areas, a PCR hood must be used since the template addition will be taking place in the BSL2 or BSL3 laboratory where the DNA extraction was performed. The PCR hood should be dedicated to molecular work and used to prevent any exogenous DNA, contaminants or inhibitors from entering the reaction tubes. A BSC dedicated to molecular work can also be used for template addition, however it should not be the same BSC where the DNA extraction work was performed.

If the master mix was made on day one, before the DNA was extracted from the specimens, the PCR tubes should be removed from the - 20ºC freezer, taken to the template addition room or PCR hood in the BSL2/3 laboratory and thawed.

Cover-shoes, laboratory coats and powder-free gloves dedicated to this area must be worn, and strict procedures to prevent contamination followed (as described above for the reagent preparation area). After donning the required PPE, the bench or PCR hood and the equipment and consumables must be decontaminated with 1% hypochlorite followed by 70% alcohol. The bottles for these decontamination

solutions must be dedicated to this area, and specifically labeled to indicate that they are only for use in the template addition area. The pipette and tip-box containing the filter tips must be wiped with the two decontamination solutions, and the discard bag sprayed with 1% hypochlorite to kill off any contaminants from the discarded tips.

Due to its backbone of phosphate ions, DNA is negatively charged and may adhere to the walls of the plastic tubes. The 1.5 ml tubes must therefore be gently agitated to mix the suspensions of extracted DNA, immediately before adding the samples to the respective PCR tubes. Once adequately mixed, 5 μl of each sample should be added to the corresponding tube containing 45 μl of master mix and then mixed by gently pipetting up and down a few times. The PCR tube must be closed immediately after. Only one tube should be open at a time. The tip must be discarded into the waste bag and a new filter tip used for the addition of each sample.

Depending on the location of the thermal cycler, the closed PCR tubes can either be placed directly into the instrument, or covered before transporting them to another area. If the closed tubes are to be moved in a PCR rack, the lid of the rack must be closed to cover the tubes. If a beaker covered with aluminum foil is used, an additional piece of foil should be used to cover the tubes completely. Once covered, the DNA-containing PCR tubes can be taken to the area where the thermal cycler is located.

Before leaving the template addition area, the bench surface, pipette, tip box and other consumables used must be decontaminated. If a PCR hood was used, the items used should be decontaminated and then removed from the hood. Once the items have been removed, the hood or the bench surface must be decontaminated using the freshly prepared 1% hypochlorite for 20 minutes, followed by 70% alcohol.

Figure 35 illustrates the template addition procedure and subsequent loading of the thermal cycler where the DNA will be amplified.

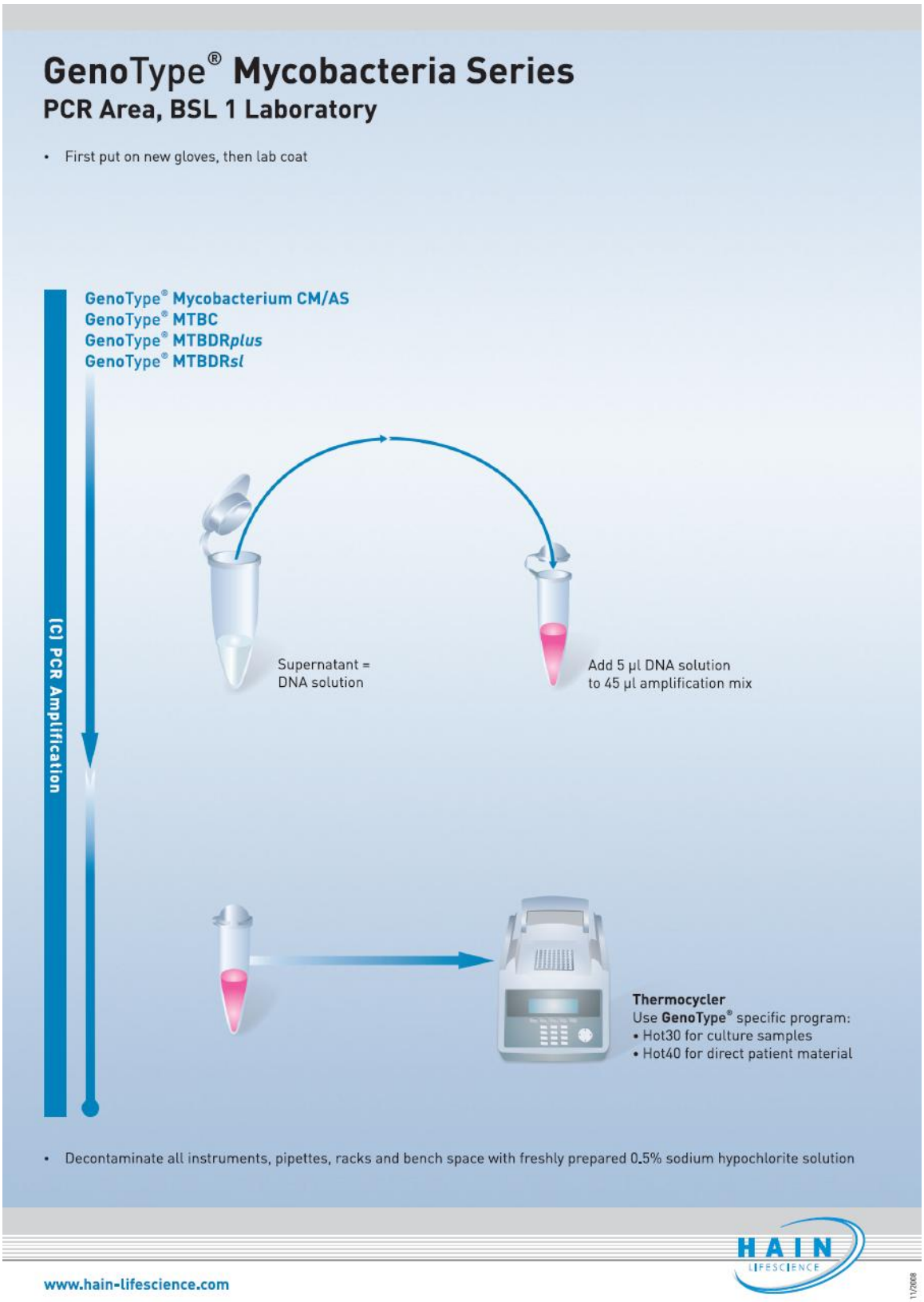

Figure 35: Work-flow diagram illustrating the steps of the template addition procedure. Here the DNA that was extracted in the BSL3 BSC is added to the aliquoted master mix that was prepared in the reagent preparation area.

AMPLIFICATION IN THE THERMAL CYCLER

The PCR tubes containing the DNA should have been previously labelled according to the type of specimen (either direct processed specimen or isolate from solid or liquid media). This information is essential to select the appropriate PCR conditions for the thermal cycler. Before the PCR tubes are placed into the thermal cycler, they should be mixed slightly and spun down for 5 - 10 seconds in a mini-centrifuge.

The following 40 cycle (10 + 30) thermal cycler program should be used if DNA from direct processed specimens is being amplified:

- Denaturation at 95°C for 15 minutes;
- **10** cycles of denaturation at 95°C for 30 seconds and elongation at 58°C for 120 seconds;
- An additional **30** cycles of denaturation at 95°C for 25 seconds, annealing at 53°C for 40 seconds, elongation at 70°C for 40 seconds;
- Final extension at 70°C for 8 minutes.

For DNA from cultured isolates, a 30 cycle (10 + 20) thermal cycler program is required:

- Denaturation at 95°C for 15 minutes;
- **10** cycles of denaturation at 95°C for 30 seconds and elongation at 58°C for 120 seconds;
- An additional **20** cycles of denaturation at 95°C for 25 seconds, annealing at 53°C for 40 seconds, elongation at 70°C for 40 seconds;
- Final extension at 70°C for 8 minutes.

Once amplified, the PCR tubes must be carefully removed from the thermal cycler, and placed into the PCR rack or the foil-covered beaker, and covered appropriately. Care should be taken to prevent the opening of any tubes, since amplicons from

open tubes may be aerosolized and contaminate the machine and surrounding area. Before taking the PCR tubes to the hybridization /detection area, the thermal cyclers must be decontaminated with a solution that will not harm the surface of the instrument (such as Contrad®), followed with 70% alcohol. The PCR tubes containing the amplicons must never be taken back into the reagent preparation or specimen preparation areas: they must be taken to the hybridization and detection area only.

Hybridization and detection procedures should be carried out immediately after the amplification procedure. If it is necessary to postpone the hybridization until the following day, the amplicons must be stored in a dedicated area at 4ºC. The amplicons may be stored for a maximum of one week. However, the intended use of LPA is to provide results with a rapid turn-around time, so the final steps should be performed as soon as possible after amplification.

After the hybridization and detection processes have been completed and the results found to be satisfactory, the amplicons should be placed into a biohazard bag and discarded in a dedicated sealable waste bin. The bins must never be filled to overflowing, and the contents of the bins must eventually be removed and destroyed by autoclaving or incineration. The beaker that was used to store the PCR tubes containing amplicons must be autoclaved and the PCR rack must be decontaminated in 1% hypochlorite and wiped clean with 70% alcohol before re-use.

HYBRIDIZATION AND DETECTION

The hybridization procedure should be performed directly after the amplicons are taken out of the thermal cycler, however the amplicons may be kept at 4ºC over night and hybridization performed the following day.

Before starting the final procedure, ensure that all the materials and tools required to perform the hybridization procedure are readily available. The checklist that was created specifically for this area should be used to determine which items are required. Powder-free gloves and laboratory coats dedicated to this area must be used. The work area should be organized so that one works from the amplicons to

the tray, from the tray to the TwinCubator®, from the TwinCubator® to the score sheets, and from the score sheets to the worksheet where the interpreted results will be recorded. In addition, there must be a “clean” area on the bench where the strip preparation can take place.

Before starting the first step, the work area must be decontaminated with freshly diluted 1% hypochlorite for 20 minutes, followed by 70% alcohol. Once decontaminated, the work area can be covered with clean paper towels. A discard container lined with a plastic bag and containing a small amount of freshly diluted 1% hypochlorite should be assembled. **Figure 36** gives an illustrated over-view of the hybridization process using the manually operated TwinCubator®.

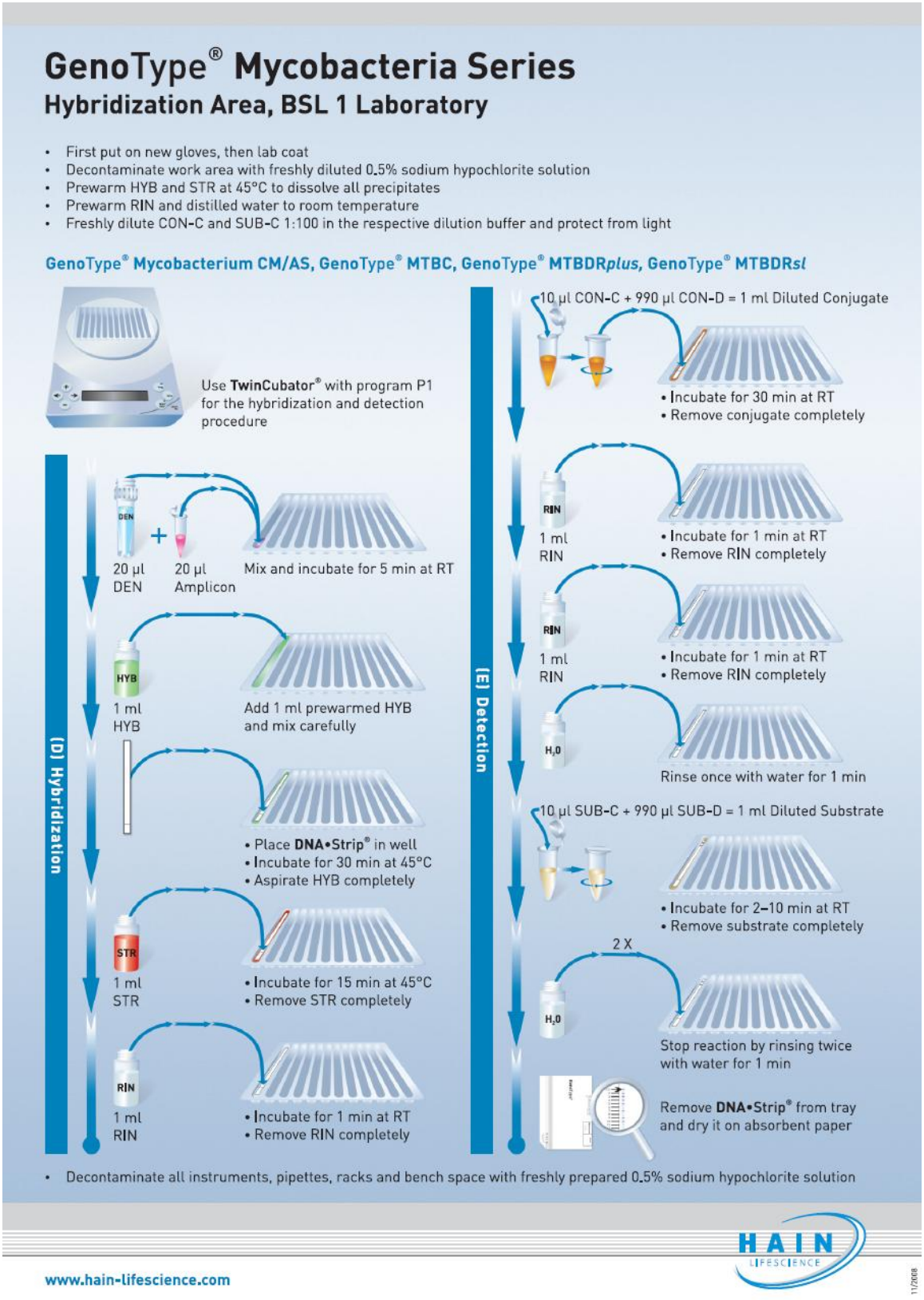

Figure 36: Work-flow diagram illustrating the different steps of the Hybridization and Detection procedure using the Twincubator.

Preparation of the strips

It is recommended that a dedicated bench or section in the hybridization area be used to number the new strips. This area should not be in close proximity to where the denaturation will be performed. Clean tweezers or forceps dedicated to the handling of the new strips should be used. When removing the new strips from the tube, the tube should be tipped slightly and gently tapped so that the strips begin to slide out. The strips should be grasped on the shorter side of the blue line, removed from the tubes one at a time, and gently placed on a clean sheet of paper. To number the strips, the tweezer's point should be placed on top of the blue line to stabilize the strip and the strip numbered using waterproof ink. The numbering on the strip must correspond to the numerical order of the specimens on the worksheet.

Denaturation of DNA

In order for the hybridization to take place, the double stranded DNA amplicons must be denatured into single strands, thereby allowing for the formation of amplicon-probe hybrids. To begin this procedure, a new or clean TwinCubator® tray should be removed from the kit and placed on the bench top. This same tray will be used for the next three steps: denaturation, hybridization and detection. Using the same filter tip, 20 µl of the denaturing buffer (DEN) should be dispensed into the corner of each well of the tray. By carefully opening the PCR tubes one at a time and using a new tip for each, 20 µl of the DNA amplicons should be added to the denaturing buffer (DEN) and gently mixed by pipetting up and down at least 5 times. It is critical that the solutions be mixed very gently: splashing would contaminate adjacent well(s) and excess force might puncture the tray. If the tray is unknowingly punctured, buffers and solutions might leak into the hybridization platform, resulting in amplicon contamination. Once all the amplicons have been added to the denaturing buffer and mixed, the denaturation should proceed for 5 minutes at room temperature.

Operation of the TwinCubator®

The TwinCubator® must be set on the program that is specific for the LPA (see the user manual and the Equipment chapter). **Figure 37** shows the TwinCubator® control panel. To power-up the TwinCubator®, the start button (A) must be pressed. Once the start button has been pressed, the back arrow (B) should be pressed immediately, in order to pause the machine. Halting the timed steps in the procedure will allow the TwinCubator® platform to warm to 45ºC, the temperature required to begin the hybridization procedure.

Figure 37: The TwinCubator® control panel. This instrument allows for manual hybridization of the amplicons to the probes on the strip. The Start button (**A**) is used to power-up the machine, and the Reverse arrow button (**B**) is used to pause the machine immediately after the start button has been pressed. Once the machine has reached 45ºC, the Forward arrow button (**C**) must be pressed to begin the first step in the hybridization process. Button **C** will be used throughout the procedure to pause and begin all the steps, including pauses needed for aspirating and dispensing solutions and buffers. Refer to the user manual and Chapter 2: Infrastructure Requirements for more information.

Before starting the incubations, the inside of the glass lid of the TwinCubator® may be covered with aluminum foil. Condensation will likely be present on the foil after each incubation step and should be wiped off between steps. Each of the following

incubation, washing and rinsing steps should be carried out in a volume of 1 ml on a shaking platform - either the TwinCubator® or the automated hybridizer. The same tray is used throughout the procedure and the previous buffers or solutions must be aspirated before the new ones are added. At the end of each incubation step, an alarm will sound. The forward arrow (C) should then be pressed to pause the run and the current buffer/solution removed. The next buffer/solution should then be added and the forward arrow (C) pressed to begin the next step. The machine is programmed to time each step correctly, as well as indicate which buffer is required for the subsequent steps.

After the 5-minute denaturation step, 1 ml of the pre-warmed hybridization buffer (HYB) should be added to the wells using a P1000 pipette. By adding the buffer to the opposite end of the well from the denaturation solution, the same filter tip can be used for all wells. The tray should then be carefully tilted back and forth so that the purple denaturation solution and green hybridization buffer are well mixed. The tray can now be placed on the TwinCubator® and the strips added to each well. The labeled strips should be grasped with the tweezers on the numbered end, placed halfway inside the well with the numbers facing upwards, and carefully released so they slide under the liquid. Care must be taken to ensure that the tweezers or forceps do not come into contact with the solution in the wells. For successful hybridization, the strips must be completely covered by the liquid. If a strip is turned over or incompletely covered, it must be carefully re-positioned using a clean filter tip.

When the machine reaches 45ºC, the cover should be closed over the tray, and the forward arrow button (C) pressed to start the 30 minute hybridization step. **Figure 38** illustrates the denaturation and pre-hybridization steps.

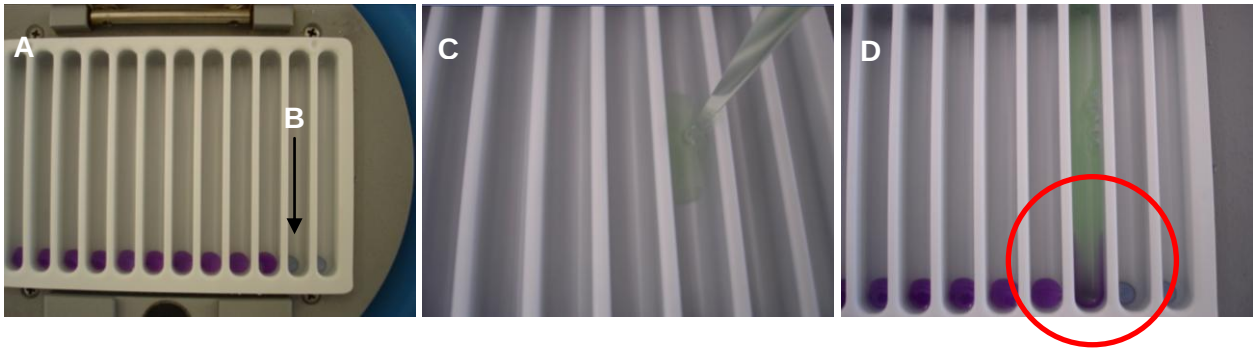

Figure 38: Initial pre-hybridization procedures. (**A**) The blue denaturing buffer (DEN) is loaded into a corner of the tray using the same tip. After the denaturing buffer has been added, the purple coloured DNA product is added to the DEN. The colour change is a good indicator of where the present specimen was added and (**B**) where to load the next specimen. A new filter tip must be used for each specimen. After the 5-minute denaturing time, pre-warmed hybridization buffer (HYB) must be added at the top part of the well, so the amplicons do not come into contact with the filter tip (**C**). Immediately after adding the HYB to all the wells, mix the solutions by gently tilting the tray to ensure a homogeneous distribution of the denatured amplicons in each well before adding the numbered strips.

An alarm will indicate when 30 minutes has elapsed. The forward arrow (C) should then be pressed to pause the run. The glass panel lid should be opened and the condensate that formed during the incubation wiped off. The HYB buffer should be aspirated from each well using either a Pasteur pipette or the P1000 pipette with a filter tip. The Pasteur pipette or filter tip, and the spent buffer, should be discarded into a plastic bag or 50 ml tube containing a small amount of 5% hypochlorite. The liquid should not be decanted into a sink or container at this stage in the procedure, as the liquid contains unbound amplicons that may contaminate the work area.

Stringent wash

After the green hybridization buffer has been aspirated, 1 ml of the pre-heated red stringent wash buffer (STR) must be dispensed into the tray, either by individual pipetting or using a multi-channel pipette. When a multi-channel pipette is used, the filter tips must be kept directly above the wells so that the strips do not come into contact with the tips. If a single channel P1000 pipette is used, a clean filter tip must be used for each well. Once all the strip-containing wells have been filled, the glass

panel lid should be closed and the forward arrow (C) pressed to start the stringent wash step.

During this 15 minute incubation at 45^{o}C, any non-specifically bound amplicons will be removed from the probes on the strip. When the alarm sounds, the forward arrow button must be pressed to pause the run. The glass panel lid should be opened, the condensate wiped from the lid or the aluminum foil, and the STR buffer aspirated and disposed of with a Pasteur pipette or a P1000 pipette into 5% hypochlorite. As described previously for the HYB buffer, the used STR wash buffers must not be removed from the tray by tipping it over and decanting. Only after the first rinse can the liquid from all the wells be decanted together into a plastic container containing 1% hypochlorite.

Rinsing off the stringent wash solution

To wash off excess STR buffer, 1 ml of Rinse (RIN) is added to the strip-containing wells. The glass panel lid must be closed and the forward arrow button pressed to begin a 1 minute wash step. When the alarm goes off, the right arrow button should be pressed to pause the run. The lid should be opened and wiped clean of any condensate. The RIN solution can be aspirated by transfer pipette, P1000 pipette or simply decanted into a container with 1% hypochlorite. The tray must be carefully removed from the hybridization platform so that no splashing occurs. From this step onwards, there is no need to use different filter tips for the addition of solutions.

Conjugation

After the RIN solution has been discarded, 1 ml of the previously prepared Conjugate solution should be dispensed into each well. As stated above, there is no need to change tips. The glass panel lid must be closed and the forward arrow button pressed to begin the 30 minute incubation on the TwinCubator®. The temperature reading on the TwinCubator® should by now be almost down to room

temperature. When the alarm goes off, the forward arrow button should be pressed to pause the run. The lid should be opened and wiped clean of condensate, and the liquid in the tray decanted into a container with 1% hypochlorite.

Removal of the unbound conjugant

The strips must then be washed twice with 1 ml of Rinse solution (RIN) to wash off the excess CON solution. Once the RIN has been dispensed into the strip-containing wells, the lid closed and the forward arrow button pressed, a 1 minute rinse in the TwinCubator® will begin. When the alarm sounds, press right arrow button to pause, decant the RIN solution and add the second 1 ml of RIN into the strip-containing wells. Repeat the 1 minute wash in the TwinCubator®, and decant the second RIN wash liquid.

Final rinse with distilled water

To wash off the RIN solution, 1 ml of sterile distilled water (dH_2O) should be added to each strip-containing well, and a 1 minute wash performed on the TwinCubator®. When this wash is finished, decant the distilled water completely.

Colour development

After the distilled water rinse has been discarded, 1 ml of the Substrate solution (prepared earlier) should be dispensed into each well. Once again there is no need to change tips. Close the glass panel and press the forward arrow button and allow the strips to incubate for 5 - 15 minutes on the TwinCubator®. When the alarm goes off, press the forward arrow button to pause the run. Open the glass panel and aspirate or decant the Substrate solution.

It is important to note that the reaction time of the colour development may vary. If the intensity of the banding pattern is strong after only a few minutes, the reaction should be stopped. If the intensity is still weak after 15 minutes, the strips should be allowed to incubate longer. However, care must be taken not to allow too long an incubation as artifacts may develop, which may lead to incorrect interpretation of the result(s).

Stopping colour development by removal of substrate

In order to stop the colour development, the substrate must be removed, and the strips washed twice with sterile distilled water (dH_2O). Dispense 1 ml of dH_2O into the strip-containing wells, close the glass panel lid and press the forward arrow button for a 1 minute wash. When the alarm goes off, press the right arrow to pause the run, decant or aspirate the wash and add the second 1 ml of dH_2O into the strip-containing wells. Close the glass panel and press the forward arrow button and allow washing for 1 minute. When the alarm goes off, press the right arrow to end the run, and aspirate or decant the wash.

It is critically important to remove the excess conjugate and substrate solutions. Failure to do so will likely result in increased background staining, making the strip very difficult to interpret.

Transfer of hybridized strips

The developed strips must now be dried and transferred to the GenoType® MTBDR*plus* scoresheet. A pair of clean or disposable tweezers, dedicated to removing hybridized strips, should be used to remove the strips from the TwinCubator® tray and place them onto absorbent paper. It is recommended that the strip be partially dried and then placed onto the scoresheet. The water will act as a “fixative” and prevent the strip from “jumping” towards the cellophane tape that is used to fix the strips onto the scoresheet. Once taped and completely dried on the

scoresheet, the strips are ready for interpretation. The Interpretation and Reporting chapter should now be consulted to determine the results of the LPA.

Alternatively, the GenoScan® automated scanner can be used. The strips must remain in the tray and dry completely before they can be scanned. Any wetness on the strip(s) will deflect the beams of the scanner and either prevent the scanner from recognizing any of the strips in the tray, or result in badly scanned strip(s).

Decontamination of the hybridization area

The hybridization area is considered the “dirtiest” area, since it poses the greatest risk of contamination to the other dedicated areas. It is therefore essential that the supplies, instruments and decontamination bottles be labeled accordingly. These should never be used in any of the other areas, once they have been installed and used in the hybridization area. It is also essential that the area be thoroughly decontaminated after the work is completed. Dedicated laboratory coats, worn for the hybridization and detection procedures, must stay in this area. Gloves must always be changed if they become contaminated with amplicons. The two different tweezers or forceps, dedicated to either handling of the unhybridized strips or the post-hybridization transfer of strips to the scoresheet, must be clearly labeled and decontaminated after use.

Once the hybridized strips have been interpreted and the results determined, the PCR tubes (containing the remaining amplicons) must be discarded into a plastic bag, sealed and then discarded into a dedicated sealable plastic drum or biomedical waste box, which should be kept far away from the areas used for reagent and strip preparation. The pipettes required in the hybridization area are a P1000 and a P200. These can be numbered as pipettes 7 and 8 respectively (**Figure 39**).

Figure 39: Area specific numbering of the pipettes. Shown here are the pipettes that are used in the hybridization area.

The work area, pipettes, racks and instruments must be cleaned with freshly diluted 1% hypochlorite for 20 minutes, followed by 70% alcohol. The tray should be cleaned with Contrad® and/or 70% alcohol, followed by a final wash with sterile distilled water. Cleaning the tray with hypochlorite may leave residual bleach in the tray, which would destroy the DNA amplicons during the next use. If hypochlorite decontamination is performed, it is therefore recommended that sterile distilled water be used to thoroughly rinse the strip trays and tweezers afterwards. These supplies can also be subjected to UV-irradiation to eliminate any residual amplicons. Once decontaminated, the dedicated trays and tweezers or forceps must be placed in a sealed plastic bag.

It is recommended that this area have dedicated spray flasks or beakers of 1% hypochlorite and 70% alcohol for the cleaning of surfaces, instruments and tools. The hybridization area should also have dedicated tip boxes and a plastic container filled with 1% bleach for decontaminating the used PCR and 1.5 ml tube racks. The two tube racks are the only items that can be taken back to the reagent area (for the PCR racks) and specimen preparation area (for the 1.5 ml tube racks). Nothing else must ever be returned to these two areas.

Preparation and use of the GT-Blot automated hybridizer

The same pre-heating procedures are to be followed when using the GT-Blot 48 automated hybridizer. The two pre-heated ready-to-use buffers, the prepared conjugate and substrate solutions, the rinse aid and the de-ionized water must be placed into the colour-coded slots of the machine. The colour-coded suction heads must then be placed into the corresponding solutions.

The number of wells can be selected, up to a maximum of 48 per run. When started, the machine pre-heats the two hybridization buffers at 45ºC for 15 minutes. Once the pre-heating is completed, the tray with the denatured amplicons and numbered strips must be placed into the machine. The HYB buffer will therefore be added after the strips have been loaded into the tray. It is essential that the denaturation solution in the well does not come into contact with the strip before the hybridization buffer is added. The machine will automatically aspirate and dispense the subsequent hybridization buffers and solutions.

Once the hybridization is complete, the strips can either be air-dried or dried using the heat transfer between the hybridization tray and the hybridization platform. The strips can then be placed onto the scoresheets and fixed with cellophane tape. As described above, it is recommended that this be carried out while the strips are only partially dry. If the GenoScan® is used, the fully dried strips can be scanned into the Blotrix® software, which will generate an automated read-out of the banding patterns. However, the final result on the read-out must be verified by eye, since the machine may occasionally misread or misnumber the banding patterns on a strip.

GENERAL COMMENTS

Carry- & cross-over contamination

If there are mixed susceptible/resistant-banding patterns on a single strip it might be due to cross- or carry-over contamination. However, hetero-resistant strains may produce a mixed banding pattern on a strip. It is recommended that the strips flanking the strip in question be investigated to see if they have banding patterns with similar mutations. If the flanking strips are both susceptible or the mutation bands differ from those on the strip with an unusual pattern, the result indicates the presence of a hetero-resistant strain. The Interpretation and Reporting chapter should be consulted for more details on hetero-resistance.

Workflow

It is recommended that the specimen preparation step be performed on a separate day, or earlier in the day, from the rest of the procedure. The extracted DNA can be stored in a 4ºC fridge or frozen at - 20ºC until required. If the DNA is extracted on day one, then the reagent preparation, amplification and subsequent hybridization can be done on day two. This re-enforces a unidirectional workflow within the laboratory. There should be a BSC that is exclusively used for DNA extraction, and a PCR hood that is exclusively used for DNA template addition to the tubes containing the PCR master mix. Reagent preparation should always be done at the start of the work day and can be done on day one, provided that it is done before starting the DNA extraction. If the reagent preparation and DNA extraction are both performed on day one, the tubes containing the PCR master mix can be removed from the - 20ºC freezer early on day two and the previously extracted DNA added and amplified. This would allow the procedure to be completed earlier in the day, improving the overall turn-around time.

Positive and negative controls

It is recommended that the Quality Control samples be placed in the last three PCR tubes in the amplification procedure and the last three strips of the run during the hybridization procedure. The third to last control must be the positive control; the second to last, the extraction negative control; and the last one must be the negative master mix control.

The H_{37}Rv laboratory strain should be added as the positive control during the DNA extraction procedure. If an H_{37}Rv laboratory strain is unavailable, a susceptible strain of known genotype can be heat-killed, sonicated and used as the positive control. If there are no bands on the positive control strip, it can be concluded that the amplification procedure failed. If this control exhibits a mixed pattern, this indicates cross- or carry-over contamination.

The negative controls test for contamination of the molecular grade water stock used to resuspend the pellet before the heat kill (specimen preparation area), and the molecular grade water that was used to bring the master mix solution to volume (reagent preparation area). The negative control strips must only have the CC and AC bands, and no other bands should be visible. The presence of the TUB band is an indication of contamination. Positive results with other bands of these controls may indicate cross- or carry-over contamination.

Contamination accountability

It is very important that the laboratorian(s) who carry out the various steps involved in the LPA sign the relevant worksheet. If there is contamination or a problem with the assay, this can then be traced to a specific area and to the person who performed the work. A record must therefore be kept for each batch, indicating which laboratorian:

- Decontaminated the direct patient material using the NaOH-NALC method;

- Read the smears;
- Extracted the DNA from the specimens;
- Prepared the master mix;
- Added the template DNA;
- Carried out the hybridization of the amplicons.

Chapter 5:
NEW DEVELOPMENTS

With the introduction of newer technologies aimed at diagnosing TB and/or its associated mono/MDR-drug resistance susceptibility pattern directly from sputum/decontaminated sputum, a new version of the MTBDR*plus*® LPA has been developed. The MTBDR*plus*® version 2 (v.2) is aimed for use on pulmonary and extra-pulmonary direct decontaminated smear positive and smear negative sputums, which will be of great benefit to patients who are co-infected with HIV/AIDS.

MTBDR*plus*®: Version 1 vs. Version 2

There are three major differences between the methodology used for version 1 and version 2 of the MTBDRplus® LPA, and these differences are associated with the extraction, master mix preparation and template addition areas.

DNA extraction

The first difference is that the need for the present manual extraction method by means of sonification, as outlined in chapter 4, **must** now be replaced with the manual GenoLyse® chemical extraction method.

Genolyse®

The GenoLyse® method allows for the manual extraction of genomic bacterial from direct patient material. Upon receipt the GenoLyse® kit components are ready-to-use (**Figure 40**) and DNA can be extracted within 20 minutes. The method of

extraction is almost similar to the one described in Chapter 4 (**Figure 41**). The difference using this method is that after the first centrifugation step, the pellet is resuspended into 100ul of the yellow lysis buffer (A-LYS) by physically dislodging the pellet my means of consecutive up and down pipetting with the lysis buffer and/or vortexing. The combined effort will result in a higher yield of DNA. Once resuspended, the specimen is to be incubated/heat-killed for 5 min at 95°C in either a hot-air oven, water bath or dry-block. Once the 5 minutes has elapsed, the lysate must be spun down briefly, after which an equal amount (100ul) of the neutralization buffer (A-NB) must be added and vortexed for 5 seconds. After vortexing, the neutralized lysate must be spun down for 5 minutes at top speed. Finally, 100ul of the supernatant is to be aliquoted into a clean tube, where 5ul of the extract is to be used in the template addition (amplification) area.

Figure 40: The GenoLyse® DNA extraction kit.

Always ensure that the residual specimen is thoroughly vortexed prior to aliquoting it for the extraction procedure to ensure homogeneity of the specimen, since failure to do so might influence the sensitivity of the test. In addition, it is good laboratory practice to aliquot a working stock of the GenoLyse® buffers into smaller 1.5 ml tubes, based on the amount of specimens to be extracted, in order to prevent contamination of the stock solution.

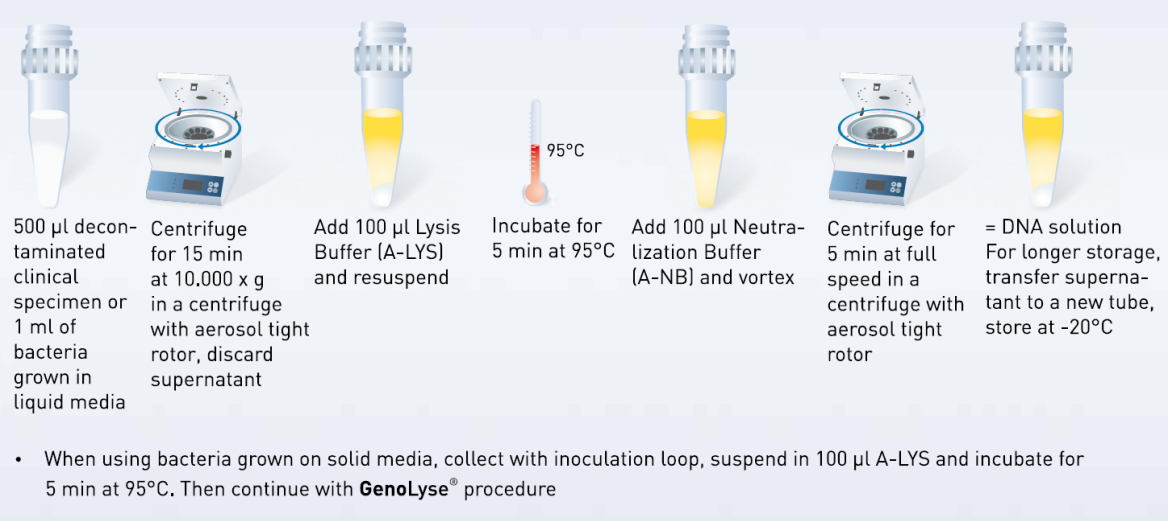

Figure 41: DNA extraction with the GenoLyse® method

The most important aspect however is that by using the GenoLyse® method there is a considerable reduction in overall cost and time, since there is a reduction in usage of consumables (less tips used), labour (faster method) and equipment (the use of an ultrasonic water bath is no longer required).

Master Mix Preparation

The second and most important difference is both the decreased possibility of introducing contamination into the pre-amplification area due to decreased liquid handling during the master mix preparation where only two pipetting steps are required to obtain a fully reactive master mix resulting in lesser time spent in this area, as well as the cost-reducing benefit of not having to purchase additional DNA polymerase. In version 2, the master mix consists only of Amplification mix A (AM-A) that contains the 10X buffer, nucleotides and DNA polymerase. Amplification mix B (AM-B) contains the MgCl2, the biotinylated primers and dye. For one PCR reaction, 10ul of reagent A must be mixed with 35ul of reagent B which results in the normal volume of 45ul, exactly the same as for version 1, but less pipetting steps are involved (**Figure 42**).

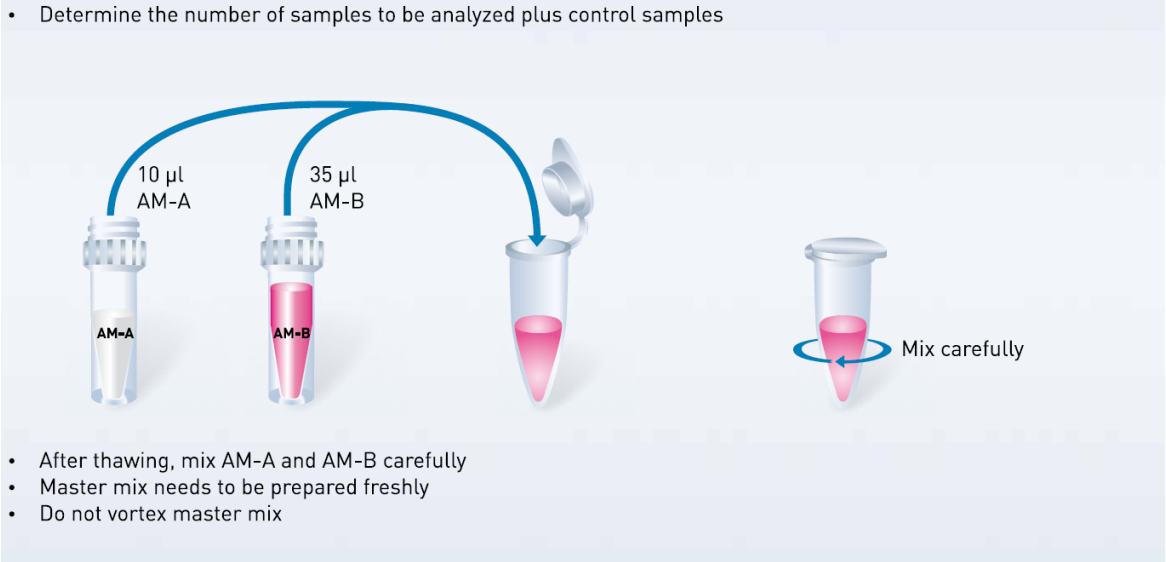

Figure 42: Preparation of master mix using the MTBDRplus® Version 2 kit

Based on the amount of specimens in the batch from which DNA is to be extracted, the corresponding amount required for the aliquoted master mix is obtained by multiplying the batch size with the respective reagent volume, plus one or two extra for correction of volume loss during the aliquoting of the master mix into the PCR tubes. Amplification mixes A and B must be mixed gently by inverting the tube(s) a few times. Never attempt to vortex the master mix.

Amplification

The third difference is that of an increase in the amount of PCR cycles for direct decontaminated smear positive and smears negative sputums where 50 PCR cycles are required instead of the 40 PCR cycles used in version 1, which will take a little bit longer. There is no difference in the amount of cycles used for cultured specimens (30 cycles as per usual).

Hybridization and Interpretation of Results

The reverse-hybridization step and the interpretation of results, remains exactly the same as that for version 1.

Infrastructure Requirements

All chemicals/reagents and consumables required for pre-amplification must be separated from the area where cultivation of bacteria, DNA addition and amplification in a thermal cycler takes place. Please refer to Chapter 2.

STORAGE OF THE MTBDR*plus*® Ver. 2 KIT COMPONENTS UPON ARRIVAL

Version 2 consists of the GenoLyse® extraction buffers, as well as kit component 1 of 2 and kit component 2 of 2. Component 1 of 2 consists of the membrane strips which are coated with specific probes, the denaturing buffer the hybridization-, stringent wash-, rinse buffers as well as the conjugate-, and substrate concentrates and diluents. Kit component 2 of 2 (the amplification mixes AM-A and AM-B) must be placed in the -20°C freezer in the pre-amplification area upon receipt of the kit (these must be separated from products that contain DNA). The hybridization buffers (component 1 of 2) must be stored in the post-amplification area, and the GenoLyse® buffers must stored in a fridge in the extraction area.

Chapter 6:
REAGENTS AND THEIR STORAGE

Expired or heat-damaged reagents should never be used. If these inappropriate or damaged reagents are used, many problems will occur, including invalid results, poor diagnostic accuracy, and delayed turn-around times. Such issues can cause significant harm to patients and to a laboratory's reputation among clinicians and other health care personnel. It is therefore essential that reagents are stored correctly, used in order of receipt, and monitored for expiration dates on a regular basis. These simple and reasonable activities are essential components of Good Laboratory Practice.

This chapter will discuss the different reagents that are used in the line probe assay (LPA) for the identification of *Mycobacterium tuberculosis* and its associated drug susceptibility pattern (**Appendix A, Table 1**). The reagents include those in the GenoType® MTBDR*plus* LPA kit and the Qiagen HotStarTaq® DNA polymerase. Other reagents used for the LPA that are not included in the kit are sodium hypochlorite, absolute ethanol (96% v/v), molecular grade water, distilled water, and a non-corrosive disinfectant such as Contrad®.

A few handy tips for high-throughput laboratories will also be described, and the chapter will conclude with examples of reagent-specific log sheets. These log sheets must be filled out and kept for all reagents used in the molecular areas. In addition to their importance for assessments and accreditation purposes, the log sheets are essential if it becomes necessary to perform a retrospective check on reagents that were used to test a specific specimen with questionable results.

GenoType® MTBDR*plus* KIT

Like other GenoType® kits from the same manufacturer, the GenoType® MTBDR*plus* LPA assay kit is produced so that the hybridization and detection

solutions are not specific for that kit. The major difference between the different LPAs is the DNA sequences of the primers in the Primer-Nucleotide-Mix (PNM) mix and the probes adhered to the nitrocellulose membrane strips. The kit components, colour-coded to aid in identification of each reagent, include two vials of blue Denaturing buffer (DEN), one bottle of green Hybridization buffer (HYB), one bottle of red Stringent wash buffer (STR) and three bottles of transparent Rinsing buffer (RIN). The Conjugate (CON) and Substrate (SUB) solutions are mixed from the concentrates (CON-C, orange and SUB-C, yellow), and from transparent diluent (CON-D and SUB-D). In addition to the buffers, there are two tubes of nitrocellulose membrane strips and two vials of purple PNM. The number of strips is dependent on the specific kit ordered (12, 24 or 96 strips). New kits should be stored at 2 - 8ºC. However, upon opening each kit, storage conditions for the different components will differ, as described in the following sections. All reagents should be labelled with the date the kit was opened.

HYBRIDIZATION, STRINGENT WASH AND RINSE BUFFER

When opening a new kit, the green hybridization buffer (HYB) and to a lesser extent the red stringent wash buffer (STR) will have formed precipitates or crystals due to the kit's storage at 2 - 8ºC. Once opened, these two buffers, along with the rinse buffer (RIN), can be stored at room temperature (**Figure 43**). Stability and heat-stress experiments have indicated that even after the storage of these three reagents at 45ºC for three months, good quality results were obtained with their use. After the HYB and STR buffers have reached room temperature, they should be shaken until visual inspections determine that all crystals have dissolved. Before the actual hybridization process can begin, the HYB and STR buffers must be pre-heated to between 37 and 45ºC, either in a waterbath or in the automated hybridization machine. The CON and SUB components must continue to be stored at 2 - 8ºC after a new kit is opened, but should be taken out before starting the hybridization process, so that they reach room temperature while the first steps in the procedure are being performed.

Figure 43: Ambient temperature storage of the HYB and STR buffers (requiring pre-heating before the hybridization process) and the RIN buffer. The lids are dated to ensure the reagents are not outdated and are used in the order received.

CONJUGATE AND SUBSTRATE SOLUTIONS

The diluents and concentrates of the CON and SUB solutions are to be stored at 2 - 8ºC. The solutions must be made up fresh for each run and this can be done either before or during the hybridization procedure. The concentration ratios are the same for both the CON and SUB solutions: 10 µl of the concentrate added to 1 ml of the diluent. These volumes need to be multiplied by the number of tests performed, e.g. 48 tests will require 480 µl of concentrate and 48 ml of diluent. The diluted SUB should be stored in the dark after preparation, either in a closed drawer or wrapped in foil.

DENATURING BUFFER AND STRIPS

The denaturing buffer and strips can be stored at 2 – 8ºC in a plastic box or the lid of an old tip box, labelled with the date the kit was opened.

PRIMER-NUCLEOTIDE MIX (PNM)

Once it has been removed from the kit, the PNM solution is usually stored at - 20ºC. However, as the unopened kit is stored between 2 - 8ºC, the PNM solution can also be stored at this temperature, as shown by in-house stability and stress experiments. It is important to note that the PNM of all the different GenoType® kits are colored purple and could easily be mistaken for each other. If the wrong solution is used, the results obtained would be inconclusive, and time and expensive reagents would have been wasted. A strategy to prevent this from happening is to assign kit-specific drawers in the - 20ºC freezer, e.g. the top drawer can be allocated to the GenoType® MTBDR*plus* kit and the bottom drawer to the Mycobacterium CM kit. In addition to this, the PCR rack where master mixes are stored in the - 20ºC freezer can also be given a dedicated colour, e.g. a blue rack might indicate that the master mix is for the GenoType® MTBDR*plus* assay, while another colour would indicate that the mixes are to be used for another assay.

HotStarTaq® DNA POLYMERASE

The DNA polymerase used in the LPA must be shipped from the supplier on dry ice, and, upon receipt, should immediately stored in a constant temperature freezer at - 20ºC. Should the DNA polymerase (*Taq*) arrive in the afternoon when staff members have already worked with DNA and/or amplicons and thus cannot re-enter the pre-amplification area, the package may have to stay at room temperature overnight. One night at room temperature will not affect the activity of the enzyme, since it has been shown to retain its full activity for up to 2 weeks at room temperature. When moving the *Taq* polymerase to the pre-amplification area, contamination preventive measures must be adhered to (wearing gloves, overshoes etc.), and the vials should be dated, initialed and stored in the - 20ºC freezer until use.

SODIUM HYPOCHLORITE AND ABSOLUTE ETHANOL

It is essential that all areas have their own dedicated pair of spray bottles, one containing 1% sodium hypochlorite and the other 70% ethanol.

The most common sodium hypochlorite commercial stock concentrations are either 5% or 3%. These solutions must be diluted to prepare the working stock of 1% (e.g. 100 ml of 5% sodium hypochlorite plus 400 ml of distilled water = 500 ml of 1% sodium hypochlorite). The concentration of the commercial sodium hypochlorite can be checked with pH dipsticks, and should never be less than 2%. Sodium hypochlorite is light sensitive and will lose effectiveness if continually exposed to light. The stock solution must therefore be stored in a dark or light-tight container. Should the TB laboratory order the commercial sodium hypochlorite in 20 L drums, the stock solution can be transferred into a clean 5 L brown glass bottle for ease of use. The working stock must be made up fresh each week and stored at room temperature, preferably in dark spray bottles.

Absolute ethanol (96%) can be stored at room temperature and does not need to be in a dark bottle. The 96% ethanol stock is to be used to make up the 70% alcohol working stock (e.g. 700 ml absolute ethanol plus 300 ml distilled water = 1L of 70% alcohol). This solution can be stored in spray bottles at room temperature.

MOLECULAR GRADE WATER AND DISTILLED WATER

To ensure that the PCR reaction will not be inhibited by exogenous contaminants, molecular grade water (MGW) must be “Type 1 water” as described by the National Committee on Clinical Laboratory Standards (NCCLS). NCCLS Type 1 water is purified molecular grade water that is deionised, double distilled, autoclaved, and free of any endonucleases, ribonucleases and proteases (causing “nicking” activities). MGW can be stored at room temperature in a DNA free environment. Type 1 water usually comes in 1 L bottles, and should be aliquoted into sterile 125 ml bottles to prevent stock contamination. The mouth of the sterile bottles should

be flamed when aliquoting the MGW to ensure sterility during the transfer. The aliquoted MGW can also be autoclaved in the 125 ml bottles, and smaller aliquots can be made into 1.5 ml tubes. This strategy is critical for preventing contamination that may occur when bottles of MGW are repeatedly opened.

Distilled water can be obtained from a laboratory that has a water purification system, such as the Millipore® system. If the water purification system is far away from the area of use, it is suggested that a 25 L container with a stopcock be used to store the distilled water (**Figure 44**). However, algae may form on the inside of the 25 L container after a period of time standing on the bench. The container should be checked regularly for algae or other contaminants. If needed, the container should be cleaned with sodium hypochlorite and rinsed thoroughly to remove any left-over sodium hypochlorite that may interfere with the hybridization procedure.

Figure 44: 25 L container with stopcock, containing distilled water.

DECONTAMINATION REAGENTS

Decontamination reagents, such as Contrad® should be kept at room temperature and stored in a secure location next to the sodium hypochlorite and absolute ethanol stock solutions. It is suggested that these solutions be placed in a container lined with sand, in case a chemical spill should occur.

REAGENT LOT NUMBERS

Quality assurance in the laboratory requires that the lot numbers of all reagents used to perform a diagnostic test be documented on Reagent Log Sheets. This practice allows for reagent-related troubleshooting and rapid identification of manufacturing errors that may have led to analytical failure of the test. Each of the reagents used in the pre-amplification room, such as the 10X buffer, $MgCl_2$, and Qiagen HotStarTaq® DNA polymerase, will have individual reagent lot numbers. These different lot numbers must be documented on a reagent log sheet dedicated to this area.

With regards to unopened kits of the GenoType® MTBDR*plus*, it is necessary to date and sign the kit when received (**Figure 45A**). Both the lot number on the outside of the kit (**Figure 45B**), and that of the strips found inside the kit, are to be documented on the reagent specific log sheet. If detailed information is needed on the components of a specific kit (**Figure 45C**), Hain Lifescience can be contacted, but must be provided with the specific lot number of the GenoType® MTBDR*plus* kit.

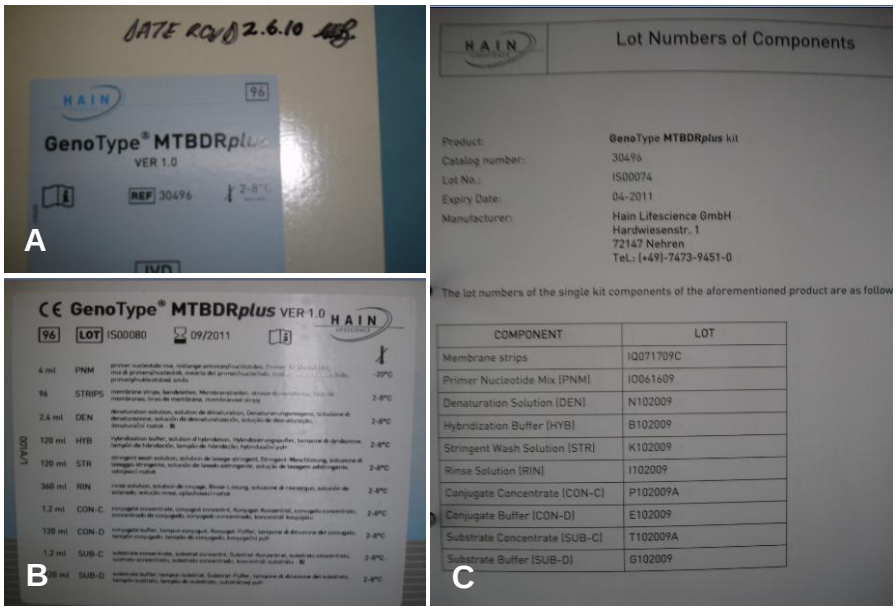

Figure 45: GenoType® MTBDR*plus* reagents lot number documentation procedures. (**A**) Dating and initialling of stock received, (**B**) Kit LOT number of the kit (LOT ISO 0080), (**C**) Example of component lot numbers for the different reagents to be found in kit LOT ISO 0074). The expiration date is listed on this page and must be documented elsewhere.

The second reagent lot number required is that of the strips. The sticker on the vial contains all the relevant information about the strips and can quite easily be removed and adhered to a reagent log sheet (**Figure 46**).

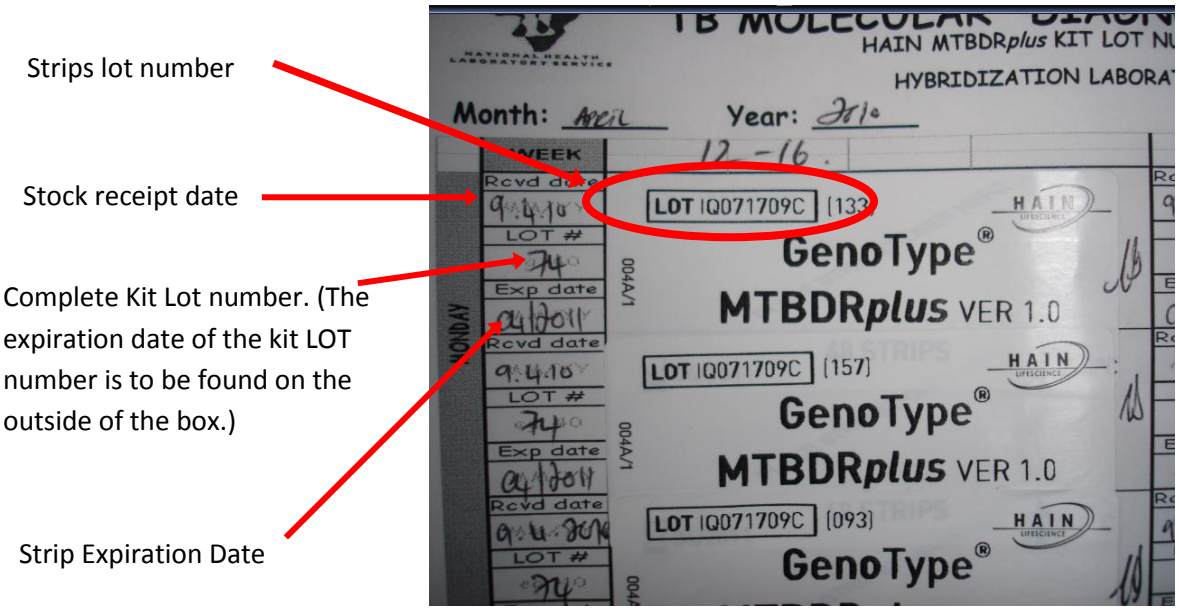

Figure 46: Example of the GenoType® MTBDR*plus* kits' essential LOT numbers requiring documentation. The lot numbers of the strips are seen on the sticker that is peeled and pasted onto the log sheet.

All this information can be documented on the GenoType® MTBDR*plus* reagent log sheet for the post-amplification area (**Appendix A, Figure 1A & 1B**), as well as the reagents used for the Mycobacterium CM kit (**Appendix A, Figure 2**). An example of the reagent log sheet for the reagents used in the pre-amplification area is shown in **Appendix A, Figure 3**. The lot numbers of the MGW and decontamination reagents, initials of the person who made the working solutions, and dates prepared should also be documented.

It is important to note that the log sheets described in this section are more appropriate for high-throughput laboratories where more than 100 LPAs are done on a daily basis. If the laboratory does not perform as many tests per day, the lot numbers can be written out by hand in the appropriate blocks of the log sheet or, alternatively, the laboratory can develop and customize reagent log sheets to meet their needs.

ROTATION OF STOCK

Sufficient stock should be on hand for at least two weeks. It is useful to monitor and document monthly stock usage, and order the amount needed between orders plus two more for unforeseen increased workloads. Another important means of stock control is to document the amount of reagents left at the end of each day (**Appendix A, Figure 4**).

GenoType® MTBDR*plus* kit

When receiving GenoType® MTBDR*plus* kits, the date received should be written on the box and initials placed next to the date. The lot numbers of the unopened kits must be checked and those with the lowest lot numbers should be placed on the top or most convenient shelf of the refrigerator, and those with higher lot numbers on the bottom shelves, e.g. lot ISO 0075 must be used before lot ISO 0080, since the lower numbered lot will expire before the higher numbered lot. As the kits are used and new stock arrives, the older stock should be moved up to the top shelf and the new stock placed on the bottom shelf to ensure that the older stock is used first. Any additional Reagent Sets for the GenoType® MTBDR*plus* kits should be rotated and used in the same manner. When opening a new kit, all the reagents should be dated and signed on the lid, and stored in separate trays or containers according to each reagents temperature requirements.

DNA polymerase

A - 20ºC freezer is required for the pre-amplification laboratory. For storage of reagents, it is recommended that three plastic container boxes (e.g. cryogenic 10x10 boxes) and a mini cooler be placed in the freezer. The mini cooler is an insulated container that will keep reagents cold when removed from the freezer and placed on the work bench or in the PCR hood. The reagents that come with the DNA polymerase can be stored in the 10x10 containers. It is recommended that the

reagents be placed directly into these containers upon arrival, rather than leaving them in the original package. This way, the remaining stock is visible and can be assessed whenever a tube is taken out and used, and essential components of the master mix can be ordered before the stock is depleted. If reagents are out of stock, there will be a delay in the turn-around time and a backlog of work will build up. It is important to place orders well in advance by taking regular stock inventories and being aware of the suppliers' ordering processes and delivery schedules.

PCR reagents can be unpacked into the plastic freezer boxes as follows: the first can be used to store the 10X buffer, the second for the $MgCl_2$, and the third for the DNA polymerase. The reagents must be placed into these containers in an organized way so that it is known which tubes should be used first (tubes received recently will expire later than those received previously). A sticker from the original box with the expiration date may be pulled off and placed inside the plastic box as a reminder **(Figure 47).**

Figure 47: (**A**) Stocking of DNA polymerase into 10x10 boxes upon arrival with Reagent lot numbers included, indicating the expiration date to manage stock rotation. Note that the enzymes are packed into separate batches due to the different expiration dates of the two different deliveries. The enzymes with the earliest expiry date must be used first. (**B**) Expiration date information of the DNA polymerase. (**C**) The reagent information and expiration dates of the enzyme and the associated buffers and co-factors are found in the inside of the box's lid and can be peeled off and placed into the 10X10 box as seen in Figure 9A. (**D**) Division of DNA polymerase stock, GenoType® MTBDR*plus* pre-made master mix and CM pre-made master mix.

SAFEGUARDING OF STOCK SOLUTIONS

If small numbers of LPAs are tested in each run, it is recommended that smaller volumes of hybridization solutions are aliquoted into 15 ml or 50 ml tubes, after all crystals have dissolved. This applies to other stock solutions as well (e.g. the MGW discussed previously) and will aid in preventing contamination of stock solutions.

TIPS FOR THE HIGH VOLUME LABORATORY: PLANNING AHEAD

Strips

Strips for the LPA can be pre-marked and stored in a light-tight container until they are ready to be used, either later that day or the following day. This will allow the operator to begin the hybridization procedure as soon as the amplicons are ready, and will therefore reduce the turn-around time for the test results. The exposure to room temperature will not reduce the signal strength of the strips, if used within the next two days. However, leaving pre-labelled strips at room temperature for extended periods of time is not recommended.

Conjugate and substrate solutions

In a high-throughput laboratory that uses an automated hybridization machine, such as the GT-Blot 48, all of the CON-C and SUB-C vials (120 µl) can be added to their respective CON-D and SUB-D (120 ml) bottles. If there are left-over solutions, these can be placed back into the refrigerator and used the following day. After adding the concentrate solutions to the diluent bottles, it is recommended that a small volume of the diluent be added back to the empty concentrate vial, the solution mixed in the vial, and then transferred once again to the 120 ml bottle. This will ensure that all residual reagent is removed from the vial. The bottle must then be closed and shaken to ensure proper mixing.

Master mix

When the laboratory is temporarily understaffed, for any number of reasons, master mixes that will last for a week or two can be prepared, as long as they are stored at -20ºC until use. Master mixes can also be made a day in advance, however staff not enter the pre-amplification area once they have extracted the bacterial DNA, added

the template DNA to the master mix or added amplicons to the denaturing solution prior to hybridization.

Chapter 7:
CONTAMINATION CONTROL

In high-throughput molecular mycobacteriology laboratories, there is a need to batch specimens so that many assays can be performed simultaneously and turn-around times can be reduced. In order to optimize performance and maximize output, contamination prevention and control must be seriously addressed. The presence of specific amplified products (amplicons), resulting from DNA in either the original specimen or from exogenous contamination, may predict the presence of *Mycobacterium tuberculosis* and any associated drug susceptibility or resistance. Chances of incorrect diagnoses due to molecular contamination problems can place patients at great risk. The most effective way to control contamination is by preventing its occurrence in the first place. Strategies for contamination prevention and control are described in this chapter.

"Contamination" in the mycobacteriology laboratory is usually associated with the growth of unwanted bacteria or fungi in either the mycobacterial growth indicator tube (MGIT) liquid media or on solid-media slopes (used for conventional drug-susceptibility testing). In addition to contaminating organisms, sample-to-sample contamination (cross-contamination) may also occur. Such contamination can result in the misdiagnosis of *M. tuberculosis*, and can have a negative impact on the clinical outcome of the patient. However, when performing the polymerase chain reaction (PCR)-based GenoType® MTBDR*plus* line probe assay (LPA), "contamination" refers to the erroneous introduction of previously amplified DNA products (amplicons) or other contaminants or inhibitors into the reaction tube. Whatever the source of the contaminant, an incorrect diagnosis of *M. tuberculosis* and its drug susceptibility pattern is likely to occur.

This chapter will discuss PCR contamination and how to prevent and control it both before and during amplification of DNA templates. It will also aim to provide direction on how to eliminate PCR contamination in the different areas of the molecular diagnostic laboratory. In addition, strategies on how to perform adequate but not excessive monitoring and screening of all pre- and post-amplification areas for

potential contaminants will be discussed. **Appendix B** contains information on avoiding contamination during reagent preparation and on procedures for the cleaning of equipment used in the LPA.

Although the World Health Organization (WHO) indicated that the ideal configuration for performing the LPA is the use of four separate rooms or areas, their guidelines also indicate that it is acceptable for the amplification (area 3) and post-amplification (area 4) areas to be combined. The material in this chapter will be presented based on laboratories that have four areas dedicated to the LPA. However, since many laboratories in high-burden countries have only three areas, this material should be understandable and readily adaptable to those circumstances.

CLEAN, CLEAN, CLEAN

The main challenge for laboratories performing PCR is the maintenance of a clean and healthy working environment by ensuring that all contaminants are eliminated from the four work areas. The preventable contamination control measures discussed below should be implemented and incorporated into already existing Quality Assurance (QA) measures. The laboratory manager, as well as the technical and support staff, must all adopt a pro-active approach towards contamination control. This will not only reduce the risk of contamination within the PCR facility, but will also help with the smooth running of the laboratory: having to deal with a contamination problem results in a back-log of specimens to be tested. Initially, the turn-around time (TAT) of the laboratory will be negatively affected and, once the contamination problem is resolved, a great deal of time will be required to process the accumulated specimens.

The major sources of contamination of DNA samples and PCR reagents in a laboratory are amplified DNA products that are spread throughout the laboratory on aerosols and dust. Other contaminants include DNA from the technicians' skin and hair roots. This concern emphasizes the need for PCR staff to wear powder-free gloves, over-shoes and a hairnet, especially if the manipulation of DNA and DNA products are done in the absence of a hood or contained workspace. In addition, the

potential for contamination is the main reason that the different areas of the laboratory must be well-separated, as described in Chapter 2: Infrastructure Requirements.

Pipette control

Trafficking of pipettes between areas designated for specific tasks must be avoided at all costs. The most common type of contamination is the introduction of **previously amplified sequences** (amplicons) into a new reaction. This can occur when pipettes previously used in the post-amplification area are taken to the pre-amplification area and used to prepare the master mix, or are taken to the template addition area and used to add template DNA to the master mix. To avoid this, reagent master mixes must never be prepared using pipettes from areas 1, 3 or 4. It is especially important to avoid pipettes that have been used to manipulate extraction, amplification or post-amplification products.

Note: it is a good idea to keep a clean set of pipettes on hand in each area in case there is a pipette malfunction or breakage during use.

Pipette-related contamination is caused when a contaminated pipette or contaminated tip introduces amplicons or DNA into other samples. This problem can be prevented by using sterilized filter tips and by wiping the pipette after each day's use with 1% sodium hypochlorite (followed by wiping with 70% alcohol, so that the bleach does not corrode the pipette). Autoclaving the pipette may be necessary if contamination is obvious. It is important to remember that each specimen requires a separate tip and the tip must be changed after the pipetting of each sample.

Sample-to-pipette contamination occurs when samples or aerosols enter the core of the pipette. To prevent liquids and aerosols from entering the pipette body, the pipette should be held vertically when pipetting, and aerosol-free filter tips should be used. Also, the pipette's push-button should be released slowly to further prevent aerosols.

Sample-to-sample (carry-over) contamination occurs when the remains of one sample mixes with the next sample inside a re-used pipette tip. Both of these instances may cause a false test result.

Avoiding PCR contamination

As already mentioned, contamination of new PCR reactions with amplicons from previous reactions is the most common source of false-positive results. The best approach to avoid this problem is to develop a comprehensive contamination control program (CCP) prior to implementing the LPA. General CCP requirements that must be followed in order to aid in the prevention of contamination are outlined and discussed below.

Building blocks of a comprehensive CCP:

1. Space and time separation of pre- and post-amplification procedures
    a. Four (or three) rooms that are physically separated from each other must be dedicated to the four different procedures as per infrastructure requirements.
    b. The master mix should be prepared first thing in the morning by a staff member before he/she performs other procedures such as the template addition, amplification and hybridization. Optimally, a staff member who does not perform these procedures should prepare the master mix.
2. All areas must have their own dedicated supplies of consumables relevant to the work done in that specific area.
    a. Separate supplies of pipettes, pipette tips, PCR tubes, and reagents must be kept specifically for PCR and not be used for any other procedure. “On-hand” stock, as well as back-up stock of all the consumables and reagents should be available in each room.

3. Good quality disposable Nitrile and/or powder-free gloves must be worn at all times and changed frequently.

    a. If the powder used in latex gloves enters the PCR tubes, the reaction may be inhibited. Also, DNA contaminants may be carried along with the powder.

    b. It is essential that new gloves be worn when entering each PCR area, and used gloves be removed and disposed of when the work has been completed and before exiting each area. Wearing gloves is especially important when taking out strips that are to be marked prior to hybridization, handling concentrated DNA samples and hybridization reactions, decontaminating work areas and tube racks before re-use, and whenever a splash occurs.

4. Certain reagents, consumables and equipment such as distilled water, 1.5 ml tubes, PCR tubes and tips should be sterilized, if possible, in a **clean** autoclave before use.

    a. When buying pipettes, those that are either fully or partially autoclavable should be selected to ensure that possible contaminants can be eliminated.

5. Dedicated laboratory gowns and over-shoes should be worn, especially in the room for reagent preparation, in order to prevent carry-over contamination from contaminants that the technical staff could have picked up via their clothing and foot-wear in other areas of the laboratory.

6. It is preferable that master mix preparations and template additions be set up in a biological safety cabinet or PCR hood.

    a. The biological safety cabinet or PCR hood must be dedicated for the use of trained personnel who perform the LPA. If the laboratory does not possess a biological safety cabinet or PCR hood, the work can be done on the bench in a separate room dedicated to this process.

b. Reagent preparation and template additions must be done in separate areas (areas 2 and 3) and those areas must be completely isolated from area 4, where the PCR products are detected.

7. Cleaning as an important means of contamination control.

    a. Meticulous cleaning with freshly prepared 1% sodium hypochlorite and 70% alcohol of surfaces, pipettes and instruments should be done before and after performing each procedure.

    b. Alternatively, other decontamination reagents mentioned below can be used instead of sodium hypochlorite.

8. Measures must be taken to avoid contaminating stock and working solutions of reagents.

    a. When practical, reagents should be aliquoted soon after receipt into the smaller amounts needed for each batch of specimens.

    b. Only the amount of PCR and hybridization reagents needed for each day’s work should be prepared.

9. Positive and negative controls must be carefully prepared and used with each PCR batch.

    a. One or more negative controls containing molecular grade water instead of DNA template must always be included when performing PCR (even if only one specimen is being tested). Ensure that the last two reaction tubes in the run are the positive ($H_{37}Rv$) and negative controls.

    b. Molecular grade water used during suspension of the DNA template should be used as a negative control; likewise, molecular grade water used to bring the master mix to volume can be added as an additional negative control.

    c. Reagent or water contamination has occurred if banding patterns other than the two internal controls are seen in any negative control.

d. A mistake such as “number-dropping” can be easily detected if a positive control ($H_{37}Rv$) is placed in a fixed spot in the middle of a batch (i.e. number 24 in a batch of 48). If the result is interpreted as anything other than fully susceptible to RMP and INH, a laboratory error has occurred.

10. Use of ultra-violet light (if possible)

    a. UV-irradiation will destroy all DNA fragments inside the PCR hood or biological safety cabinet, as well as any fragments on the pipettes. However, care should be taken to ensure that the template DNA is never be exposed to UV-irradiation since this treatment will break up the DNA and result in a false negative result or weak signal on the strip.

11. Adding reagents and DNA template to the PCR tubes.

    a. The master mix must be aliquoted into the PCR tubes in area 2 before working with DNA or amplicons in other areas. Because this is a clean room and each tube will receive the same volume of the master mix, a single pipette tip can be used for all tubes.

    b. The DNA template can be added to the PCR tubes containing the master mix in either the DNA extraction area or the amplification area. The template DNA must always be added last.

    c. When adding the DNA to “8-strip” PCR tubes, only one row should be opened at a time. The pipette containing the DNA template should not be placed over any open tubes other than the one receiving the sample. By moving the pipette directly over the entire row, there is a risk of exposing other open tubes to the wrong sample.

    d. Single PCR reaction tubes that are opened one at a time further minimize the chances of contamination. However, the use of single tubes is time consuming and generally more suitable for low-throughput laboratories.

12. It is suggested that the LPA not be used as a diagnostic tool in a tuberculosis (TB) research laboratory, but in a dedicated PCR facility. Work performed in such a laboratory is likely to generate potentially contaminating amplicons that

may be difficult to control should contamination arise. Such contamination can result in incorrect diagnoses and serious implications for patients.

Prevention of sample contamination

Sample contamination is a critical problem for nucleic acid-based screening assays due to the extreme sensitivity of PCR. There are a number of ways that this problem can occur:

1. **Carry-over contamination** occurs when either the DNA from the specimen that is being extracted (in area 1) enters an adjacent tube(s), or when amplicons (in areas 3 and 4) enter the PCR tube or detection well. Amplicon contamination, more than other types of contamination, is likely to lead to incorrect interpretations and end in negative impact on patient care.

2. Sample contamination can also result from external sources such as **contaminated surfaces** (floors, counter tops, instruments). Laboratory policies must ensure that PCR areas are access controlled to prevent trafficking by all staff members who might unknowingly carry contaminants from areas 1, 3 and 4 to the PCR clean area 2.

3. **Contamination due to a mistake by the operator** usually occurs when the attention of the technician performing the assay has been diverted by external sources or by personal thoughts. For example, the operator could be preoccupied enough to add two DNA specimens to the same PCR reaction tube, or overlook obvious splash-over from one hybridization well to another. To help avoid such situations, it is suggested that there be no distractions, such as a radio or extensive private conversations, in any of the four work areas.

Area-specific sample contamination

Pre-amplification area (Area 2):

The pre-amplification area (master mix preparation area) should only be used by the operator assigned to perform this task. The amplification and post-amplification duties should be done by another operator. However, with staffing shortages, the same operator may perform all three procedures, so long as the master mixes are made first, followed by DNA addition and finally amplification and hybridization. Of course, it is critical that operators do not re-enter the pre-amplification area after working in the amplification and post-amplification areas. To prevent re-entry, a notice such as “NO ENTRY AFTER 10 AM” should be posted.

For the **pre-amplification area**, the following rules should always be adhered to:

- The pre-amplification area is to be used for one purpose only – preparation and aliquoting of the PCR master mix.
- It is recommended that reagents used for the master mix be stored in this area whenever possible.
- Pipettes and other instruments and supplies needed for master mix preparation must be kept in this area and are to be used exclusively for pre-PCR activities.
- No thermal cyclers or amplicons should ever be brought into this area, and no reagents or supplies should be brought back after being removed.
- There should be dedicated cleaning supplies and brooms for this area, which should also be stored in the room. Cleaning should be performed by PCR laboratory staff, and not by general cleaning personnel. This will prevent accidental introduction of PCR products, dust, and other potential contaminants from other areas.

Laboratory clothing:

- Disposable shoe covers should be stored in the ante-room of the pre-amplification area and put on in the following manner: Cover the first shoe and step with the covered shoe into the pre-amplification area, keeping the

uncovered shoe on the outside. Once balanced, lift the exposed shoe, cover it, enter the area and close the door behind you.

- No *used* laboratory coats, surgical gowns, gloves or opened boxes of gloves are to be taken into this area.

Reagent handling:

- Always use only positive displacement pipettes and aerosol-free tips (barrier filter tips).
- Reagents for the master mix that are stored in the freezer must be allowed to thaw completely. After thawing, the contents must be mixed well before pipetting to ensure uniform concentrations of all the components.
- Reagents should not be shared or used with different kits. The components of each reagent have been specifically formulated and reagents from different kits will not be compatible.
- Each reagent tube should be closed and moved aside before working with the next reagent on the list to rule out contamination of the preceding reagent with subsequent solutions. This also ensures that a reagent will not be overlooked or added twice.
- If multiple rounds of PCR are to be conducted on the same day, all master mixes must be prepared and aliquoted into the reaction tubes all at once (in area 2) and then moved to the area where the DNA template will be added. It is important that the correct number of reaction tubes be prepared and then moved to the amplification area, since it will not be possible to go back into the pre-amplification area once DNA is added to the PCR tubes.

Amplification and post-amplification area(s):

- As mentioned above, one should never, under any circumstances, re-enter the pre-amplification area (area 2) on the same day that one has been working in

the amplification and post-amplification areas (areas 3 & 4). Amplicons circulating in these areas can stick to clothes, hair, shoes, etc.

- Before and after each experiment, all surfaces should be decontaminated by wiping with dilute bleach (10%) followed by 70% ethanol.

- Care should be taken when opening PCR tubes following amplification. In order to prevent the release of liquid near the top of the tube or on the inside of the cap, the tubes can be swung down hard by hand, or quickly centrifuged in a small micro-centrifuge. Then the caps should be carefully opened either one by one, or one strip at a time.

- If the GT-Blot 48 automated hybridization system is used in the laboratory, the waste tubes must be placed into a 25 L capacity drum. Thus, all the liquid waste containing massive quantities of amplicons will safely drain into the drum. To ensure that these un-hybridized amplicons are destroyed and the contamination risk is reduced, it is recommended that approximately 500 ml of undiluted sodium hypochlorite be poured into the drum. In addition, the bottom of the drum must be placed in a large plastic tray that could capture any over-flow from the 25 L drum. This precautionary measure prevents the escape and spread of amplicons throughout the laboratory, and also eliminates the chance of staff being injured by slipping on soapy liquid waste. The same guidelines are recommended if the GT-Blot 20 and a smaller drum are used instead.

- Normally, a low-throughput laboratory will use the TwinCubator® manual hybridization and detection system. The contamination prevention strategy for this system depends on the size of the batch of specimens to be tested. To prevent contamination of the “stock” solution of each reagent, the desired amount of each reagent should be aliquoted into 50 ml tubes before beginning the procedure. When removing the waste liquids from the wells following the hybridization, washing and detection steps, the liquids must be discarded into 50 ml tubes containing a quantity of undiluted sodium hypochlorite. This is an approach similar to the decontamination of liquid wastes in the GT-Blot systems.

- Great care must be taken to avoid storing or manipulating post-amplification products (area 4) close to the reagents that will be used in the pre-amplification-

and amplification areas (areas 2 & 3). For example, in the amplification and post-amplification laboratory, it is very important that the liquid waste bucket is kept far away from the “strip preparation area” as well as the “clean area” where the racks are washed and stored. The sealable amplicon waste bucket can be placed underneath a designated “liquid hybridization waste” sink.

- It is highly unlikely that contamination of reagents would occur during the commercial manufacturing process. However, there have been cases where commercial preparations of *Thermis aquaticus* (*Taq*) polymerase have been found to be contaminated with bacterial DNA. Fortunately, this should not affect the ability of the kit to amplify DNA from *M. tuberculosis*.

Storing reagents and other consumables:

When the **DNA polymerase** is delivered it must be stored at - 20ºC immediately upon arrival. The styrofoam box should be wiped clean with undiluted sodium hypochlorite before taking it into the pre-amplification area. As usually done upon entering this area, shoe covers, gloves and a clean laboratory coat should be worn. Once inside the area, the box should be wiped again with both sodium hypochlorite and 70% alcohol. A scalpel blade, dedicated to this area, should be used to open the box. The small DNA polymerase boxes should be removed, wiped and placed in the - 20ºC freezer.

Re-stocking of consumables for all the different laboratory areas should be done on a weekly basis to ensure that a constant supply is available in each area. This can be done before the weekly scheduled PCR facility clean-up at the end of the week.

CONTAMINATION! NOW WHAT?

Monitoring for DNA contamination is an important part of the molecular diagnostic laboratory, and laboratory policy requires immediate action and investigation if contamination is identified or suspected.

Sentinel test for PCR contamination

Other than including the negative (water) control as described above, monitoring should also include regular sentinel tests to confirm that there is no amplified or genomic DNA present in the four PCR areas.

Sentinel testing is performed periodically to monitor for potential contamination within all the areas of the PCR facility. Detecting the root cause of contamination is necessary to be able to eliminate the problem. Once the contamination has been dealt with, the existing procedures can be adapted to prevent further incidents and thus improve the day-to-day performance of the laboratory.

The recommended procedure is as follows:

1. Three or more 1.5 ml tubes should be filled with 100 µl of sterile molecular grade water, and left uncapped in each of the four areas at different locations (e.g. inside the hoods, on the benches, near the thermal cyclers, etc.).
2. At the end of the day, the tubes should be collected and closed.
3. The next day, a portion of the water in each tube should be tested in the PCR reaction, followed by the LPA. Positive and negative controls should be included in the batch.

It is suggested that sentinel testing be done in all areas on a quarterly basis. The hybridised strips should be kept together with the log sheet, documenting where each of the open tubes had been placed. Sentinel testing immediately identifies a contamination problem area should any of the strips come up as “positive”. Such a

problem necessitates the need for immediate changes in current laboratory procedures to prevent aerosol contamination.

A positive sentinel test

Guidelines are given below as to how to deal with a positive sentinel test:

1. The laboratory director or supervisor must be notified immediately if any area has a positive sentinel test.
2. Specimen testing must be stopped until the root cause of the contamination has been found and corrected.
3. The laboratory director or supervisor should initiate a thorough investigation to identify the source of contamination.
4. All reported areas of contamination MUST be thoroughly cleaned with 10% bleach and other sentinel tests performed in these areas.
5. To identify the source of contamination, the investigation may require additional sentinel tests of other laboratory surfaces.
6. The laboratory director or supervisor should review all test results since the last negative sentinel test and the current positive test. If necessary, corrected or amended reports should be sent to the institution submitting the specimen.
7. When the repeated sentinel tests are negative and the investigation has been completed, the laboratory director or supervisor will indicate that specimen testing can resume.
8. Laboratory procedures and policies should be reviewed and modified as needed to help prevent future incidences of contamination.

Internal reagent controls

The **AC** band on the LPA serves as the positive control for the amplification reaction and is included in order to detect the presence of inhibitory substances that could mask the detection of DNA. The negative control contains all reagents used except template DNA, and thus potential contamination can be identified by the presence of the **AC** band, the **TUB** band, and/or any of the *rpoB* bands in the negative control.

Decontamination control measures

Contamination can and will occur at times and may be extremely difficult to recognize. The following inactivation or sterilization protocols have been developed:

1. **Chemical:** sodium hypochlorite or 1M HCl
2. **Enzymatic methods:** It is possible to remove contamination by pre-treating the master mix with a restriction enzyme or DNase treatment to digest the amplified PCR product. This must be done according to strict protocol to ensure that the DNA template is not digested.
3. **Photo-chemical (UV-irradiation):** PCR primers are more resistant to ultraviolet irradiation than target DNA. It has been reported that pre-exposure of a PCR mixture to UV light, for example from a germicidal lamp, can very efficiently eliminate contamination.

Decontamination procedures

The following processes should effectively destroy exogenous DNA:

- **Chemical/Enzymatic degradation:**

These methods are the most reliable and cost-effective ways to get rid of contamination. Surface decontamination of the workplace and contact areas, including the walls and floor should be carried out using the following agents:

- 10% sodium hypochlorite, followed by 70% EtOH.
- Contrad®, followed by 70% EtOH.
- Ultraseptin®, followed by 70% EtOH.
- Incidin®, followed by 70% EtOH.

Enzymatic methods that can be incorporated into the decontamination procedure may include the treatment of the reagents with restriction enzymes or by making use of Uracil n-glycosylase (UNG), which hydrolyses the Uracil incorporated into amplicons. However, most diagnostic mycobacteriology laboratories, which are starting or already have a molecular division, would not normally use these expensive reagents.

According to the U.S. Centers for Disease Control and Prevention (CDC), chlorine solutions gradually lose strength; so diluted solutions should be made fresh regularly, if not daily. Cleaning with bleach must be followed 70% ethanol (EtOH) to eliminate the possibility of introducing bleach into the sample and to remove the bleach so it does not corrode surfaces and instruments. EtOH alone, even 70% strength as employed in microbiology settings to prevent transfer of pathogens, is not sufficient to rid a surface of exogenous DNA.

- **Decontamination with UV irradiation:**

Another means of decontaminating hoods, reagents, pipettes, tubes, etc., is exposure to UV light. Most biological safety cabinets are equipped with a UV light source. It is generally accepted that UV exposure at 254nm for a minimum of 5 minutes is sufficient to disinfect and destroy extraneous DNA on surfaces. Laboratory SOPs for PCR hoods often include UV exposure steps as long as 30 minutes before and after each use. Wiping with bleach and alcohol is still warranted, since only contaminants on exposed surfaces will be eliminated by the UV light.

Recommended UV irradiation:

- >10 min with 100 uJ/min UV (254 nm) for tubes and reagents (not the enzyme)
- >8 h with 400 uW/cm^2 UV (distance <1m) for decontamination of work surfaces

Reagents, consumables and tools

Applicable consumables and tools such as forceps, microcentrifuge tubes, PCR tubes (if not sterile when purchased), racks, containers, and other items should be autoclaved in a clean autoclave prior to use. It is a good practice to apply autoclave tape to items being sterilized to test the autoclave's efficacy and differentiate between items that have and have not been autoclaved. The staff must pay careful attention to the heat stability of plastics that are to be autoclaved. Some plastics can break down during autoclaving and become more porous and prone to harboring contaminants. Most commercial filter tips are sterile when purchased. A new aliquot of sterile molecular grade water must be used to eliminate water-based contaminants. All other reagents must be assessed for possible contamination as well.

GOLDEN RULES FOR CONTAMINATION PREVENTION

Before attempting to use the GenoType® MTBDR*plus* LPA, and/or any other molecular mycobacterial identification product, it is essential that the laboratory meet the specific 4 (or 3) room specifications as set out in Chapter 2: Infrastructure requirements. In addition, the division of labour between the pre- and post-amplification areas will greatly minimise the chance of contaminants carried by the operators. Keeping the areas well separation and careful attention on the part of all personnel is the key to preventing contamination. The appropriate technical skills, careful reagent handling, as well as well planned procedures and daily work flow are also essential components. Even though chemical decontamination with sodium hypochlorite and alcohol is an effective deterrent, these other critical elements must be in place to avoid contamination problems.

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Chapter 8: QUALITY ASSURANCE

ISO 15189 provides guidelines on several Quality Assurance issues including: the collection of patient samples; the acceptable turn-around-times from specimen receipt to reporting of results; and the role of the laboratory in training and education for health care providers, both at point-of-care and in the laboratory. Application of these standards will ensure that good laboratory practices are followed in each laboratory. Regulatory and professional bodies within each country should monitor all laboratories to ensure adherence to the quality standards listed in ISO 15189.

During assessment by a regulatory body, when laboratories are found to be adhering to the standards in ISO 15189, the status of the laboratory should be documented through an accreditation process. Accreditation indicates to the health care community that a laboratory can produce competent results on a consistent and sustainable basis. Accreditation should be granted in stages dependent on the capacity of the laboratory. Importantly, the accreditation status should be in effect for a limited period of time, so that the laboratory is assessed on a regular basis and is responsible for maintaining the quality of testing by continuously improving services and moving up in accreditation status.

Quality Assurance (QA) programs consist of activities within all sections of the laboratory that are needed in order to ensure that testing is being performed according to ISO 15189 standards. QA depends on Internal Quality Control (IQC), External Quality Assessment (EQA) and an effective Quality Improvement (QI) program. To successfully implement the line probe assay (LPA), a QA program must be in place for the diagnostic mycobacteriology laboratory. This section describes in detail the elements of a QA program that are essential for accurate LPA testing and interpretation.

SMEAR QUALITY AND INTERPRETATION

The QA of the LPA begins with proper training of laboratory staff, both tuberculosis (TB) microscopists and those who perform the LPA. As mentioned in other chapters, the successful result of the LPA is determined by accurate reading of the smear. A clear indication that the preparation or interpretation of the smears is not being performed accurately is the lack of correlation between the LPA results on processed sputum specimens and the culture results. Also, if smears from processed specimens are incorrectly reported as positive, much time will be wasted in resolving weak or negative LPA results. Furthermore, it is very important that the acid-fast bacilli (AFB) observed on the smears prepared from isolates grown in liquid media should be arranged as the serpentine cords typical of *Mycobacterium tuberculosis*. If the microscopist sees AFB organized as “scattered” rods, this morphology should be noted on the worksheet for subsequent follow-up as a potential NTM. Specimens should not be tested in the LPA unnecessarily if a NTM is suspected.

MOLECULAR LPA QC

The purpose of a Quality Control (QC) system is to ensure that the results of each test are accurate. Because PCR is able to detect very small quantities of DNA, special attention must be paid to QC procedures. Failure to adhere to the QC procedures can result in lack of ability to detect either false positive results due to contamination with DNA and amplicons, or false negative results due to contamination with inhibitors.

INFRASTRUCTURE

Infrastructure forms the cornerstone of QA within the laboratory’s PCR facility. Physically separating the areas used for the different molecular procedures allows

for unidirectional workflow and reduced risk of contamination. Molecular operational activities require that the specimen preparation area (DNA extraction lab), reagent preparation area (pre-amplification lab), template addition, amplification, and hybridization/detection area (post-amplification lab) be individually enclosed and separated from one another. The reagent preparation area requires positive pressure to prevent introduction of contaminants, whilst the specimen preparation and hybridization/detection areas require negative pressure to adhere to the BSL-3 guidelines on biosafety, and to keep the exogenous DNA and amplicons in the area itself. Under no circumstances can anything from the specimen preparation area be taken into the reagent preparation area. Nor can anything from the amplification and hybridization/detection areas be taken into the specimen or reagent preparation areas. Chapter 2: Infrastructure Requirements provides more details on these dedicated facilities.

To ensure that the physical barriers against contamination are effective, it is necessary to perform quarterly sentinel tests within these areas (See Chapter 6: Contamination Control). The strips of the LPA-tested sentinels should be documented on a LPA score sheet that is dedicated to the quarterly sentinel testing. This documentation will then serve as proof that environmental quality control checks are in place. If the sentinel testing is positive for contamination, immediate corrective and/or preventative action should be taken to clear the area(s) of the contaminant(s). Proof of the corrective and/or preventative action also needs to be documented.

TRAINING OF STAFF

As with microscopy, the QA of the LPA starts with appropriately trained staff. Laboratory staff performing the LPA must be deemed competent in performing molecular analysis before working on patient specimens. Training must begin with liquid handling skills and validation of the accuracy of the pipettes. The required documentation of staff training must include the dates for the start of the LPA training and the demonstration of competence to perform the assay independently, and include dates for training received on safety and general laboratory QA. Once

deemed competent to perform the LPA, each staff member should test specimens sent for LPA analysis for either an Inter-Laboratory Comparative Analysis (ILCA), or External Quality Assessment (EQA). Accurate results on these specimens will provide documented proof of ongoing competency. In addition to training on the LPA itself, training is also required on the proper use, care and maintenance of all equipment used in the assay. For example, if the thermal cycler has not yet been programmed, or if it has been programmed for a different number of cycles than needed for the specimens being tested, the individual performing the LPA must be able to accurately program the instrument.

In addition to training and competency records, staff personnel files must contain personal information including educational background and professional registrations as well as all other certificates of qualification. Health evaluations and information on any TB exposure may also be included. The personnel file will determine if the person is qualified to perform the LPA according to ISO 15189 requirements.

DIVISION OF LABOUR

In order for the molecular TB laboratory to function optimally, to maintain the best possible turn-around times, and to lessen chances for contamination, it is recommended that the LPA should not be performed from start to finish by a single staff member. Instead, the assay process should be broken up so that different staff members are performing separate parts of the procedure in the different rooms. Because separation of the work may make it difficult for staff to remain competent in performing all steps in the procedure, staff should rotate to different areas on a regular basis, for example weekly.

STANDARD OPERATING PROCEDURES (SOPs)

All methods used in the laboratory for the LPA must be saved as “master copies” in the laboratory’s Standard Operating Procedures (SOPs) manual. Each SOP must include a description of the OBJECTIVE or PURPOSE of the method, the PRINCIPLE, the RESPONSIBILITY of the person(s) who performs the procedure, the PROCEDURE itself, the TYPE OF SPECIMEN tested, the number of QC controls and corrective actions for QC failures, LIMITATIONS of the method, and the MAINTENANCE, VALIDATION, and CALIBRATION of the equipment. All of these sections will differ for each specific SOP and some sections may not apply to all SOPs.

Step-by-step descriptions of the procedures (Bench Operating Procedures [BOPs] based on the SOPs) performed at a particular bench or room must be kept at each work site. A flow chart (ideally a copy that has been laminated) with detailed steps for each procedure can be placed on the wall over the work area in addition to having bench copies available.

QA DOCUMENTATION

QA documentation will include manuals or files created to store procedural documentation of all aspects of the laboratory, and will serve as documented proof of QA within the laboratory during an accreditation investigation. It is recommended that the documentation consist of the following sections:

1. **Standard Operating Procedures** (SOPs)
2. **Equipment**: one file for each group of instruments
3. **Internal Quality Control**: containing documentation of the quarterly sentinel tests, with the strips included as proof, in addition to other IQC documentation
4. **External Quality Assessment**: containing the results from inter-laboratory comparative analysis and panel testing from participation in EQA programs

5. **Reagents**: documenting the reagents used during the month and their respective lot numbers
6. **Safety**: containing the first aid kit inventory for each month and the eye wash log sheet indicating weekly replacement of the saline solution
7. **Audit**: containing problems or non-compliances to ISO 15189 found during assessments by internal auditors and the accreditation body
8. **General**: containing the cleaning and maintenance log sheets of the dedicated areas
9. **Templates**: containing all the operational templates used within the laboratory
10. A **Laboratory Quality Management manual** may also be included, with contents applicable to the overall management of the laboratory.

SOP Manual

This manual should be categorized into four sub-sections:

1. **General:** containing the detailed description of each method including sampling, organograms, workflow diagrams, publications, and other relevant information.
2. **Test Method**: containing Bench Operating Procedures (BOPs) for all tests performed.
3. **Equipment**: containing SOPs on the use, maintenance, calibration, etc. of all the laboratory equipment.
4. **Information Technology**: containing SOPs on how to use the Laboratory Information Management System (LIMS), e.g. how to create a worksheet, enter a result, or review a result.

All “master copies” of the SOPs used in the laboratory must be kept in the SOP Manual. These SOPs must be updated on an annual basis in case there was a

change in the procedure during the year. SOPs must also be reviewed and signed by all staff annually.

Equipment Manual

All equipment in use must have a manual or section of a manual containing records pertaining to each “event” in the life of that particular piece of equipment. This file must contain all the details of each instrument in the form of an “Equipment history log sheet”, which in turn must contain the instrument number, serial number, inventory number, model name and number, date received, date placed, and current location. In addition, the manual must contain the validation and calibration reports, temperature chart records if applicable, service and repair records, maintenance records, respective SOP for the particular instrument, as well as the instruction manual for easy access should the machine develop problems.

Equipment should be grouped according to type and thus all microfuges should be in one section, all thermal cyclers in another, and so on for all the equipment used. Each type of equipment will likely have various sub-sections as shown in **Figure 48**. Servicing schedules for the different apparatuses must be placed on a notice board nearby to serve as a reminder that periodic services are required. The equipment SOPs must also state the number and types of services that are required each year for each tool.

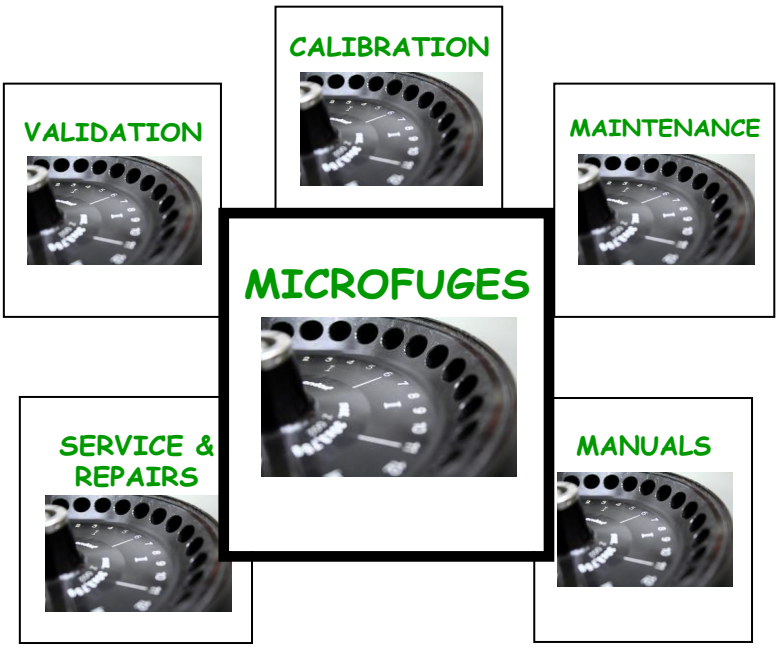


Figure 48: Example of the Microfuges Manual with its dedicated sub-sections. The log sheets pertaining to each piece of equipment must be filed in the corresponding sub-division.

The recommended sub-sections for each type of equipment such as micro-centrifuges should include the following:

1. Equipment list.
2. An individual history log sheet for each piece of equipment, containing information about the model number, serial number, current location.
3. SOPs: containing instructions on the use, maintenance, validation, of the instrument.
4. Service and repairs: containing all the service reports and supporting documentation (proof that the instrument has been serviced as required by the manufacturer).
5. Validation: containing the validation results after a service or repair to verify that the instrument is functional.
6. Calibration: containing the calibration certificates given by the qualified service technician.

7. Decontamination SOP: containing the decontamination procedures specific to an instrument and/or the laboratory decontamination SOP.

8. Decontamination clearance certificates: these certificates guarantee that the piece of equipment to be serviced has been decontaminated according to the manufacturers' instructions and that it does not pose any biohazard to the service technician.

9. Manuals: containing the operating instructions as specified by the manufacturer. If the manual is too heavy or big, the front page can be copied and placed in the file, stating that the manual can be retrieved from the cabinet in which it is kept in the laboratory.

The Equipment manual with sections for all the types of equipment is an essential part of QA, since ISO 15189 requires that the records of all the technical contents of the laboratory are kept. Proper maintenance ensures that the useful life of the equipment is extended. The documentation of servicing and maintenance found in the manual can serve as an invaluable tool when troubleshooting is required. The manual also provides information on the equipment used for specific specimens should problems arise.

Reagent Manual

The GenoType® MTBDR*plus* kit (Hain Lifescience) and the HotStarTaq® (Qiagen) are commercially produced and thus a copy of the manufacturers' instructions and specifications must be kept in the Reagent Manual. Also, monthly reagent log sheets used to document all the different reagents and their lot numbers must be kept in the manual. This will include the reagent log sheets of the DNA polymerase and master mix associated reagents, the lot numbers of the strips inside the GenoType® MTBDR*plus* kit, the itemised reagent log sheet (available from Hain Lifescience) with the lot numbers of the hybridization solutions, and the stock control log sheet for a particular month. All this information is needed when trying to troubleshoot problems and identify possible sources of contamination.

SPECIFIC RECOMMENDATIONS FOR PROCEDURAL QC

Note: For ease of instruction, the batches described below will consist of 93 clinical specimens and 3 controls. Numbers used in the actual performance of the LPA will vary according to the workload in each laboratory.

In the laboratory, it is required that each patient receive a unique patient identifier number as an essential safeguard against specimen mix up. Even when specimens have unique numbers, there may be a mix up with the batch numbers or with the tests requested. This problem may be noticed only after the results have been sent to clinicians, who will be initiating treatment based on the laboratory results. This error can be very serious, especially if a patient who is actually infected with MDR-TB is diagnosed as TB negative, or vice versa, a TB negative patient is put on treatment for MDR-TB. Thus the QC procedures described below are critically important to ensure that the results on patient specimens are accurate.

The thermal cycler capacity determines the maximum number of specimens to be batched together onto each worksheet. If the thermal cycler has a 96-well capacity, a maximum of 93 clinical specimens and 3 controls can be tested during a single 30 or 40 cycle PCR run. Usually, a thermal cycler run takes approximately 2 - 2.5 hours, and thus one can perform up to three batches of 93 clinical specimens (279 patients) per day. The positive and negative controls will always constitute the last 3 spaces in the batch irrespective of the number of specimens to be tested. Because different types of specimens can be tested, there should be different worksheets created for each type of specimen, and different PCR runs for a batch of processed patient material (sputum) versus a batch of cultured isolates. When thinking about the correct number of amplification cycles for the different specimen types, it is good to remember that “less is more”: because there is “less” DNA extracted from sputum specimens than from cultured isolates, “more” (40 cycles) will be required for sputum specimens than for isolates (30 cycles). This does not apply to the hybridization and detection processes since the test parameters during those steps are the same for all specimen types.

QC during DNA extraction procedure

Two sets of clean screw cap 1.5 ml tubes will be needed for each specimen during the DNA extraction procedure. These tubes are to be marked and labelled according to the organization of the batch on the worksheet (see Chapter 4: LPA Procedure) as follows. The tubes containing the live *bacilli* or patient specimen can be marked only with the number from the worksheet. The second tube, which will contain the extracted DNA, will have both the worksheet number written on the lid and the unique patient identifier number written on the side, or affixed as a label (**Figure 49**) It is recommended that the first tube in every batch of extracted DNA should be labelled with a coloured sticker (**Figure 50**), and the worksheet number written on it.

Figure 49: (**A**) The tube (here containing extracted DNA) will have the unique patient ID on its side and the corresponding worksheet number on its lid (circled in red). The number on the lid corresponds to the number of the screw-cap tube containing the live *bacilli* or patient specimen, which in turn corresponds to the number on the worksheet. (**B & C**) The batch number of the specimens that were extracted is written on a larger label that is taped to the side of the rack. The rack that contained the live bacilli would have had an identical label on it. The date on which the extraction was done, and the signature of the person who did the extraction must be on the label. The Extraction positive control (H37Rv) and the Extraction negative control (ENC) are numbers 94 and 95 respectively. Tube number 96 acts as place holder for the master mix negative control (MNC). Please note that the unique patient identifier numbers in example (**A**) consists of 8 digits (SGT 30397***), and the specimens are not to be confused as all being SGT30397.

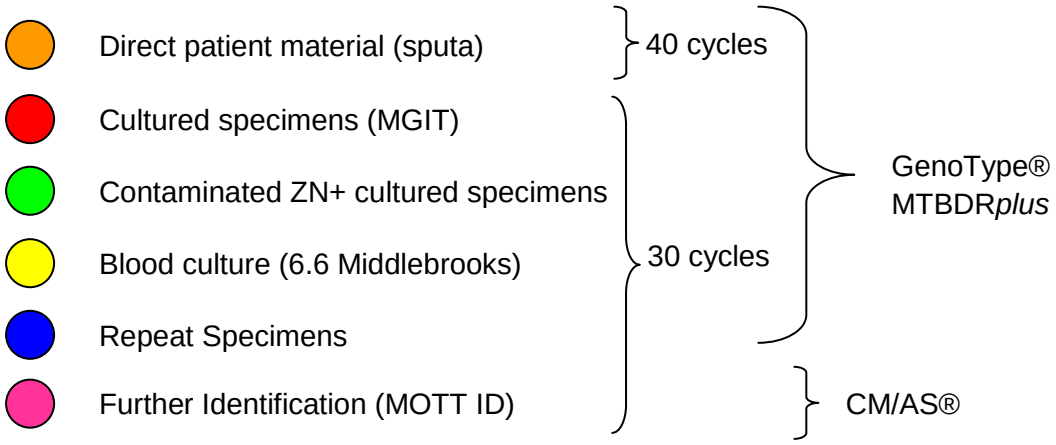


Figure 50: Examples of colour-coding of the different specimen types, and test method required for the specific colour.

It is important that each specimen should be tested correctly according to the request from the clinician. Communication within the laboratory is an important part of each laboratory's QA program. If, for example, the clinician has requested identification of any AFB present, the DNA of a specimen that was smear-positive but negative for *M. tuberculosis* on the GenoType® MTBDR*plus* LPA should be labelled appropriately to ensure testing in the CM/AS kit (if available) for identification of NTM. Failure to indicate that the CM/AS kit is needed would result in the specimen being unnecessarily tested again in the GenoType® MTBDR*plus* LPA, and wasting valuable time and reagents.

As described in Chapter 4: LPA Procedure, it is recommended that the first "space" in all racks be left open for the subsequent moving of tubes after completing each transfer or addition of specimen, reagent or DNA. The space where the first tube was will then be open to receive the second tube after transfer or addition has taken place. This should be done for all racks of tubes during each step in the procedure. In addition, there should be enough finger space so that each tube can be picked up individually. **Figure 51** shows an example of how the tubes should be placed into the tube rack.

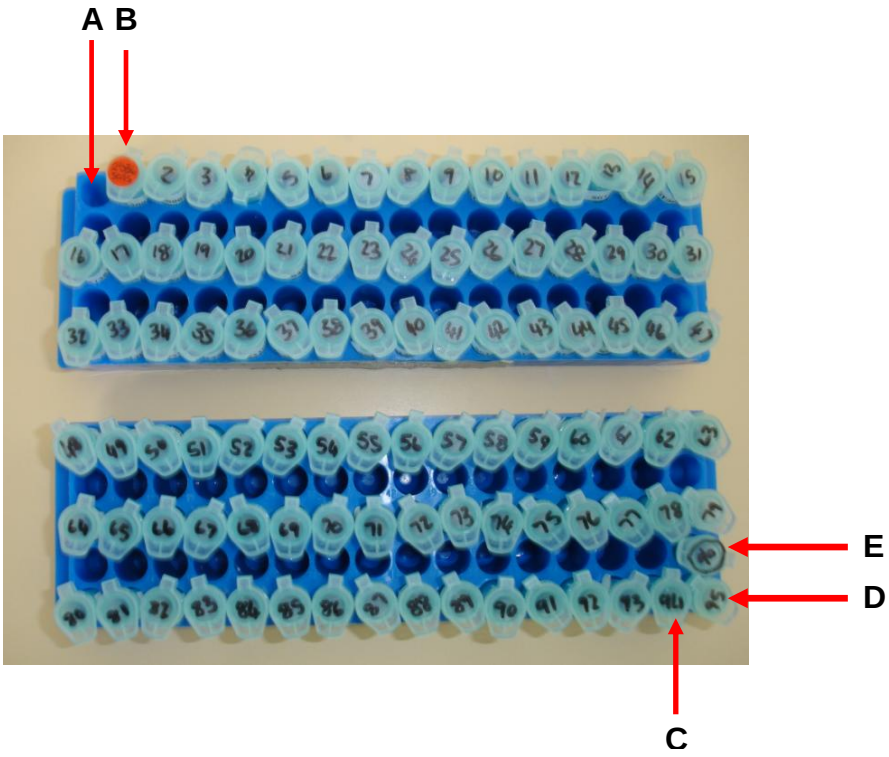


Figure 51: Example of a tube rack containing marked tubes that contain DNA extracted from a batch of 93 specimens. Note that provision has been made for ample "finger-space" between the specimen rows. (**A**) After adding the DNA from the first tube into its respective PCR tube, the tube can be placed into the space provided as indicated by the arrow, thus differentiating between DNA that has been added and DNA that still needs to be added. (**B**) The first number in every batch is marked with a specific coloured sticker to indicate the type of specimen. The label also contains the number of the worksheet (PCRTB 2010). The red sticker indicates that the DNA was extracted from cultured isolates, indicating that 30 PCR cycles must be used. (**C**) Tube 94 is the extraction positive control ($H_{37}Rv$). (**D**) Tube 95 is the extraction Negative Control (ENC) with water used to re-suspend the pellets. (**E**) Tube 96 (black circle on the lid) is the master mix negative control (MNC).

Extraction Equipment:

Equipment readings should always be taken before beginning the extraction process. This applies to the BSC, centrifuge, hot air oven (or heat block or water bath) and sonicator. It is important to stress that a faulty BSC should not be used. If other instruments are out of range, they should be adjusted or replaced if necessary. Pipettes need to be re-calibrated or replaced as soon as possible if they are not

delivering the correct volumes. Failure to check the functionality of the instruments prior to processing could result in wasted specimens, time, consumables, or perhaps all of the above. For example, if it were established at the end of the procedure that the hot air oven or heat block used for DNA extraction had been set at 70ºC instead of 95^{o}C, the results of the entire procedure would be questionable, since weak signals would likely be seen on the strips due to insufficient cell lysis and low levels of DNA. Also, some *bacilli* may have survived the heat-kill step and staff may have been unknowingly exposed to infection.

QC during the preparation of the master mix

The QC required for this area relies mainly on:

1. The proper functioning of the pipettes (which need to be calibrated on a quarterly or bi-annual basis);
2. The homogeneity of the different reagents used;
3. The ability of the refrigerator/freezer to maintain - 20ºC.

Visual inspection of the reagent volumes when drawn up inside the filter tips will also help with QC, thus ensuring that the volumes added to the master mix tube are correct. Reagents such as the 10X buffer and $MgCl_2$ must be properly thawed to ensure that the concentrations of the components are evenly distributed throughout the vial. A common problem that occurs during the preparation of the master mix is that the operator forgets to add the DNA polymerase. It is advised that the master mix be held up to the light while adding the DNA polymerase in order to visually verify that it has been added to the purple PNM buffer solution (**Figure 52**).

Figure 52: Visual inspection of the addition of DNA polymerase to the PCR master mix. (**A**) Tip containing the DNA polymerase is inserted in the middle of the tube to prevent contamination. Note that the purple PNM mix is homogeneous prior to addition of the enzyme. (**B**) When adding the DNA polymerase to the PNM mix, the enzyme starts out with a higher viscosity than that of the PNM mix and one sees a cording effect. (**C**) The “cords” then sink to the bottom of the tube. The PNM master mix will become heterogeneous again once the tube has been gently inverted a few times.

The DNA polymerase needs to be stored at - 20ºC, and thus the freezer in the pre-amplification area needs to be monitored by daily checks to ensure that the temperature stays at - 20ºC. It is recommended that insulated coolers previously stored at - 20ºC be used on the bench-top to keep the DNA polymerase cold while preparing the master mix. The lot numbers and expiration dates of the DNA polymerase and the PNM mix need to be documented on reagent log sheets dedicated to this room. When a new lot of polymerase is received, quality control specimens should be tested using the new reagent. The new polymerase must be accepted only if the results are comparable to those from previous lot numbers. A permanent marker pen and a normal black-inked pen, dedicated for the use of the labelling of the PCR tubes and PCR rack, as well as the log sheet documentation,

are needed in this area. Pens or markers from other sections of the laboratory should not be brought into the reagent preparation area.

The numbering of the tubes (containing the 45 µl of aliquoted master mix) must be standardized to maintain continuity throughout the entire process. This numbering is essential in case the PCR rack containing the tubes with the DNA or amplicons is accidentally dropped, and the tubes need to be re-arranged into the correct order. If the tubes are numbered, they can be correctly rearranged in the rack, thereby eliminating the chances for specimen mix-ups. **Figure 53** illustrates a recommended method for numbering these small tubes in a 96-tube PCR rack.

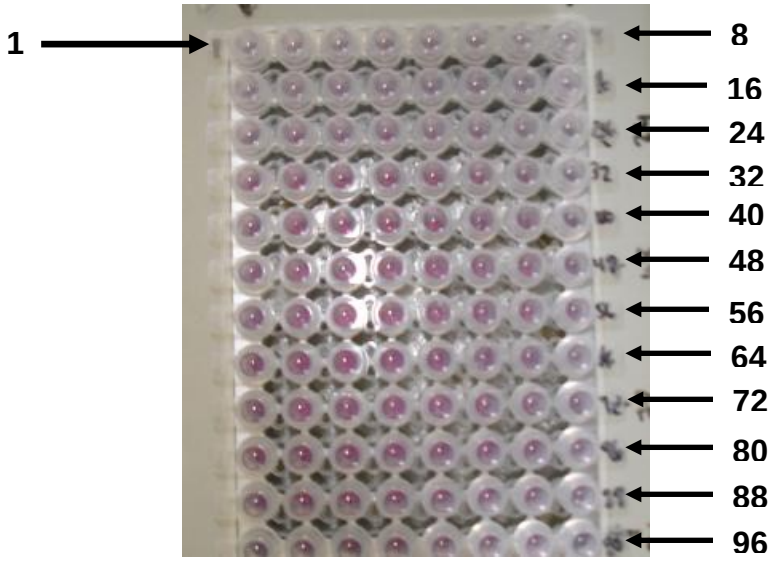


Figure 53: Numbering of the PCR reaction tubes on the sides of the lid. To ensure consistency these numbers must always remain in this format if 93 clinical specimens are going to be tested.

Always make sure that all the PCR reaction tubes have received the PNM master mix. To see if one or more tubes have been accidentally skipped, the PCR rack can simply be held upright above ones head and visually inspected from below (**Figure 54**)

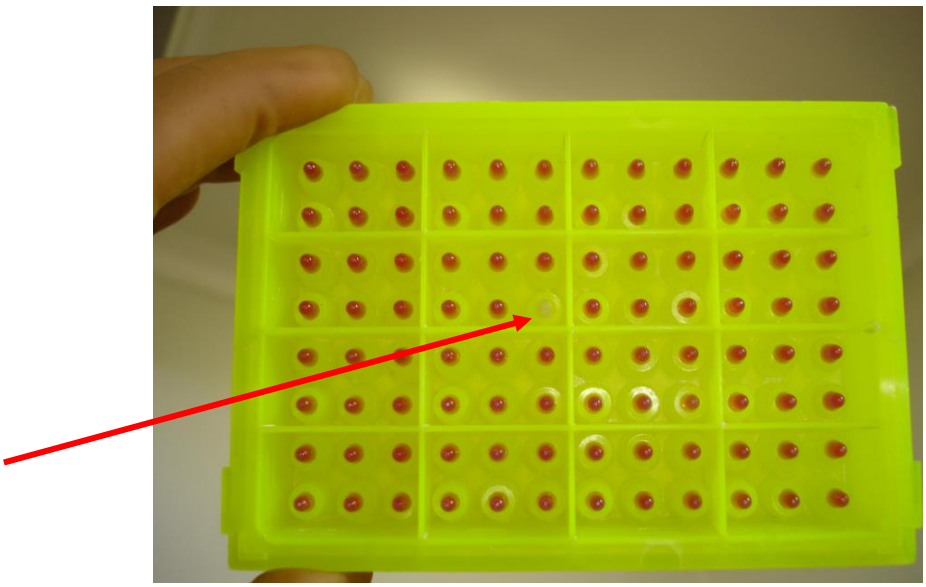


Figure 54: Visual verification that 45 µl of the master mix has been added to all tubes. By carefully lifting the PCR rack with the PCR tubes containing the master mix, it is easy to spot a skipped tube. Note that one tube has been skipped in this example. If the operator does not check for skipped tubes, one specimen in the batch of 93 specimens will not be amplified, delaying the turnaround time for that specimen.

The person who prepared the master mix must initial the side of the lid of the PCR rack and also write the date when the mix was made (**Figure 55**).

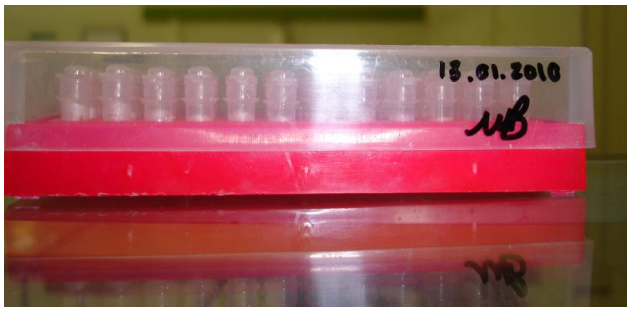


Figure 55: Initials of the person who prepared the master mix. If the reagent preparation was done in advance, the aliquoted PCR tubes must be stored at - 20ºC and used soon after removal from the freezer.

QC during DNA template addition

It is critically important to ensure that the thermal cycler program selected for each run corresponds to the type of specimen to be amplified. Cultured isolates should never be amplified for 40 cycles, since the DNA polymerase will be depleted within the first few cycles due to the increased amount of DNA present. In addition, the caps of the PCR tubes should not contain any liquid when loaded into the thermal cycler. If liquid is present in the cap, the volume in the reaction mixture will be reduced, and will have a negative influence on the amplification efficiency. It is also advised to check the PCR reaction tubes for any bubbles that are caught between the bottom of the reaction tube and the reaction mixture (**Figure 56**). Failure to get rid of the bubbles will result in uneven temperature distribution within the reaction tube.

Figure 56: Examples of what to look for during visual inspection of the PCR reaction tubes before they are placed in the thermal cycler. (**A**) No liquid should be in the domed-caps. If seen, the tubes can be briefly spun down in a mini-fuge or swung down by hand. (**B**) All bubbles at the bottom of the PCR reaction tubes must be cleared by lightly tapping the bottom of the tubes on the bench (without getting any of the liquid in the lid), or spinning in a mini-fuge.

If more than one thermal cycler is used, they should be numbered, e.g. thermal cycler 1 and thermal cycler 2. It is also recommended that dedicated numbers corresponding to the numbers on the PCR tubes be assigned to the wells of the thermal cycler (**Figure 57**). The first PCR tube, containing the DNA of the first specimen on the worksheet, must always be placed into well number one of the thermal cycler. This ensures consistency for all subsequent PCR reactions. Once the

reaction tubes have been loaded and the lid of the thermal cycler is closed, the thermal cycler program should be checked to ensure that the volume to be amplified is set to 50 μl.

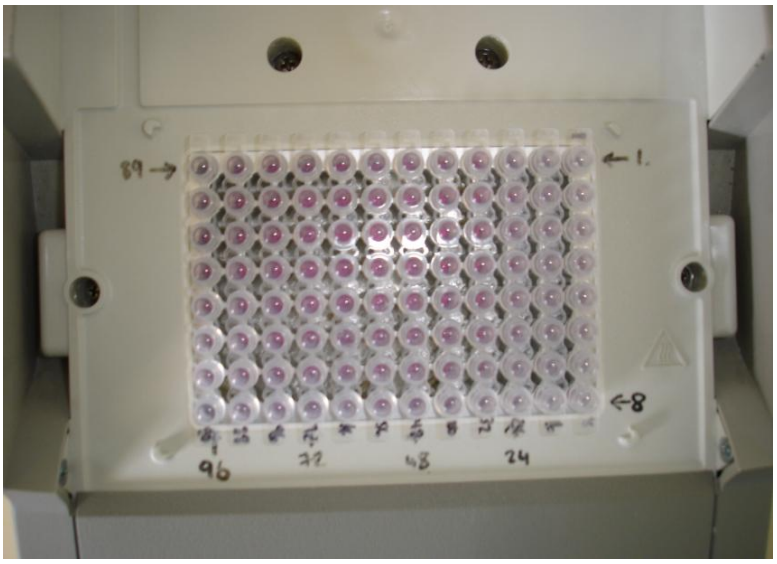


Figure 57: Example of a thermal cycler indicating the designated wells for the respective PCR tubes. The numbering of the PCR tubes must correspond to the numbering on the thermal cycler.

Once the thermal cycler is running, the information that was transcribed onto the tape on the side of the Eppendorf racks containing the extracted DNA (figure 2) should be transcribed onto the lid of the PCR rack (**Figure 58**). The person who added the template DNA to the PCR reaction tubes must also initial and date the top of the lid.

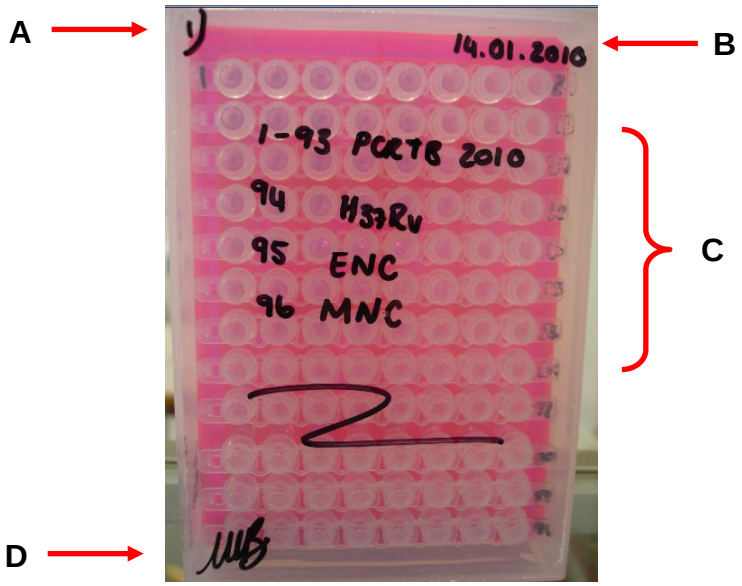


Figure 58: Example of labelling the lid of the PCR rack so it contains all the information required for proper QC: the identification number of the thermal cycler; the date that the DNA templates were added; the worksheet number of the batch of specimens; the controls; and the staff member who added the template DNA. (**A**) The thermal cycler identification number should be on the top left of the lid of the PCR rack. (**B**) The date of the amplification should be on the top right-hand side of the lid. In this example, the worksheet number (e.g. PCRTB 2010) that was written on the tape and placed on the side of the tube rack was transcribed onto the lid of the PCR rack in exactly the same sequence. (**C**) Example of the worksheet information that was transcribed onto the PCR rack after the DNA was added. Note that different batches extracted from the same type of specimen can be merged into the same PCR run, granted that the same number of cycles is required. For example, this merged run can contain cultured isolates; isolates from solid media that were inoculated from different sources. However, it is recommended that smaller batches must each have a positive control and negative controls. (**D**) The person who added the DNA to the tubes with the master mix must sign at the bottom left corner of the lid of the PCR rack.

QC during the hybridization and detection procedure

One of the key factors of internal quality control is consistency in the numbering process. The strip marked with the number 1 must correspond with the first specimen that was extracted, amplified and added to the denaturing buffer in the hybridization well. A dedicated pair of forceps must be used to handle the un-hybridized strips. The strips should be placed on a clean sheet of paper after they

are numbered. It is important to note that the strips are positively charged and static energy between two strips will cause them to “stick” together. Care should be taken to avoid placing two strips that are stuck together into the same well. If using the automated hybridizer (GT-Blot 48), the strips that are marked from 1 to 48 should be used for the first half of a batch of 96, and the strips marked from 49 to 96 should be used for the second half of the batch. The automated hybridiser can also be used for less than 48 specimens. The number of wells into which hybridization buffers are dispensed can be adjusted, and thus if there are 30 specimens to hybridize, the machine can be set to dispense the hybridization solutions only into the first 30 wells. The rest of the wells will be filled with distilled water to ensure an even temperature across the heating platform. If fewer specimens are amplified, the TwinCubator® (12 strip capacity) can be used with one or two hybridization runs depending on the number of specimens. Each run must use a clean tray in the TwinCubator®.

Testing the quality of the strips:

Pooling amplicons from known positive reactions will enable monitoring of the reaction bands on a new lot of strips. Thus, amplicons from a fully susceptible strain and an MDR strain can be mixed and hybridized. A mixed banding pattern should result and indicate that all probes on the strip are working properly.

“Tips” for accurate addition

Coincidentally, a box of filter tips also contains 96 tips, and thus batches of 96 specimens or isolates will require two boxes of 200 µl filter tips (for resuspension of the pellet during extraction and removal of the supernatant) as well as 2 boxes of 20 µl filter tips (one for template addition to the PCR master mix and the other for addition of the amplicons to the denaturing solution). If there are tips left over in the box after the procedure has been completed, it is possible that the operator used one tip twice, resulting in cross-contamination, or that a PCR reaction tube/hybridization well was missed, resulting in “number dropping”. Thus, the used and unused tips can function as “guides” when performing liquid handling. For

example, when adding DNA to the master mix, the number of empty holes in the tip box must correspond to the number of DNA tubes that have moved up a space. Likewise, the number of unused tips still inside the box must correspond to the number of tubes with DNA that still needs to be added to the master mix. Exceptions can happen if a tip becomes contaminated or falls off the pipette. It is suggested that a spare rack of tips be kept on hand to replace the missing tip so that the tip numbers and organization continue to correspond to the number of tubes.

Bringing it all together

Once the amplicons are loaded into a specific machine, in the exact order in which the DNA was amplified, the information that was transcribed onto the side and lid of the PCR rack (**Figure 59**) can be transferred to a tracking list for the LPA (**Figure 60**).

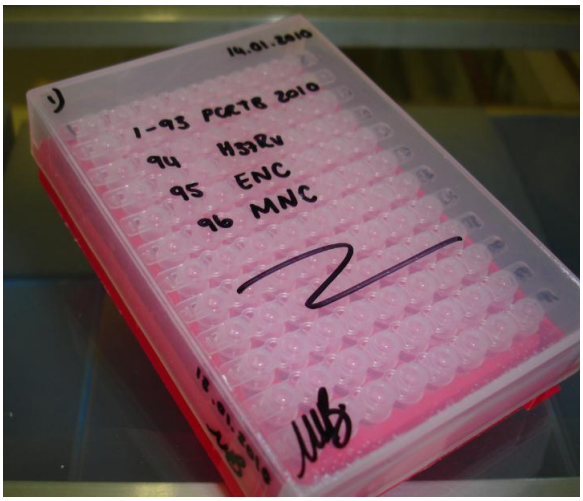


Figure 59: Example of the PCR rack containing all the information required for QC of the pre-amplification and template addition areas. Note that the PCR master mix was prepared a day in advance and stored at - 20ºC.

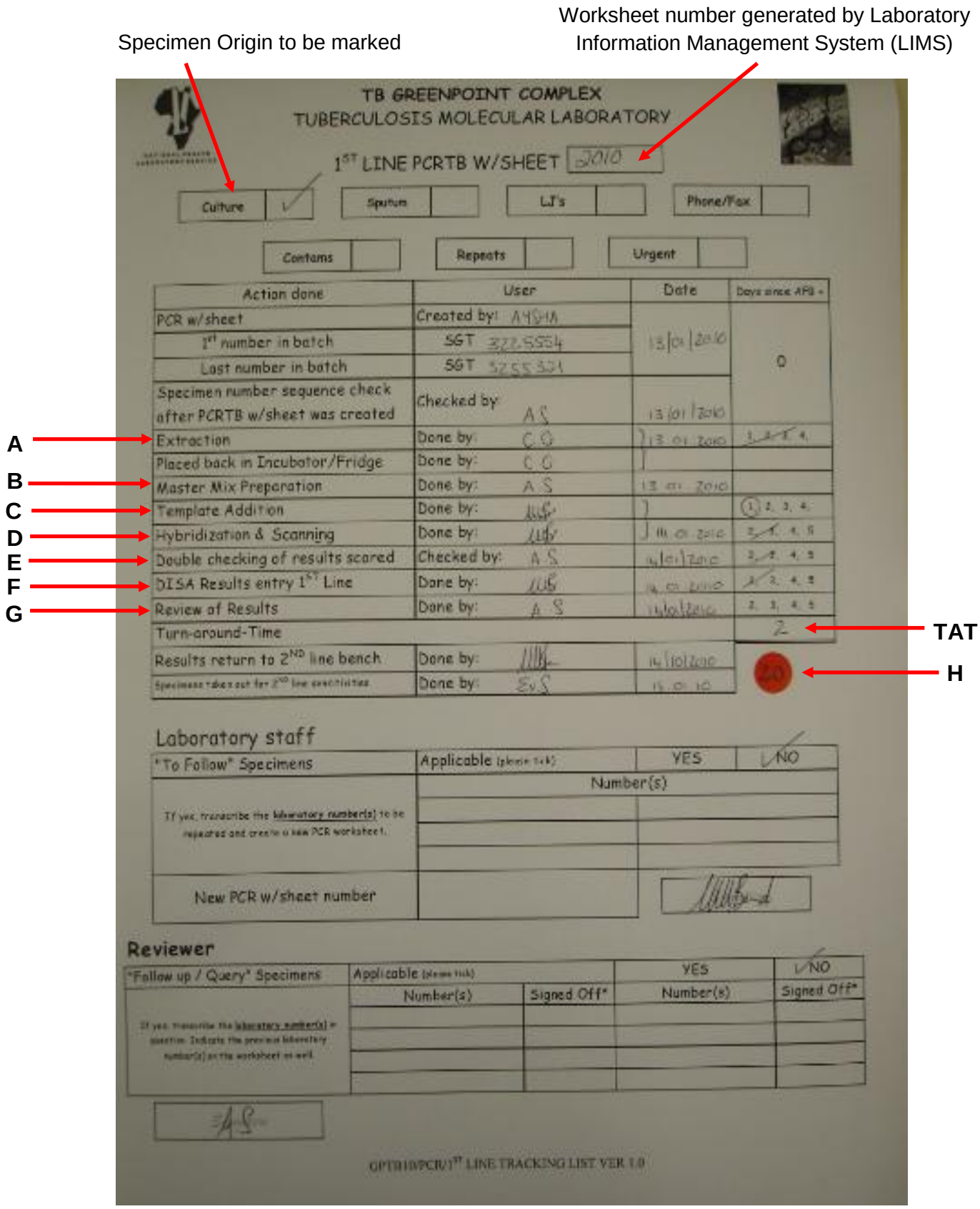


Figure 60: Example of the tracking list used for quality control. The upper section is for the actual test procedure, whilst the two bottom sections are for repeat examinations requested by the reviewer for various reasons. A step-wise documentation procedure indicates (**A**) the individual who extracted the DNA, (**B**) prepared the master mix, (**C**) added the template DNA, (**D**) hybridized the amplicons and interpreted the results, (**E**) double checked the interpreted and transcribed results, (**F**) entered the results into the LIMS, and (**G**) reviewed the results. (**H**) The red dot indicates the amount of resistant specimens requiring second line drug susceptibility testing. The extracted DNA on these (stored at 4ºC) can be tested the next day with the GenoType® MTBDR*s*l kit.

The tracking list ensures the traceability of each of the specimens and should be attached to the front of the worksheet that was created for the specimens before they were extracted. The thermal cycler number and the hybridizing machine number must be documented on the score sheets containing the transferred hybridized strips (**Figure 61**).

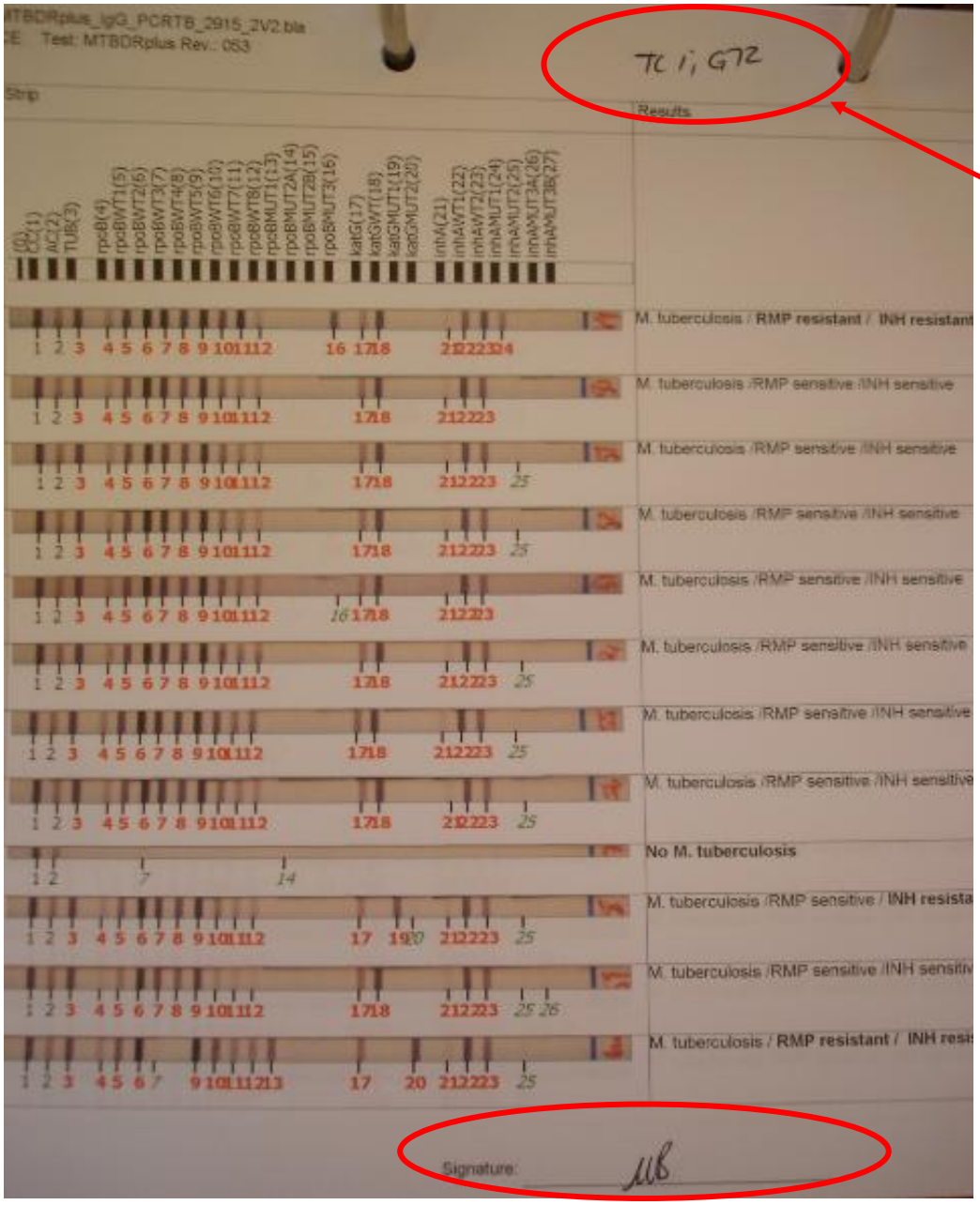


Figure 61: The specimens on this datasheet were amplified in Thermal Cycler 1 and hybridized on GT-Blot 2. The specimens can be traced to these machines. Their manuals will contain the service documentation (functional validations) as required by ISO 15189. The individual who interpreted the results or scanned the strip into the GenoScan® reader should sign at the bottom of the page.

SYSTEMATIC USE OF POSITIVE AND NEGATIVE QUALITY CONTROLS

In order to demonstrate competency for the LPA, positive and negative QC samples must be performed on a routine basis for each batch of specimens. A batch of samples is defined as a group of samples that are processed, amplified and hybridized at the same time, under the same conditions, using the same PCR master mix, thermal cycler and hybridization solutions on the same hybridization platform. It is required that each batch of specimens tested with the LPA must have an "extraction positive control" (ATCC strain $H_{37}Rv$), an "extraction negative control", and a "PCR master mix negative control" (**Figure 62**). The extraction positive control ($H_{37}Rv$) checks the extraction procedure and also verifies that the LPA is capable of detecting the *M. tuberculosis* complex, as well as all the WT (wild type) regions associated with a pan-susceptible strain of *M. tuberculosis*. It must be stressed that cross-contamination can occur with the positive $H_{37}Rv$ control and thus it is required that this control, along with the negative controls, be placed at the end of the batch. The "extraction negative control" (molecular grade water that was used to resuspend the pellet during the extraction procedure) monitors for contamination throughout the entire LPA procedure (DNA extraction, master mix preparation, template addition, and hybridization). The "PCR master mix negative control" (molecular grade water that was used to bring the master mix to volume) verifies that no contaminating nucleic acids / amplicons have been introduced into the master mix.

The amplification control (AC) on the LPA strip functions as both the internal "PCR positive control" and the "inhibition positive control". A positive AC band verifies that the PCR master mix and reagents used during the hybridization procedure were prepared correctly. The AC band will not appear if there are PCR inhibitors in the extracted material. The strips for the two negative controls must be positive only at the conjugate control (CC) and AC bands.

Figure 62: The $H_{37}Rv$ extraction positive control is shown in strip number 9 and the extraction negative control is shown in strip number 10. The PCR master mix control is not shown.

In-house controls

The GenoType® MTBDR*plus* LPA kit does not have a positive control included in the kit and so an in-house strain is required for the *M. tuberculosis* positive control. The original reference strain can be obtained from the ATCC or a well-characterized pan-susceptible strain should be used. The isolate should be "sub-cultured" onto 5 replicate slopes to make "stock cultures". Each of these in turn can be "sub-cultured" 5 times on a monthly basis to make the "working cultures" that are used with every batch. The strain can be used for as long as it is viable and demonstrates the correct morphology and susceptibility patterns. If the intensity of the banding pattern starts to deviate from what is normally seen, a new working stock culture must be used. Care must be taken not to make excessive sub-cultures from the same vial: this may result in variations due to spontaneous mutations. Also, isolates that have been opened many times may become contaminated.

Corrective actions

Upon failure of the positive control, all samples in that batch should be considered invalid, since all negative patient samples may be false-negatives. If this happens, the batch needs to be repeated from the beginning of the extraction procedure through to the end of the hybridization and detection. Before beginning to repeat the procedure, all equipment, stock control specimens, reagents, etc., need to be checked for abnormalities and problems corrected. It may be that the *M. tuberculosis* H_{37}Rv control strain was omitted during the processing, or that the DNA extraction was poorly done.

When contamination is seen in one or both of the negative controls, all work must be stopped immediately and an analysis done to identify and eliminate the source of contamination. All areas and equipment must be thoroughly decontaminated and additional environmental checks (sentinel testing) must be performed. It may be that the unidirectional work-flow between the dedicated areas was corrupted and contaminants were introduced into the work area(s). Once the source of the problem has been identified, the person responsible for the contamination of the controls must be counselled and a corrective action document prepared. To ensure that the contamination event does not happen again, **preventative** action steps should be instituted immediately.

If the contamination source cannot be identified, extra measures need to be taken at each step of the procedure. For example, if a batch is contaminated, the reagents should be replaced with new reagents and the LPA repeated. If the second run is still contaminated, the pipettes should be replaced with a new set of pipettes and the run repeated. If this run is clean, it is likely that the pipettes were contaminated and the problem can be solved once the contaminated pipettes are decontaminated and autoclaved.

Additional PCR negative controls can also be included in a run to further pinpoint the source of contamination. These include:

1. Equipment blanks, where sterile molecular grade water is passed through the equipment and processed as a PCR negative control;

2. Surface swabs, where the swab is resuspended in the molecular grade water and run as a PCR negative control;

3. Sentinel testing, as mentioned previously.

Sentinel tubes should be placed where the liquid handling takes place and not in the corner where no work is being done. It is advised that the laboratory have an SOP for ”Repeat examination due to analytical failure”. This SOP should describe all possibilities that need to be evaluated when examinations fail, and should include corrective and preventative actions.

REAGENT & CONSUMABLE PERFORMANCE

To ensure consistent results between currently used reagents and newly received reagents, lot-to-lot testing must be done when the new reagent’s lot number differs from that of the reagents currently in use. It is suggested that an $H_{37}Rv$ positive control, an MDR strain, an INH-mono resistant strain and a RIF-mono resistant strain, along with two negative controls should be used to determine the efficacy of the new lot of reagents. All reagents used must be tested for contaminants, including the molecular grade water. This allows for the regular monitoring of reagent performance and needs to be documented in the Manual for Reagents. By ensuring that the reagents are reliable, the work can continue without disruptions due to issues with these supplies. Similarly, the filter tips used for liquid handling also need to be monitored, since there might be manufacturing errors that could result in incorrect volumes of liquid delivered by the tip.

ROUTINE TEMPERATURE CHECKS

All temperature-controlled instruments used for the LPA must be monitored on a daily basis to ensure proper functioning. The refrigerators and freezers where the

reagents are stored must be checked for fluctuations in temperature. If one sees that the banding patterns have changed slightly from what was previously seen, this may be due to incorrect temperatures in the refrigerator where the reagents are stored. The ambient room temperature must also be monitored, since incubation of the substrate may need to be extended in colder conditions. In addition to the routine temperature checks of the equipment, periodic maintenance and calibration of equipment are required. The maintenance should be done as outlined in the SOP for that particular piece of equipment and the calibration/servicing schedule should be adhered to strictly.

INTER-READER VARIABILITY

As discussed in Chapter 8: Interpretation and Reporting, there might be inter-reader variability when interpreting the banding patterns on the LPA. The method of interpretation of the banding patterns differs between the paper-based method, where the strips are cello taped onto the score sheet and visually interpreted, and the computer-based method, where the strips are left in the hybridization tray and scanned into a LPA specific computer program, GenoScan®, which interprets the results electronically. It is recommended that the paper-based method be mastered before switching over to the computer-based method. Even though the strips are electronically interpreted, the operator must have prior knowledge of the paper-based method to review the scanning and interpretation done by the GenoScan® reader. This is a way to double-check the computer-analysed banding patterns before electronically saving the results. After the operator is satisfied with the electronic read-out, the GenoScan® provides a printout of the scanned strips. The GenoScan® and laboratory information management system (LIMS) can be interfaced in order to directly link the automated read-out from the scanner to the LIMS, thereby eliminating potential transcription errors. In addition, the GenoScan® reader allows the operator to adjust the cut-off values of the banding patterns to compensate for background staining.

DOUBLE CHECKING OF LPA RESULTS

Laboratories must have a system in place for a two-person double checking of the strip banding patterns. The person who interprets the LPA results, either directly from the paper-based score sheets or indirectly from the electronic printouts, is responsible for the transcription onto the worksheet as well as entering the transcribed results into the LIMS. Due to inter-reader variability upon interpretation of the strips as well as transcription errors, it is required that the interpretation and transcription of the results be checked again by a supervisor in charge of molecular testing, or another competent staff member. After the supervisor has validated the results, the laboratory manager will use the LIMS to check that the results have been entered correctly. It is recommended that, upon reviewing a specimen that was found to be MDR on the LPA, the reviewer send out a report as soon as possible, indicating the patient to be MDR. This may potentially aid the initiation of early treatment.

PANEL TESTING / PROFICIENCY TESTING

The diagnostic proficiency of the molecular TB laboratory can be evaluated by participating in an External Quality Assessment (EQA) program. EQA is an important tool whereby the ability of the laboratory to accurately diagnose a panel of well-characterized specimens (spiked sputum or DNA) is measured.

Potential EQA practices for molecular LPA testing

EQA testing should cover all the steps in the LPA, from specimen preparation right through to master mix preparation, amplification and hybridization. It is suggested that panel testing of aliquots of well-characterized heat killed cultures be diluted and adjusted to 500 µl, thereby mimicking processed clinical specimens. This panel can

consist of the following strains or strains in duplicate (a duplicate panel enables to see if concordant results can be obtained on the same sample):

- A pan-susceptible strain (H37Rv)
- An INH mono-resistant strain harbouring a *katG* mutation
- An INH mono-resistant strain harbouring an *inhA* mutation;
- A RIF mono-resistant strain harbouring a common *rpoB* mutation
- A RIF mono-resistant strain harbouring an uncommon *rpoB* mutation
- MDR strains with both common and uncommon *rpoB*, *katG* and/or *inhA* mutations;
- A NTM strain.

This method is relatively cheap and safe and also allows for the easy dissemination of these panels and can be standardized for all participating laboratories. The negative aspect of such a panel is that it does not control for the NaOH-NALC decontamination process, which forms an integral part of the specimen preparation process.

Another LPA EQA panel that can be considered is that of blinded testing of duplicate clinical specimens. It can consist of 25 duplicate specimens from the same patients, labelled A and B for each patient, or 25 duplicate clinical specimens. This allows for the analysis of concordance and invalid results between the duplicate specimens, as well as the accuracy of the positive controls and the purity of negative controls. However, it may prove difficult to organize a collection of panels that needs to be sent to various sites, in addition to finding appropriate blinded selections of clinical specimens.

Current EQA Programmes

Currently there is no well-established supra-national EQA program for molecular testing for TB but some EQA programs have been implemented on national levels. In

South Africa, for example, the National Health Laboratory Service (NHLS) participates in three different types of Quality Assurance programmes. The first is an Inter-Laboratory Comparative Analysis (ILCA) where the National Institute for Infectious Diseases (NICD) and National Tuberculosis Reference Laboratory (NTBRL) sends a panel of 10 specimens consisting of non-infectious DNA for LPA proficiency testing. This panel consists of the DNA from the following strains: at least 1 pan susceptible strain; 1 RMP mono-resistant strain with a common *rpoB* mutation; 1 RMP mono-resistant strain with a less common *rpoB* mutation; 3 MDR strains with varied mutational banding patterns in the *rpoB*, *katG* and *inhA* genes; 1 INH mono-resistant strain with a common *katG* mutant; 1 INH mono-resistant strain with a common *inhA* mutant; and 2 different NTM's. It is advised that the panels should not be fixed in format and the panel of TB strains should be shuffled. The NTM's species included must also be different with each round of the EQA. However, this ILCA does not control for specimen preparation.

The second panel, sent by the Medical Research Council (MRC), consists of 20 live *M. tuberculosis* specimens that must be subjected to NaOH-NALC decontamination in order to make a smear and MGIT inoculation. Afterwards, the LPA is performed on these cultures. This method allows for the complete proficiency testing of the laboratory, since it takes all benches into consideration. The last panel, sent by the NHLS Quality Assurance department, also consists of 4 live specimens. Once again, all tests performed by the laboratory must be done on these four specimens. Two of these specimens are TB strains, whilst 1 is a NTM and the other an *E. coli*. These three EQA programmes assure that the whole LPA procedure is tested for.

It is very important that the reference laboratory preparing the proficiency test specimens should perform the test on the same platform, i.e. the LPA that will be used by participating laboratories before sending out the panel. It should also be noted that mutations in *rpoB* accounts for approximately 96% of the strains with resistance to RIF. Therefore, 4% of the strains that are found to be resistant to RIF in liquid media would be interpreted as susceptible in the LPA. This will result in a discrepancy between the results of the two assays, and may have a negative impact on the outcome of the proficiency testing. This is another reason why reference

laboratories must confirm the results for the specimens using the same method used by the participating laboratories.

The panels must be treated as routine specimens by the participants and included in the regular workload. The panels must also be performed by the personnel who routinely perform the LPA. This means that the results of the panel testing can also serve as an ongoing competency assessment of the person who performed the tests. If the results are sub-standard, the person who did the panel testing needs to be re-trained in order to ensure the he or she produces quality results.

Another approach to proficiency testing of the LPA has been developed by the FIND India office. In this approach, the proficiency of the laboratory is assessed based on the blinded duplicate testing of 50 smear-positive specimens. Laboratories are required to exhibit concordances of more the 95% on blinded duplicate specimens, with less than 10% unsuccessful amplifications and clean negative control results (no contamination). This approach allows for the easy and immediate identification of basic problems with the performance of the procedure and enables the laboratory to immediately implement corrective actions as soon as the results become available, rather than waiting for feedback from the reference laboratory.

QUALITY INDICATORS FOR LPA

In order to monitor the quality of the laboratory, the laboratory must develop, implement and maintain various quality indicators. An important indicator is the turn-around time that should be monitored in three parts. The first indicator is the time from when the specimen was taken from the patient until it was received in the laboratory (1 - 2 days). If there is a delay in turn-around time, the laboratory should consider another specimen delivery service provider that is able to deliver the specimens within 1 - 2 days. The second indicator is the time in the laboratory from the receipt of the specimen, through the decontamination with NaOH-NALC process and the reading and reporting of the smear result (1 - 2 days). The third part will consist of the time required for extraction of the smear-positive specimens through

the performance and reporting of the LPA result (1 - 2 days). The total turn-around time for these three processes should not exceed 7 days. However, if cultured isolates are used for the LPA, the turn-around time for the complete diagnostic test procedures will be extended due to the time required for growth detection. The turn-around time of the LPA could then be based on the date when the culture became positive for AFB, until the reporting of the LPA result (2 days). Monitoring these indicators allows for the determination of which specific part of the procedure is responsible for any possible delays.

It is suggested that 10% of all *M. tuberculosis* strains that are susceptible to RIF and INH in the LPA should be cultured and once growth is detected, conventional DST and a repeat LPA should be performed. If there are discrepant results between the LPA and the conventional DST, other molecular or conventional methods could be used to clarify the discrepancy.

DATA MANAGENT & STORAGE

It is important to routinely record and store **all** the raw data (both valid and invalid) obtained from the membrane strips (**Figure 63**). This information is necessary for future troubleshooting as well as for accreditation purposes. The databank must preferably be electronic, so that results can be emailed to reference laboratories, with the potential for the creation of a web-based online learning centre (e-EQA). The raw data in the databank should contain common and uncommon mutational patterns, unusual patterns due to NTMs, mixed patterns due to hetero-resistance or partially interpretable patterns, as well as invalid results due to contamination of the negative controls or weak banding patterns.

As previously mentioned, it is recommended that the supervisor and/or manager evaluate the raw data not only before sending the report to the clinician, but also before submitting it to the databank in order to ensure that both the interpretation and transcription were correct, that the controls were consistent, and that the forms are complete. This is also helpful should an independent accreditation body ask to

see the banding patterns. In addition, about 20% of all the results for a particular day must be re-checked in a blinded fashion to make sure that the results were interpreted and reviewed correctly. If any discrepancies arise, the test must be repeated and the clinician notified. After a non-conforming event, corrective action can take place in the form of re-training if the reason for it was due to interpretation of the banding pattern.

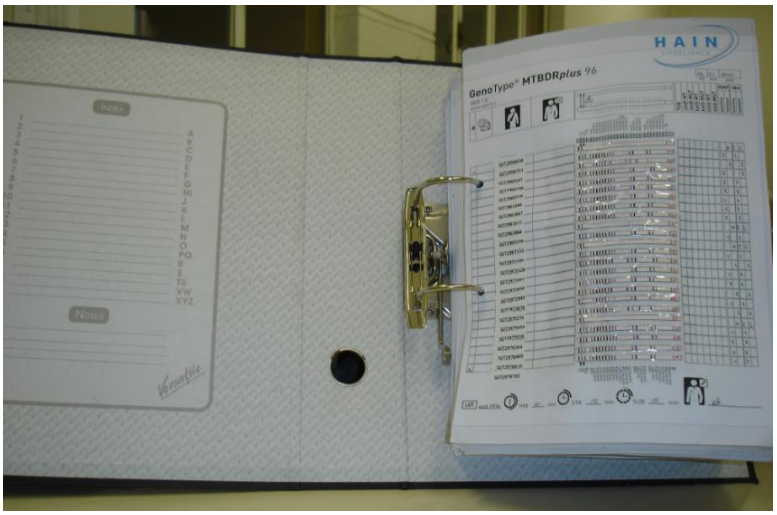


Figure 63: Example of a file containing documentation of the raw data from the LPA strips.

It is suggested that copies of the paper-based LPA score sheet be made immediately after the results have been interpreted and transcribed since the resolution of the bands tend to fade with time. If the GenoScan® is being used, the electronic files should be saved into a monthly folder and backed-up on a monthly basis (e.g. onto a CD, which should be kept in a safe place). It is recommended that these records be kept at an off-site company, where the data is fire- and flood-proof.

From this databank, an “EQA strip panel” can be created to test for proficiency in the interpretation of results in different laboratories, and can also be used as an ongoing competency tool for the personnel doing the LPA on a routine basis. The web-based e-EQA learning site can also be used to assess the competency of the personnel. This strip EQA could consist of a panel of 20 strips, 10 with valid results, 10 with invalid results and 5 with unusual results.

TRACEABILITY

It is required by ISO standards that documentation of test results must be kept for a specified period of time. If the laboratory processes high volumes of approximately 200 LPAs per day, it is suggested that monthly files of the score sheets be kept (**Figure 63**). If the testing volumes are small, the raw data can be filed quarterly. In addition to meeting the requirements set out by the accreditation standards, the storage of test results also enables the laboratory to go back into a patient's history and see banding patterns from previous specimens. These files allow for traceability of patient specimens, and also serve as a record of the internal positive and negative controls used during each run.

In addition, storage of the raw data assists the laboratory with troubleshooting. Should there be a specific problem with the assay, one can refer to these files as a starting point for the investigation. For example, if it was noticed that the strip at position number 5 on the score sheet is always un-interpretable while the rest are clear, it would suggest that the 5th well of the thermal cycler is faulty (provided the wells are assigned specific numbers). The 5th well of the thermal cycler must then be by-passed (not used).

Note: It is usually economically not viable to repair or purchase a new thermal cycler because one well is malfunctioning.

METHOD VALIDATION

The GenoType® MTBDR*plus* LPA is CE marked for In-Vitro Diagnostics, and existing validation data by HAIN LifeScience and scientific articles documenting the reliability of the test are adequate for the required validation purposes. However, each laboratory must validate the LPA against current drug susceptibility tests used in that particular laboratory before implementing the test as routine practice. In addition, the laboratory also needs to perform validations of all equipment, controls and procedures used for the LPA in order to establish if the equipment is functional;

the control strains are correct; the procedures are being followed; and, most importantly, if the results correlate with that of other laboratory tests, including conventional drug susceptibility testing. When completed, the different validation documentation can be filed into their dedicated procedure validation manual. Once the analytical requirements have been established, the performance capabilities of the LPA can be trusted and the test implemented.

IN CONCLUSION

This chapter focused on both general and specific Quality Assurance indicators, which will enable the LPA laboratory to produce accurate test results and eventually become accredited. It is advised that the complete ISO 15189 standard guidelines be consulted in order to obtain detailed clauses for assessing managerial and technical competence. If the accreditation body of the country also makes use of another approval body that has additional requirements, the laboratory must obtain these guidelines and implement their standards as well. In short, the LPA laboratory must ensure that the policies and procedures covering all testing are followed, that the appropriate IQC is implemented, and that the laboratory participates in a recognised EQA and/or inter-laboratory QC programme with regular EQA examinations.

Chapter 9: INTERPRETATION AND REPORTING OF MOLECULAR RESULTS

As with all other tools used in the diagnosis of tuberculosis (TB), the accurate reporting of molecular results relies not only on the correct interpretation of the line probe assay (LPA) banding patterns, but also on the accurate reading of the smear, either from NaOH-NALC decontaminated patient sputum specimens (Ziehl-Neelsen or Auramine-Rhodamine stain) or from cultured isolates (Ziehl-Neelsen stain). For example, if a negative smear or one that contains rare acid-fast bacilli (AFB) has been reported as positive (mistakenly reported as containing 1+ or greater AFB), the LPA performed on this specimen will be either negative or difficult to interpret. This is due to the much lower sensitivity of the LPA on smear-negative culture-positive specimens than for smear-positive culture-positive specimens (smear grading equal to or greater than 1+). Thus, the quality and ease of interpretation of LPA results is dependent on the accuracy of the laboratory performing and reading smears.

This chapter will describe the 27 reaction zones or bands found on the LPA strips. Explanations will be given for the criteria for scoring the bands on the strip to predict susceptibility or resistance to rifampicin (RMP) and isoniazid (INH). While the interpretation of the banding patterns on the completed LPA strips is the most interesting part of the assay, it can also be the most difficult. This is due to the fact that the banding patterns result from amplification of DNA from the total population of *Mycobacterium tuberculosis* cells present in the specimen. This bacterial population may contain various proportions of cells that differ in sequences in the regions of DNA found on the LPA strips. To facilitate the interpretation of banding patterns, this chapter will describe the basis for all presently known patterns found in drug-susceptible and drug-resistant strains, and also in strains with unusual banding patterns. In addition, the following issues will be discussed: performance indicators (sensitivity, specificity and reproducibility) and participation in an External Quality Assessment (EQA) program, recommended to monitor the performance of the LPA; the type of sample to be used; the conventional drug susceptibility testing (DST)

results of isolates containing the point mutations included in the LPA strips; the limitations of the LPA in regards to rare but likely significant *rpoB* mutations not found on the LPA strip (some strains with these mutations have been shown to exhibit low-level resistance to RMP); and the influence of the geographical setting on the negative predictive value of the LPA.

The GenoType® MTBDR*plus* LPA

The GenoType® MTBDR*plus* LPA is based on multiplex PCR technology, followed by reverse hybridization to detect and identify *M. tuberculosis* complex and to predict the susceptibility or resistance to the two main first-line anti-tuberculosis drugs, RMP and INH. The assay screens for the absence and/or presence of “wild-type” (**WT**) and/or “mutant” (**MUT**) DNA sequences within specific regions of three genes: *rpoB* (associated with RMP resistance) and *katG* and *inhA* (associated with high-level and low-level INH resistance, respectively). Each strip contains 27 reaction zones, as shown in **Figure 64.**

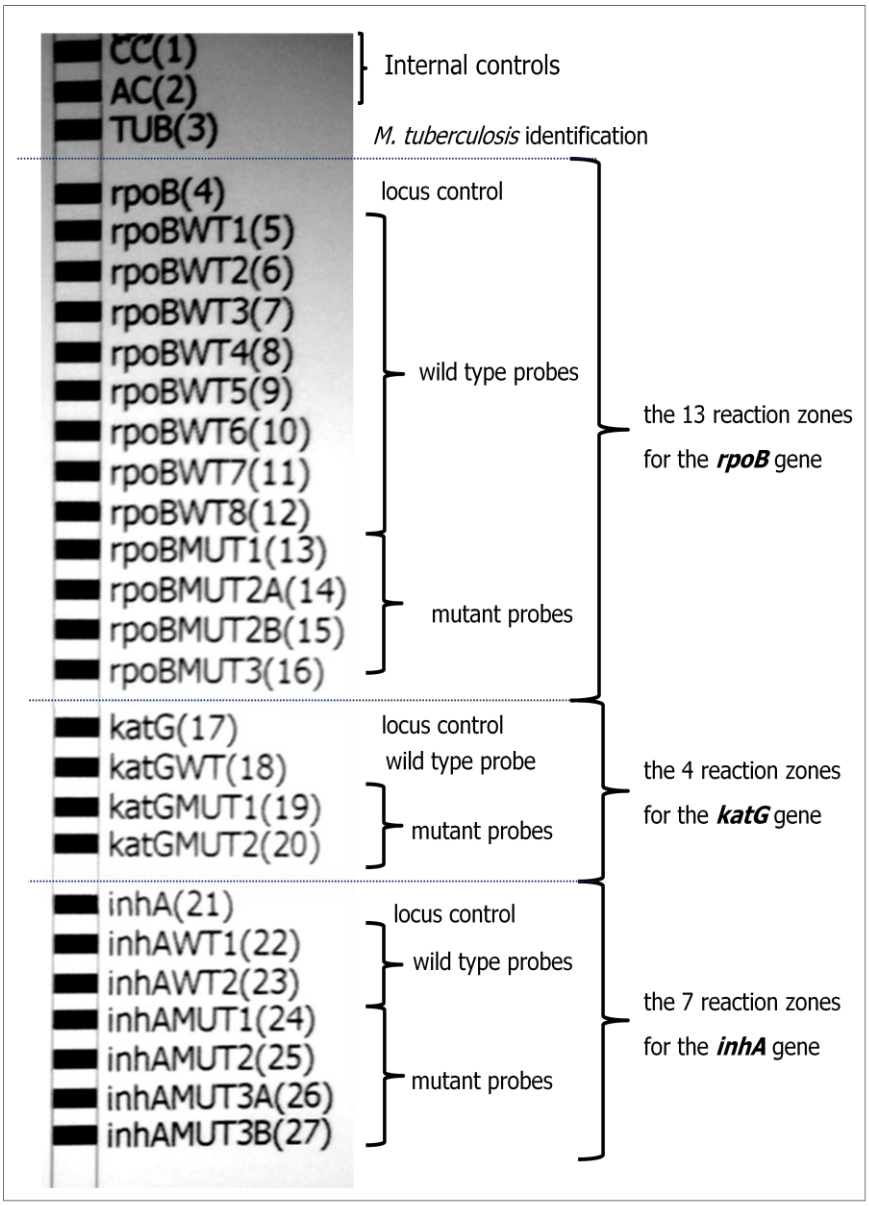


Figure 64: Description of the 27 reaction zones on the GenoType® MTBDR*plus* strip

INTERNAL QUALITY CONTROL

The LPA has two internal controls on the strip shown in **Figure 64**: the **C**onjugate **C**ontrol or **CC** (line no. 1), and the **A**mplification **C**ontrol or **AC** (line no. 2). When positive, these controls indicate that the test has been carried out correctly.

Conjugate control

The conjugate control (**CC**) documents two steps in the procedure:

1. The efficiency of the conjugate (streptavidin-horseradish peroxidase) binding to the biotinylated primers (incorporated into the PCR products that have been hybridized to specific probes on the strip) and;

2. The efficiency of the substrate (hydrogen peroxide) reaction with the conjugate to detect the positive bands (**Figure 65**).

If the **CC** band is negative, either or both of these steps must have been unsuccessful and the hybridization process must be repeated. The absence of the **CC** band may be due to expired reagents or to an analytical error during the procedure, e.g. incorrect dilutions of the conjugate and/or substrate solutions, or the substrate or conjugate solutions were left out (or one was used twice). In these cases, no colorimetric reaction would develop on the **CC** band or any of the other bands. The entire LPA procedure will not need to be repeated from the beginning since the PCR reaction mix contains sufficient volume to perform the hybridization reaction twice, if necessary. However, the entire hybridization process must be repeated. Care should be taken to avoid such mistakes since expensive reagents will be wasted and there will be a negative impact on the turn-around time in the laboratory.

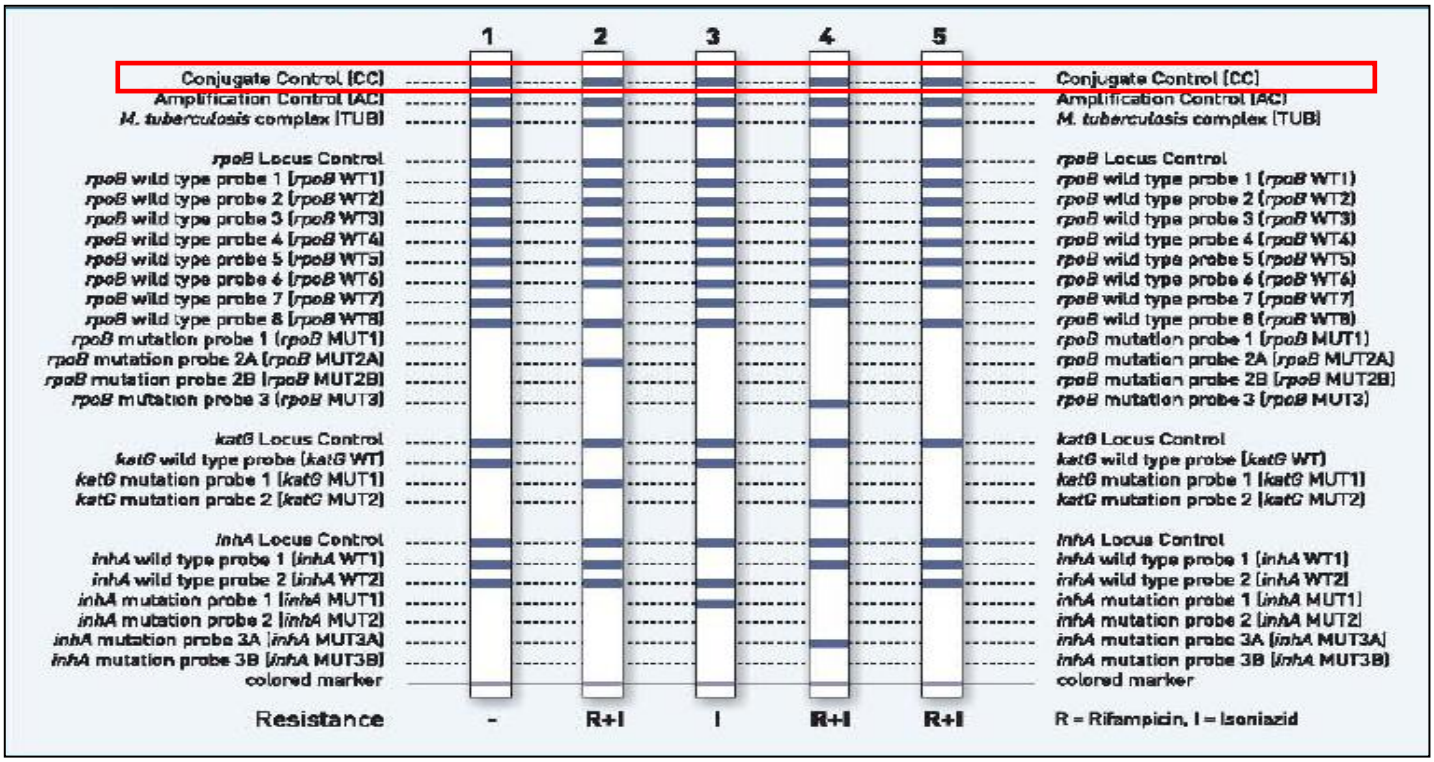


Figure 65: Conjugate Control (highlighted in red) as seen on the GenoType® MTBDR*plus* test strip.

Amplification control

The presence of the amplification control (**AC**) band indicates that the DNA extraction and PCR procedures were carried out successfully. A control product (amplicon) is generated during the PCR reaction and once denatured will bind to the AC probe on the strip (**Figure 66**).

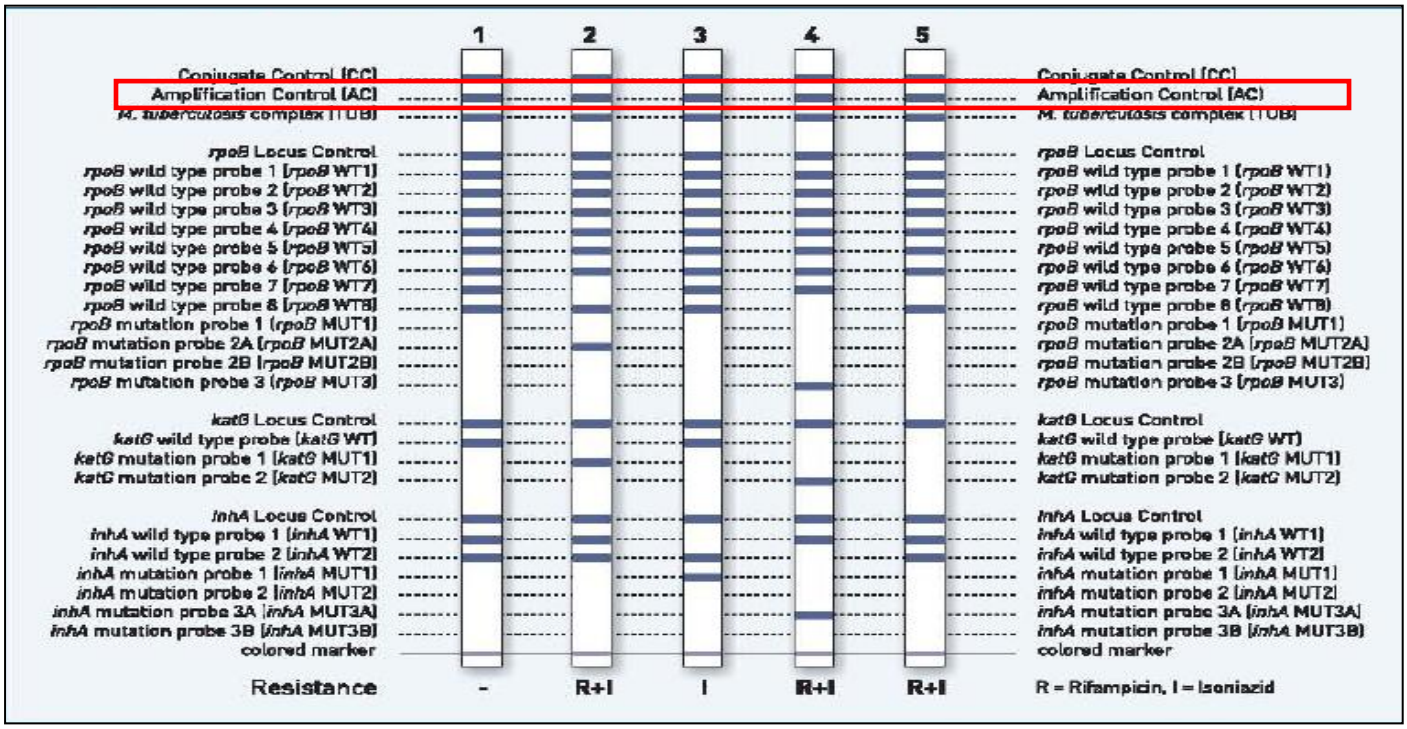


Figure 66: Amplification Control (highlighted in red) as seen on the GenoType® MTBDR*plus* test strip.

This **AC** band will be positive for all known *Mycobacterium* species. Thus, this band is not specific for the *M. tuberculosis* complex. However, if the specimen is positive for AFB, this band should be present. The **AC** band should always appear less intense than the positive test signals (**CC** and **TUB** bands). **The intensity of the AC band functions as the baseline against which all other bands on the strip are to be interpreted/"scored"** (as described below).

The only instance where the strip can still be interpreted as a valid test when the **AC** band is absent is when other bands, such as **TUB**, *rpoB*, *katG* and *inhA* locus controls and their respective bands are present. This somewhat rare result is thought to be primarily due to competition among the **AC**, **TUB**, *rpoB*, *katG*, and *inhA* primers for the limited amount of DNA polymerase that is in the amplification master mix.

If the **AC** band, along with other **WT** bands, is missing, it is likely that the PCR reaction was unsuccessful. Troubleshooting efforts should consider the following possible scenarios:

- When AC bands of all strips in a batch are missing, either a problem occurred or a mistake was made during the master mix setup (e.g. using expired reagents or

not adding the DNA polymerase), or during the PCR amplification (e.g. using a wrong program or a PCR machine failure).

- When AC bands are missing in only one or a few strips in a batch, either the concentrated pellet for these samples may have been lost during the DNA extraction process, or PCR inhibitors were present, or the DNA was not added to the PCR mix.

When troubleshooting to see if a PCR reaction was successful, the products can be visualized following electrophoresis on a 1.5% agarose gel stained with ethidium bromide. It is recommended that when first introducing the LPA procedure into a laboratory, performance of a gel should be done routinely to ensure successful amplification prior to the hybridization step. If the PCR reaction was successful, three separate bands will be observed on the gel. Only three bands will be seen since the fragment sizes of all the amplicons produced are quite similar in size.

M. TUBERCULOSIS IDENTIFICATION BAND

The **TUB** reaction band (**Figure 67**) is only present if the DNA amplified is from members of the *M. **tub**erculosis* complex. Even when other bands are present, if the **TUB** band is absent the bacterium does not belong to the *M. tuberculosis* complex. The presence of non-tuberculous mycobacteria (NTM) in the patient specimen can result in random banding patterns, with several species testing positive at the *rpoB* **WT** bands 1, 4, 5 or 8. A NTM banding pattern may actually resemble that of the *M. tuberculosis* complex, however, the **TUB** band will not be present (**Figure 68**). In busy laboratories, sufficient time must be spent analyzing each strip to ensure that the **TUB** band is present. The eye tends to recognise familiar banding patterns: some **TUB**-negative patterns could be incorrectly interpreted as a valid result for *M. tuberculosis* complex if only glanced at briefly. If there is a need to further identify a **TUB-**negative AFB, either the Common Mycobacteria (CM) or the Additional Species (AS) LPA kit can be used.

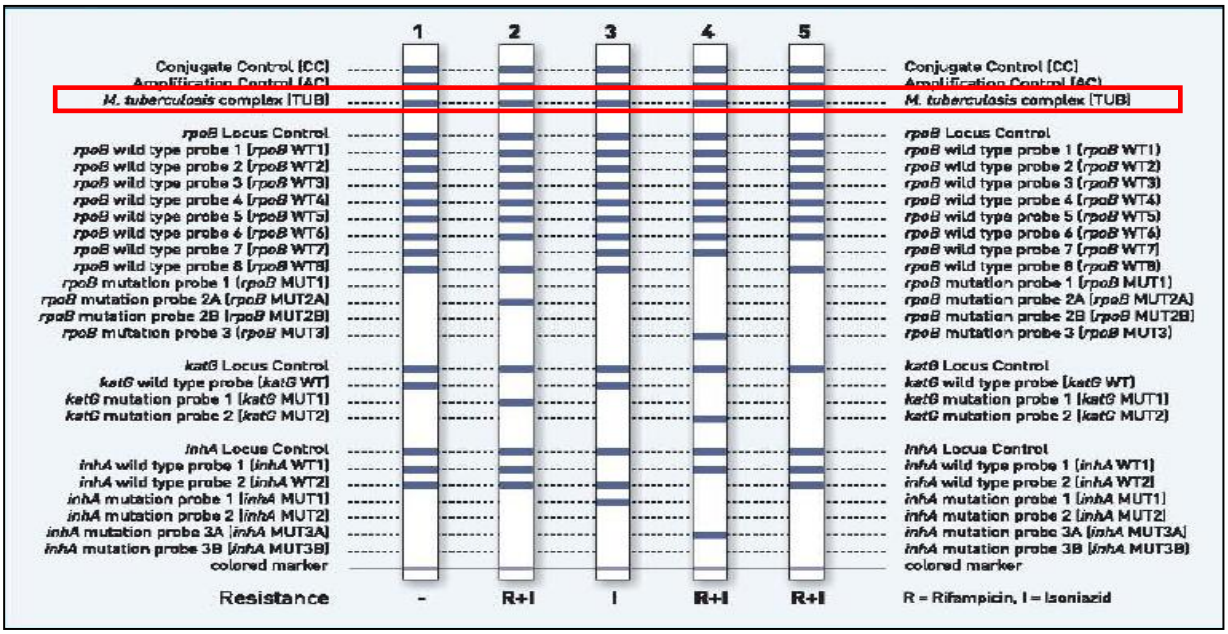


Figure 67: The **TUB** band (highlighted in red) will be present only if the DNA belongs to a member of the *M. tuberculosis* complex.

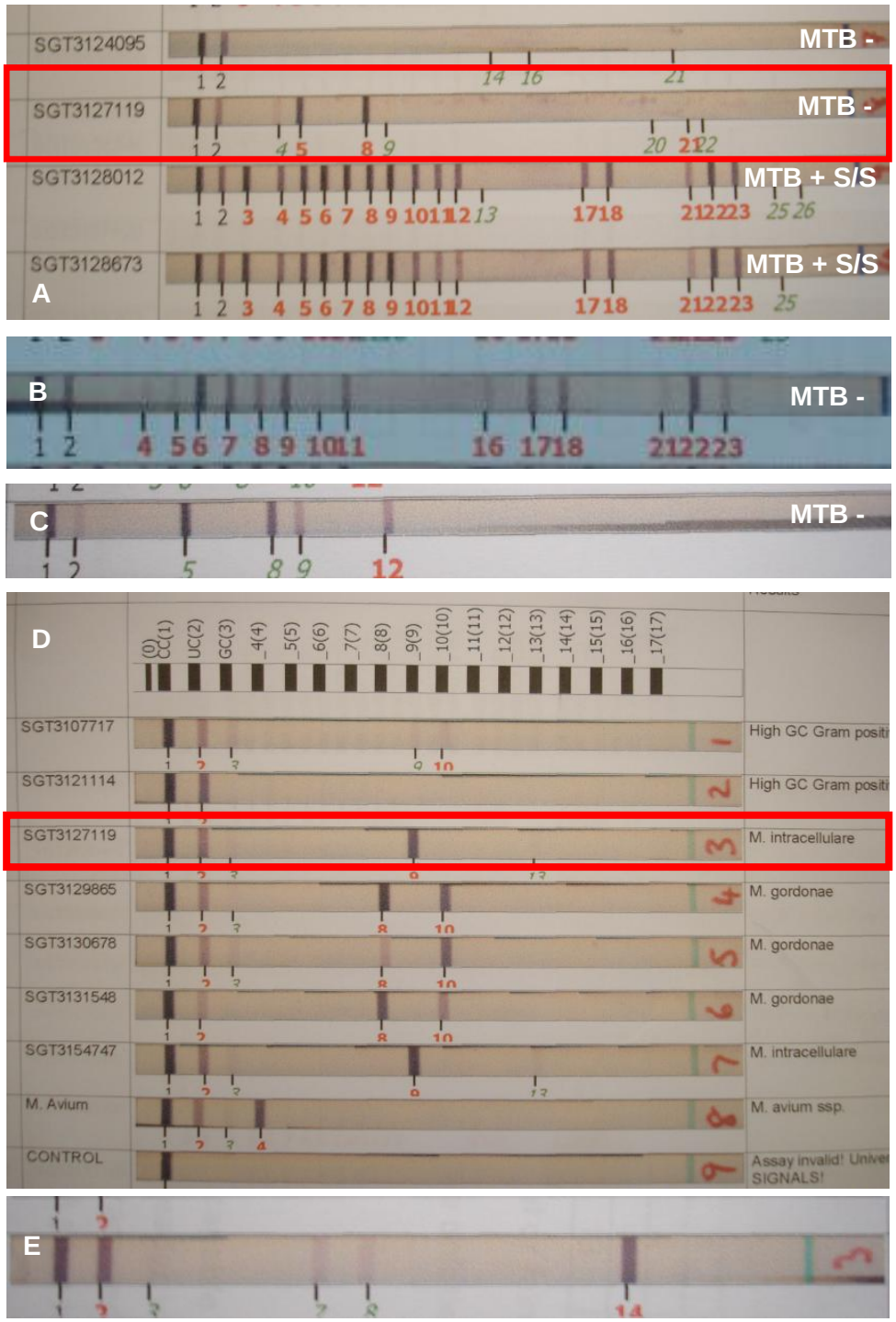


Figure 68: (**A**) The highlighted strip shows an example of the reaction zones where NTM such as *M. intracellulare* most commonly hybridize. (**B**) This example indicates how an incorrect diagnosis can be made due to NTM mimicking a "familiar" banding pattern for RMP mono-resistance. (**C**) Another example of common reaction zones for NTM. (**D**) The CM/AS kit was used to identify the NTM observed in **A** as *M. intracellulare*. Interestingly, the NTM exhibiting the banding patterns in **B** was also identified as *M. intracellulare*. (**E**) The NTM in **C** was identified as *M. peregrinum* on the CM/AS kit. MTB - = Negative for *M. tuberculosis* complex; MTB+ = positive for *M. tuberculosis complex*; S/S = Rifampicin susceptible/Isoniazid susceptible.

rpoB, katG and inhA gene LOCUS CONTROL BANDS

The gene locus control bands for the three genes analyzed on the strip (*rpoB*, *katG* and *inhA*) are located on the strip just before their respective **WT** and **MUT** bands (**Figure 69**). These bands must always be present for the assay to be considered a valid test. However, when only one gene locus control band is missing, the validation of results for the two other genes is not affected and they can be interpreted. Only the results for the gene with a missing locus control band are not interpretable. In all the cases, if the **TUB** band is absent, the organism does not belong to the *M. tuberculosis* complex even if these and other bands are present.

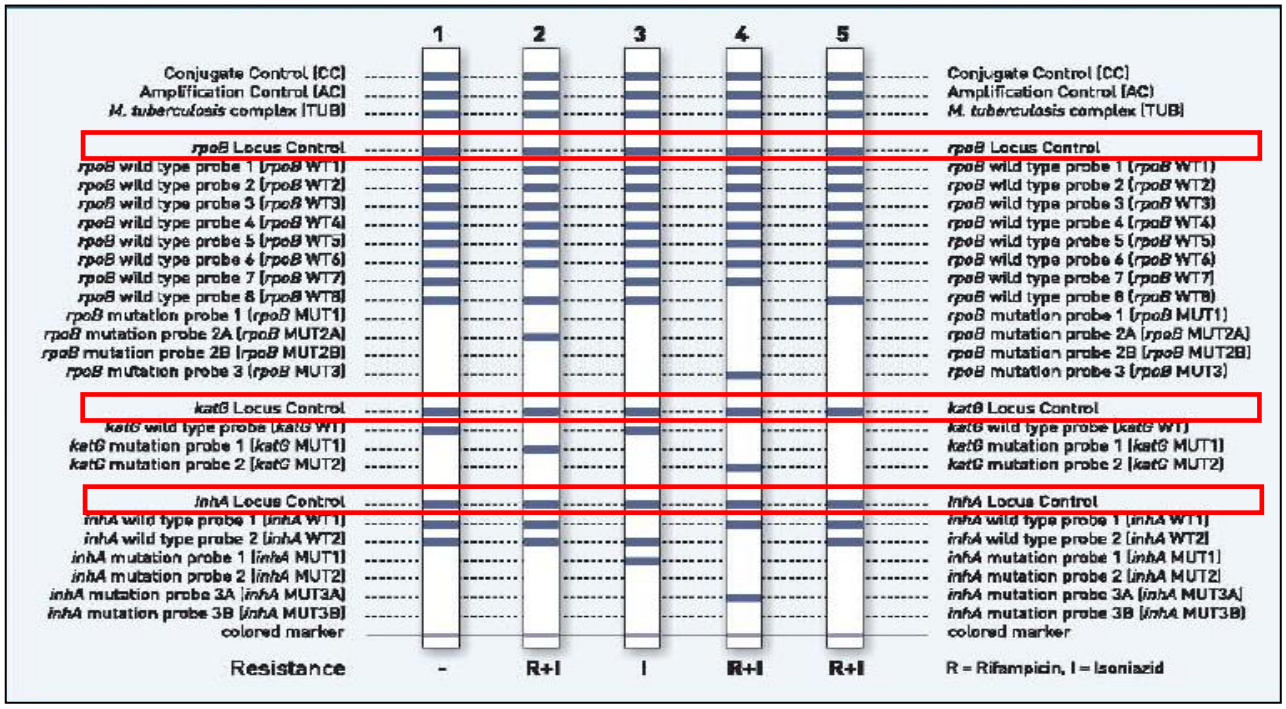


Figure 69: The three respective locus control bands for *rpoB*, *katG*, and *inhA* (all highlighted in red), where the gene specific regions are detected.

WILD TYPE (WT) REACTION ZONES

The term “wild-type” (**WT**) refers to the visible characteristics (phenotype) of the most common form of a species that has been grown in its natural environmental conditions. The phenotype in turn is determined by the organism’s genetic makeup (genotype). As relates to this assay, the **WT** phenotype is drug-susceptible *M. tuberculosis*. The **WT** reactions zones on the strip contain probes that comprise the areas containing the most common resistance-associated mutation sites of the three respective genes being interrogated. If all the **WT** probes give a positive signal, there is no detectable mutation within the examined regions, and the interpretation is that the strain is predicted to be sensitive to the respective antibiotic(s) (**Figure 70, strip 1**).

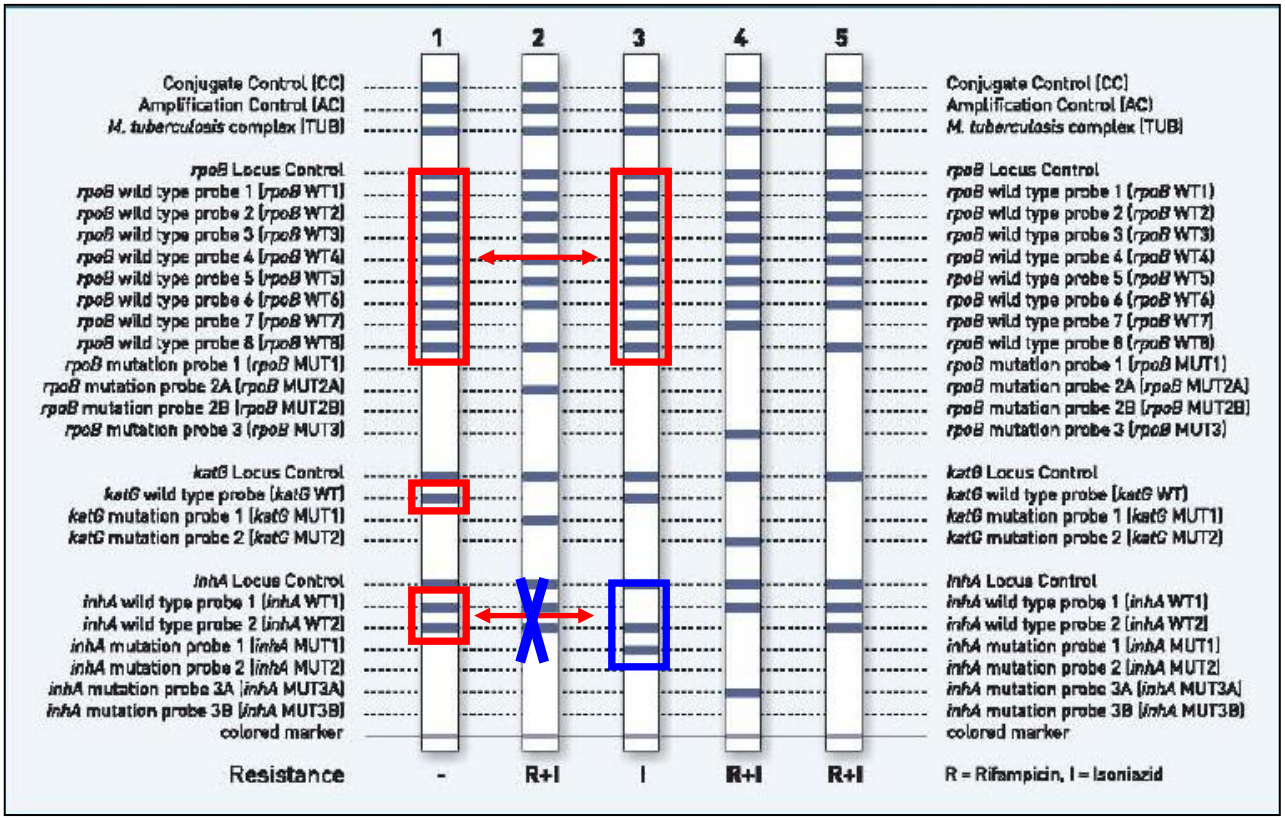


Figure 70: The banding pattern on strip number 1 shows that all the WT probes for the respective antibiotics are present (boxed in red). This pattern indicates that the strain is predicted to be susceptible to both RMP and INH. In contrast, strip number 3 indicates susceptibility to RMP, but the banding pattern of the *inhA* reactions zones are different from those observed on strip number 1, indicating resistance to INH (blue box). It is very important however, to examine both the *katG* and *inhA* banding patterns before reporting the final result for INH.

If one or more **WT** probes of a gene are missing, it is likely that there are one or more mutations within the examined region, and the strain is considered to be resistant to the respective antibiotic(s) (**Figure 70, strip 3**). However, some exceptions can occur due to silent mutations, since such changes do not result in an amino acid exchange or a modification of the phenotype.

MUTATION (MUT) REACTION ZONES

When a mutation occurs, the amplicon cannot bind to the corresponding **WT** probe on the strip because of a mismatch between the amplicon and the probe. If the mutation corresponds to one of the common resistance-associated mutations that are included in the **MUT** probes on the strip, the amplicon will “recognize” and bind to this probe, predicting that the strain is resistant to the specific antibiotic. The 8 *rpoB* **WT** reaction zones and the 4 corresponding **MUT** probes are shown in **Figure 71** (**A**).

Accordingly, if **MUT** 1 is present, **WT 3** and **WT** 4 should be absent, if **MUT** 2A or 2B are present, **WT** 7 should be absent (as shown in **Figure 71** (**B**)), and if **MUT** 3 is present **WT** 8 should be absent (as shown in **Figure 71** (**C & E**)). Conversely, if the mutation in the **WT** region is not one of the common mutations included in the MUT probes, both the **WT** and the corresponding **MUT** bands will be absent.

However, examples from specimens tested thus far have shown that exceptions to these patterns can occur. For example, **WT** 7 and the **MUT** 2A and **MUT** 2B bands can all be absent as shown in **Figure 71** (**D**). This pattern indicates that there is a mutation in the **WT** region, but that it is neither of the common mutations included on the strip. Thus, the absence of a signal for at least one of the *rpoB* **WT** probes, with or without the presence of a **MUT** band, indicates that the strain should be predicted to be resistant to RMP. The presence of less common mutations within *rpoB* that result in absent bands for either **WT** 1, 2, 5, or 6 reaction zones (which lack corresponding mutation specific capture probes) also should be interpreted as prediction of resistance to RMP.

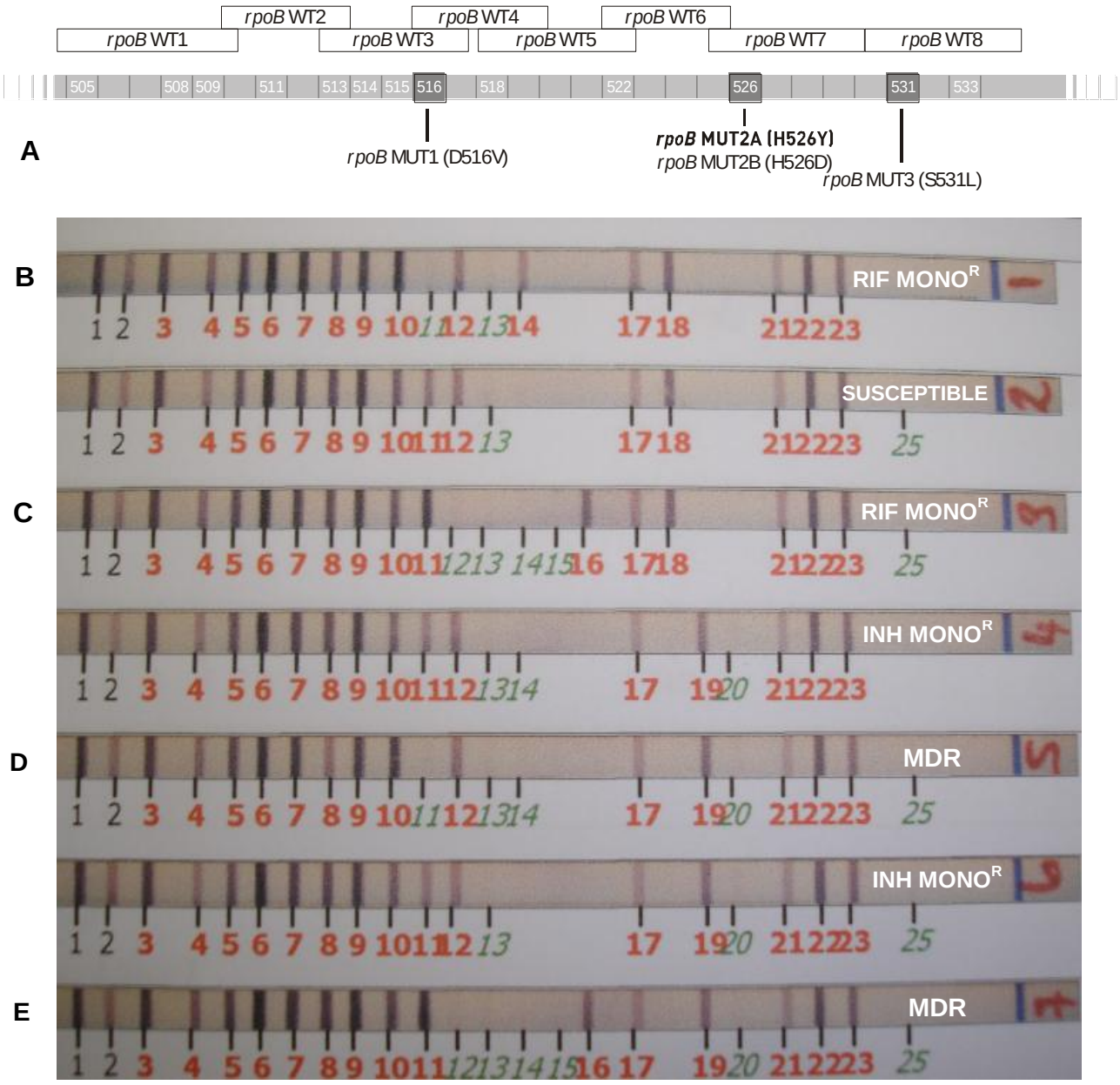


Figure 71: (**A**) *rpoB* gene region illustrating the 8 WT probes and the 4 corresponding MUT probes. Strips **B** to **E** show the WT bands with or without the corresponding MUT bands. For example, the WT 7 band (no. 11) is absent in both (**B**) and (**D**), but only strip (**B**) has the corresponding MUT 2A band (no. 14). In strips (**C & E**), the WT 8 band is missing while the corresponding MUT 3 bands (no. 16) are present. Final susceptibility results are shown in white.

Note: Red numbers indicate valid reaction zones, green numbers indicate no band or artefacts that are less intense than the AC band (no. 2) and are thus not considered as valid).

The common mutational sites at *katG* and *inhA* are not as varied as those within *rpoB*. The absence of the *katG* **WT** band is usually accompanied by the presence of either the *katG* **MUT** 1 or **MUT** 2 band, as seen in **Table 7A**. In addition, the absence of the *inhA* **WT** 1 band is usually accompanied by the presence of either the *inhA* **MUT** 1 or **MUT** 2 band, and the absence of the *inhA* **WT** 2 probe is usually accompanied by the presence of either the *inhA* **MUT** 3A or **MUT** 3B probe, as seen in **Table 7B**. Examples of possible *katG* and *inhA* banding patterns are shown in **Figure 72**.

Table 7A: *katG* probes

Missing wild type probe	Analyzed codon	Mutation probe	Mutation
katGWT	315	*katG* MUT1	S315T1
		katG MUT2	S315T2

Table 7B: *inhA* probes

Missing wild type probe	Analyzed nucleic acid position	Mutation probe	Mutation
inhA WT1	- 15	*inhA* MUT1	C15T
	- 16	*inhA* MUT2	A16G
inhA WT2	-8	*inhA* MUT3A	T8C
		inhA MUT3B	T8A

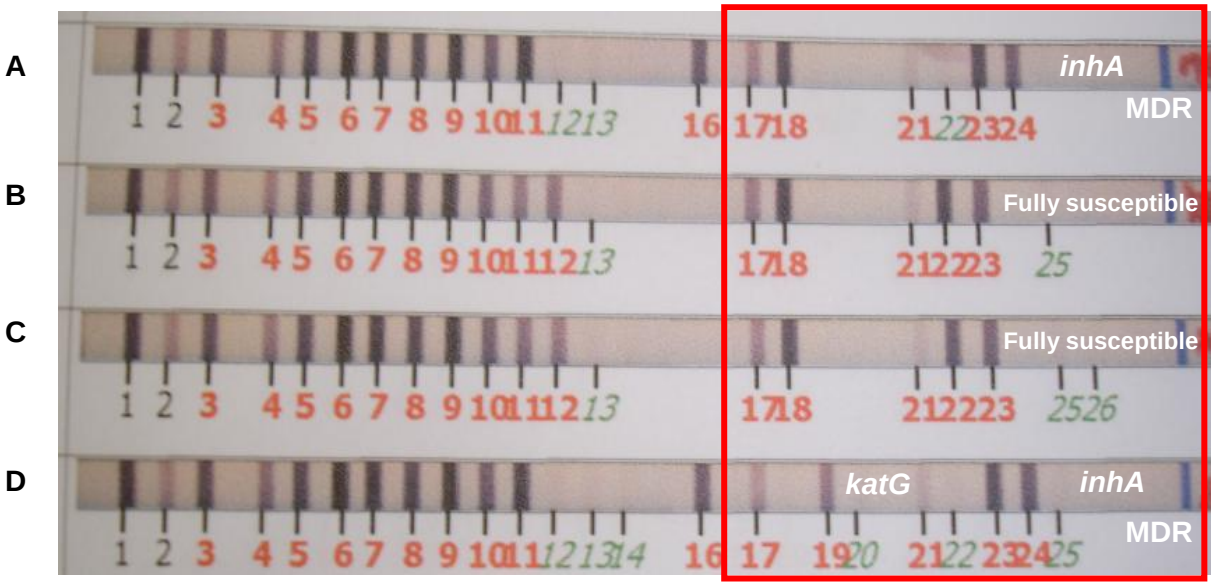


Figure 729: INH resistance-associated banding patterns (*katG* and *inhA*). (**A**) The absence of the *inhA* WT1 band (no. 22) is accompanied by the presence of the *inhA* MUT 1 band (no. 24). (**D**) The absence of the *katG* WT band (no. 18) is accompanied by the presence of the *katG* MUT1 band (no. 19), and the same mutation seen in strip (**A**) is observed for the *inhA* gene. Strips (**B**) and (**C**) both represent strains fully susceptible to the main first line drugs, RMP and INH.

PATTERN RECOGNITION OF THE *rpoB, katG* AND *inhA* PROBES

It is crucial that the banding pattern of the $H_{37}Rv$ wild type (pan-susceptible) strain, shown in **Figure 73**, be regarded as the 'baseline" pattern against which all other patterns are to be scored or interpreted. Strains with a banding pattern identical to the "baseline" must be reported as predicted to be sensitive to RMP and INH. Should any banding pattern deviate from the "baseline" patterns for *rpoB* (for RMP resistance) and *katG* and *inhA* (for INH high- and low-level resistance respectively), the strains must be reported as predicted to be resistant to the respective drug(s) as shown in **Figure 74**. Previous studies have demonstrated that the mutations in *katG* at codon 315 confer high-level resistance to INH, while mutations within the promoter region of *inhA* confer low-level resistance.

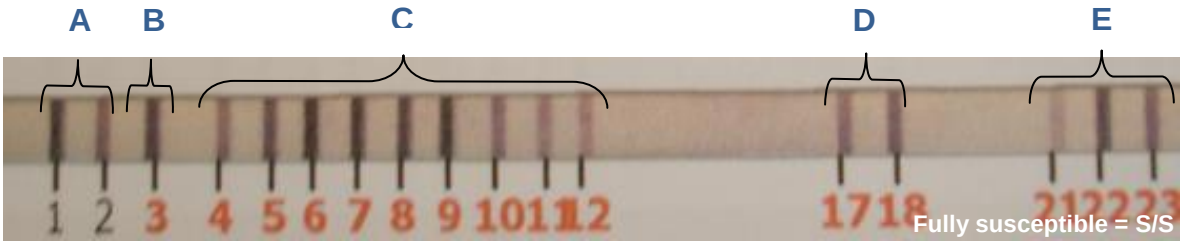


Figure 73: Banding pattern of $H_{37}Rv$ pan-susceptible *M. tuberculosis*. (**A**) The internal control reaction zones 1 (CC), 2 (AC) are present indicating that the DNA extraction, amplification and hybridization steps were successful. (**B**) Reaction zone 3 (TUB) is present, thus identifying the specimen as positive for M. tuberculosis complex. (**C**) The *rpoB* locus control (reaction zone 4) and all the *rpoB* wild-type probes (zones 5 to 12) are present, indicating susceptibility to RMP. (**D**) The *katG* locus control (zone 17) and *katG* wild-type probe (zone 18), as well as (**E**) the *inhA* locus control (zone 21) and its associated wild-type probes (zones 22 and 23), shows that the same strain also exhibits susceptibility to INH.

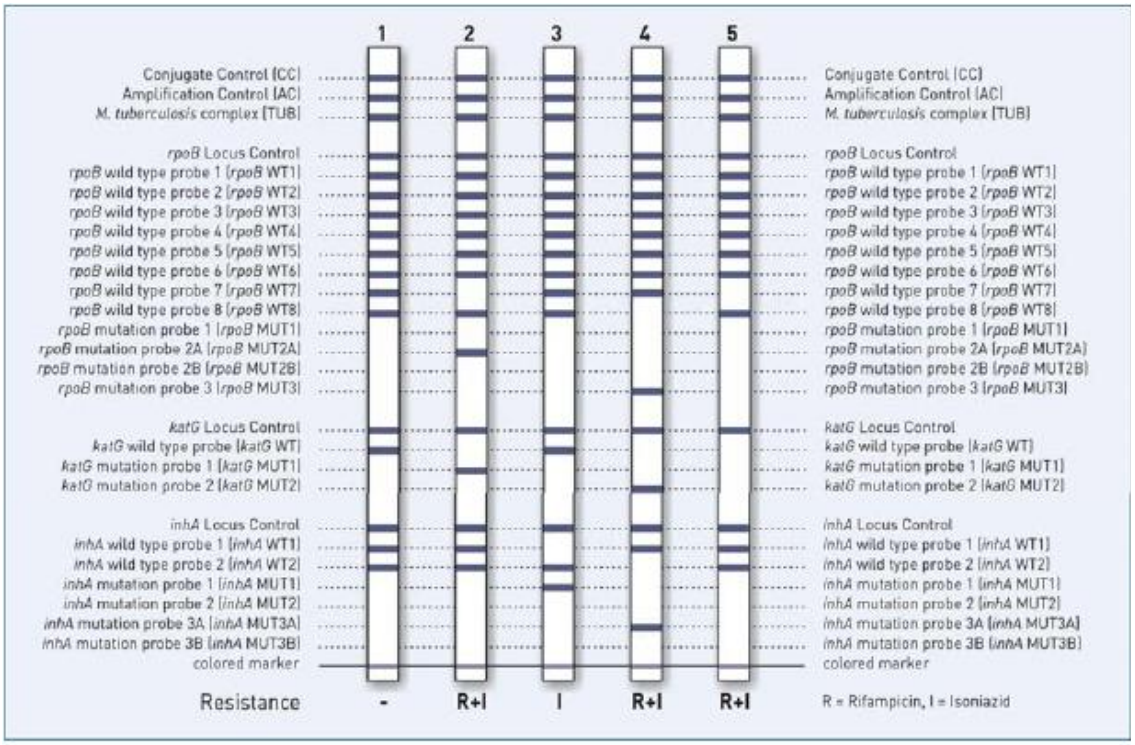


Figure 74: Illustration of typical LPA banding patterns of different strains. Strip **1:** a pan-susceptible strain (baseline banding pattern); strips **2, 4** and **5**: different patterns of MDR strains; strip **3**: an INH-mono resistant strain. Note that the banding patterns of the INH mono-resistant strain and the MDR strains differ from each other, and clearly deviate from the “baseline” banding pattern, observed on Strip 1.

KatG S315T mutation is the most common mutation in INH-resistant strains, accounting for 50–95% of INH-resistant clinical isolates. In contrast to these rather conserved mutations within *katG* and *inhA*, mutations in the *rpoB* gene occur at several sites within an 81 base pair (bp) region considered to be a mutational "hot spot". These mutations result in a protein that is still functional but that will not be bound and inactivated by RMP (thus allowing the cells to grow in the presence of the drug). Mutations in this region are present in up to 96% of all strains that are resistant to RMP (**Figure 70, strip 1**).

Note: Remember to first score the individual bands on the strip and, once finished, score that whole strip against the $H_{37}Rv$ positive control strip.

ALIGNING THE STRIPS ON THE SCORE SHEET

When the hybridization and development processes have been completed, align the **CC**, **AC** and **TUB** bands seen on the strips with the respective lines on the score sheet (**Figure 75**).

Note: To ensure this alignment is correct, each strip must be checked prior to hybridization to ensure that its size corresponds to the size of the scorecard template provided with each kit. Incorrectly sized strips will result in inaccurate interpretation of the strips.

Once properly aligned on the score sheet, the strips should be taped down using several strips of transparent cellophane tape. To facilitate the taping, it's suggested that each strip of the tape should be wide enough to cover three of the LPA strips. The resistance status of the specimen can then be determined by aligning the scorecard template with the taped LPA strips.

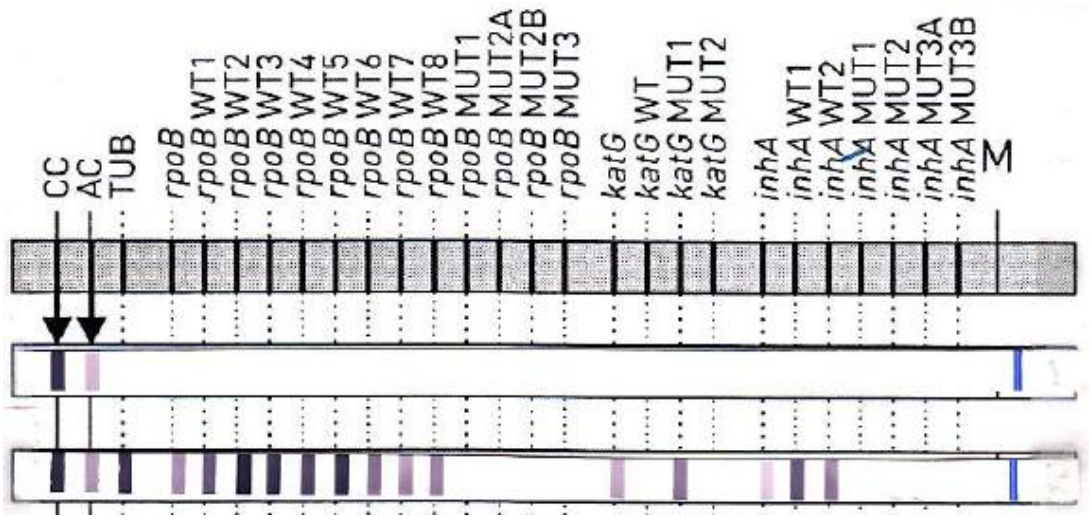


Figure 75: Two LPA strips taped down on a score-sheet with the CC, AC and TUB bands aligned with the respective lines on the score sheet.

Procedural note: *It is recommended that there be* ***two dedicated sets of tweezers****, each clearly marked with a different label, for strip transfers in the post-amplification area. These can be labelled with stickers of two different colours. For example, a pair that will be used for handling clean strips could have a silver label and the pair used for handling strips previously exposed to amplicons could have a red label. The silver labelled pair will be used for the removal of the clean strips from the kit packaging, for stabilizing the strips during labelling, and for carefully placing the numbered, unhybridized strips into the reaction wells containing the denatured amplicons. The clean tweezers should not come in contact with the liquid in the well; any needed adjustment of a strip in a well should be done with an unused pipette tip. The red-labelled pair will only be used at the end of the procedure for the removal of the hybridized and developed strips from the reaction wells. This preventative measure is critically important to avoid carry-over of amplicons that would cross-contaminate the strips prior to hybridization.*

INCONCLUSIVE RESULTS DUE TO WEAK OR NO BANDS

Inconclusive results should be an infrequent occurrence due to the high sensitivity and specificity of the PCR reaction, and the fidelity of the HotStarTaq® DNA polymerase. However, occasionally it may not be possible to clearly interpret a strip's banding pattern. When inconclusive results are obtained, the likely causes are:

1. **DNA of insufficient quantity or quality for the PCR reaction:**
   This problem results in inefficient amplification and thus is the most likely cause of weak or no bands on the strip. As a starting point in resolving this issue, it is good practice to recheck the smear to ensure that it is positive. If the smear is confirmed as positive, DNA should be re-extracted and tested from any specimen with an inconclusive result. If the result of the re-extracted specimen remains inconclusive, the different options for proceeding depend on the type of specimen being tested. If the specimen was taken from a contaminated MGIT tube, an LJ slant should be inoculated to attempt to isolate pure colonies of *M. tuberculosis* for future extraction and testing. If a processed direct patient specimen was used initially, any left-over specimen should be extracted and tested. If this is not possible due to insufficient volume, the cultured specimen should be flagged for testing with the LPA once growth is detected.

2. **Using an incorrect PCR program:**
   This is another important concern that should be considered. DNA extracted from the millions of cells in cultured isolates is much more concentrated than DNA extracted from the fewer cells found in direct patient specimens. If the 40-cycle PCR program is used to amplify such highly concentrated DNA samples (instead of the recommended 30-cycle program), the DNA polymerase may be depleted within the first few cycles and no further amplification will occur. This situation often leads to weak signals and or inconclusive results.

3. **Inadequate temperatures:**
   Weak signals may also occur especially during winter months if the ambient temperature in the laboratory is too low to maintain the correct temperature in

the GT-Blot automated hybridization machines. In this situation, it is recommended that the room temperature setting on the instrument be adjusted to 24 - 25ºC. Alternatively, the instrument temperatures for the hybridization and stringent wash steps might be set too high. If one or both of these temperatures are too high, the denatured amplicons will bind poorly or not at all to the probes on the strip, and weak or no bands will be seen.

4. **Incomplete immersion of strips:**
   Finally, the strips may not be completely immersed in the reaction mixtures during the incubation steps. If adjustments are needed after a strip has been placed in the well, a pipette tip can be used to ensure that the strip is completely immersed. After this adjustment, the pipette tip should be carefully removed from the well and discarded to avoid any potential cross-contamination.

UNEXPECTED OR UNUSUAL RESULTS

Checking the reagents used for the hybridization and detection process prior to beginning the procedure is essential to ensure valid test results. If the developed bands are faint on all strips that were processed together, the Hybridization buffer and/or Stringent Wash buffer may not have been properly pre-warmed or precipitates may be present in these reagents. If the bands are too weak to be interpreted with confidence, the run may need to be repeated. In contrast, a strong and fast colour development may occur on all bands depending on the amount of amplicons used and the specific reaction conditions. If this happens it is advisable to discontinue the substrate incubation as soon as the signals are clearly visible in order to prevent the development of cross-hybridizing bands (artefacts). Too many or too few bands may also occur unexpectedly. Strips with **all WT** and **MUT** bands, or too many bands, could be the result of contamination of wells by splash-over from a neighbouring well. Then again, lack of a **WT** band(s) without a corresponding **MUT** band could be due to uncommon mutations in the probe region as discussed previously.

HETERO-RESISTANCE OR MIXED INFECTIONS

The development of resistance is an ongoing process due to natural selection and selective pressure exerted on *M. tuberculosis* by the various drugs administered to patients. Clinical isolates of *M. tuberculosis* complex are considered susceptible to a specific drug when greater than 99% of the cells in the population tested are killed by the drug, and are considered resistant when at least 1% of the cells in the population tested are resistant to killing by the drug. Accordingly, greater than 99% of the cells within a single susceptible strain of *M. tuberculosis* have wild-type nucleotides at resistance-associated genomic sites, whereas in a resistant strain, the proportion of cells in the population with mutant nucleotides at these sites may range from 1 - 100%. Typically most patients are infected with a few cells of a single strain of *M. tuberculosis* that is either drug susceptible (with > 99% of cells genetically wild-type) or drug resistant (i.e. primary resistance, with most cells already containing a mutation at one or more sites associated with drug resistance). LPA patterns of such both types of homogeneous populations are easily interpretable as described in previous sections.

In contrast, when clinical specimens or isolates contain heterogeneous populations of *M. tuberculosis*, unexpected LPA patterns may be difficult to interpret especially when both **WT** and corresponding **MUT** bands in the strips are positive:

- In patients infected with a fully susceptible strain, drug resistance can develop gradually during (inadequate) treatment due to selection of cells with random mutations in sites associated with drug resistance (i.e. secondary resistance). In this case, as the proportion of susceptible cells decrease and resistant cells increase, a hetero-resistant population of cells will be present. These cells are primarily identical throughout the genome but a proportion of the population differs in sites associated with drug resistance.

- In contrast, patients infected with fully susceptible strains may develop mixed infections if they are co-infected with another strain that is drug resistant, resulting in a mixed population of two genetically distinct strains, one drug susceptible, and the other drug resistant.

Although the cause of a hetero-resistant population of cells in a clinical sample or isolate (either by development of resistance under treatment or by mixed infection with sensitive and resistant cells) cannot be determined using routine laboratory methods, the LPA is capable of detecting its presence since the strips contain both wild type and mutant probes for the three genes known to be involved in resistance to RMP and INH.

Figure 76 demonstrates examples of LPA patterns of hetero-resistant populations as compared to more homogeneous populations. Such patterns are to be interpreted as indicating resistance to the particular drug and should be reported as such. Rapid detection of hetero-resistance can greatly influence the clinical management of a patient by providing an explanation as to why the patient is not responding to the current drug regimen, and can also help prevent the selection of cells resistant to additional drugs. For example, it may not be effective to continue RMP treatment in a patient whose specimen exhibits hetero-resistance in *rpoB*. Although the RMP-susceptible cells will be killed, RMP will not be efficient in killing the resistant cells which may then also develop resistance to other drugs taken by the patient.

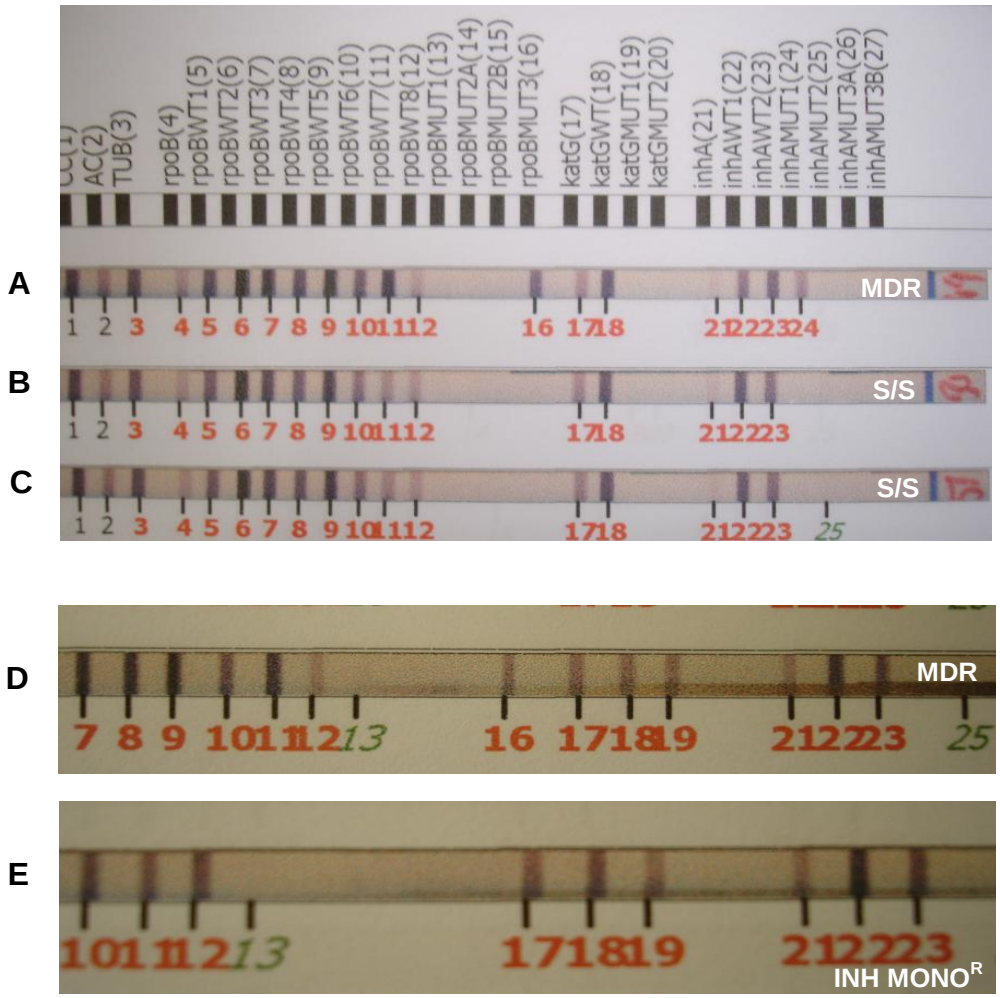


Figure 76: Hetero-resistant strains vs. strains with homogeneous populations. (**A**) Hetero-resistance to RMP is indicated by the presence of the *rpoB* MUT3 band (number 16) in addition to all the *rpoB* WT bands (numbers 5 to 12). The presence of the *inhA* MUT1 band (number 24), in addition to the *inhA* WT bands (number 22 and 23) also indicates hetero-resistance to INH. The combined hetero-resistance to RMP and INH could indicate that one population has mutations in both *rpoB* and *inhA* while the other is fully susceptible, or that the two populations present may be mono-resistant to either RMP or INH. Note that hetero-resistance can also occur for mono-resistance at any of the sites tested for on the strip. (**B** & **C**) Fully susceptible strains with no mutation bands present. (**D**) Close-up of MDR-TB specimen with mutations in genes *rpoB* and *katG* exhibiting hetero-resistance to both drugs. (**E**) Close-up of specimen hetero-resistant to INH (presence of the *katG* MUT1 band (number 19) in addition to the *katG* WT band (number 18).

CROSS-CONTAMINATION OF SPECIMENS

Cross-contamination is another possible reason why a tested specimen may result a hetero-resistant banding pattern on the LPA. The original specimen could have been contaminated with cells or DNA from *M. tuberculosis* from another patient specimen during one of the following steps:

1. The initial NaOH-NALC decontamination;
2. The inoculation of the MGIT tubes;
3. The extraction of the DNA;
4. The addition of the template DNA or;
5. The hybridization reaction.

If there was carry-over contamination during the NaOH-NALC decontamination process, the extent of this event will likely be reflected in the number of strips exhibiting hetero-resistance. Large-scale carry-over contamination can be detected with the LPA since adjacent strips will likely exhibit the same unusual banding pattern indicative of a hetero-resistant population or the presence of more than one strain of *M. tuberculosis*. Including consecutive patient specimens in the LPA as a means of quality control can help to identify problems such as those described above within the whole TB diagnostic pathway. Such specimens must always exhibit the same banding patterns on the LPA, with the identical interpretation as to susceptibility or resistance (**Figure 77**). If the banding patterns are different, there may have been some mix-up in the specimens or the specimen numbers. Thus, it is important to thoroughly investigate the reason for the discrepancy and correct the problem.

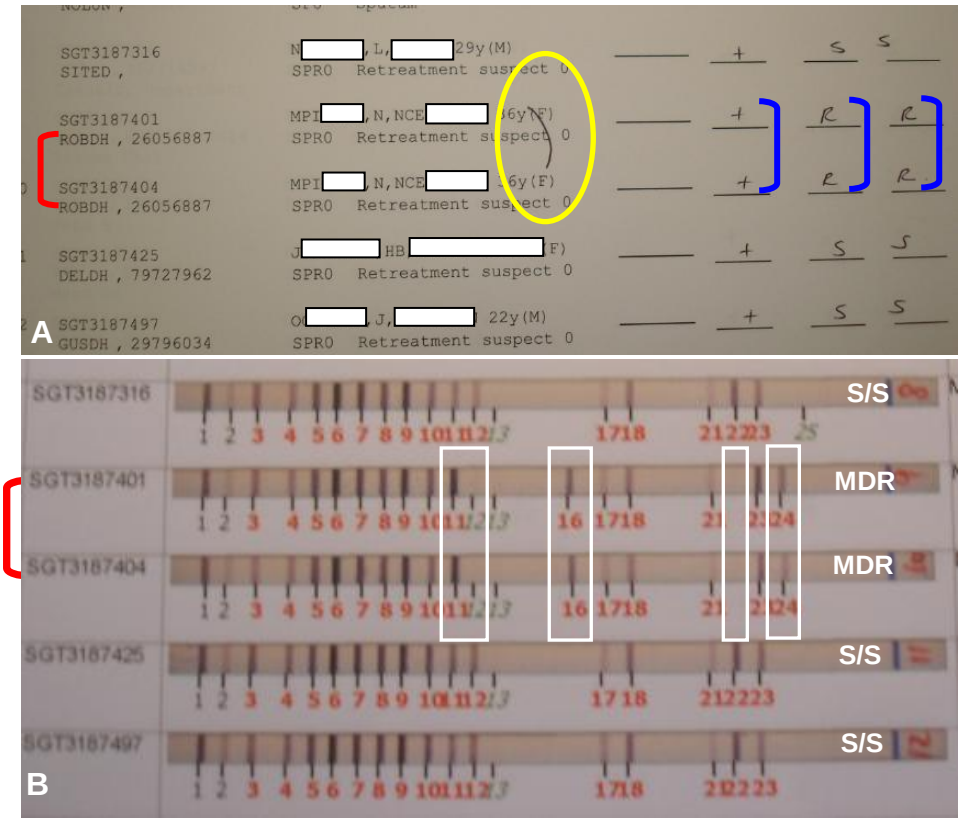


Figure 77: Example of consecutive patient specimens within a batch of specimens. (**A**) Example of the worksheet, created before extracting the DNA from the specimen, indicating that there are two specimens from the same patient (red bracket). The individual in charge of extracting the DNA should scan the worksheet for the same name and surname, and mark them (yellow ring). (**B**) The hybridized strips of the consecutive specimens exhibiting the exact same banding pattern. Note that *rpoB* WT8 (number 12) and *inhA* WT1 (number 22) are absent on both, and both have the *rpoB* MUT3 (number 16) and *inhA* MUT1 (number 24). The individual who did the LPA will transcribe the results onto the original worksheet (blue bracket). This is to double check that the sequence of the specimens during the extraction process remained correct. If a number were to be dropped in this example, the results of one of the two specimens belonging to the same patient would have been different (fully susceptible) thereby immediately alarming the diagnostician that there was an error. This system includes additional quality control, since two diagnosticians carried out the different processes and cross-checking during the LPA procedures.

Disorganization of specimens or their numbers will result in a mix-up of the sequence of specimens in a batch, often leading to incorrect results and the possibility of dire consequences for patients. Although there can be legitimate reasons for such mix-ups, these errors often occur when the attention of technical staff is focused on extraneous conversations or personal preoccupations rather than concentrating on the job at hand. In either case, it is very important to follow through with a thorough investigation of the problem. A mix-up in specimens or numbers might have occurred

during the NaOH-NALC decontamination process, the DNA extraction process, the addition of the template DNA to the PCR master mix, and if a single channel pipette was used during the addition of amplicons to the denaturing solution. All these steps should be investigated and any errors found should be immediately corrected.

MULTIPLE SPECIMENS FROM THE SAME PATIENT

The LPA banding pattern of isolates from a specific patient should be identical throughout the patient's treatment period. However, there are instances, such as the development of resistance, when the LPA banding pattern may change over time. It is therefore important for the patient that the results from previous specimens are compared to the results from current specimens and any discordant results investigated. The following situations could result in changing patterns:

- A mix-up of specimens at the clinic or at the laboratory;
- The patient's isolate is converting or has converted from drug susceptible to drug resistant, and the patient's treatment is failing;
- The patient has been re-infected with a different strain of *M. tuberculosis* either during the treatment period (i.e. re-infection with a drug resistant or MDR strain during or following treatment of a susceptible strain);
- Although rare, INH-resistant mutants can revert to a drug-susceptible phenotype in the absence of INH treatment.

The clinician must be notified and more specimens requested in order to confirm the discordant results. If a mix-up of specimens has occurred, immediate steps need to be taken to identify and correct where in the process, from clinic to end result, a mistake might have been made.

Procedural note: *It is recommended that the strips, after they have been taped and interpreted, be placed in a folder and stored in the dark. These records may play a crucial role in quality control, for example when a patient is tested more than once, it*

*should be routine practice to **refer back to results from previous specimens**. It might happen that the results from the first and/or second patient specimen may not be the same as those of the patient's third specimen. Discrepant results can be resolved by going back to strips from the patient's previous specimens and comparing them. If the first two are consistent, there may have a mix-up of the third specimen either at the clinic or in the laboratory. In this case, a new specimen should be requested and tested before a report is issued.*

REMARKS ON INTENSITY OF SPECIFIC BANDS

Bands number 6, 9, 18 and 22

With the addition of the substrate solution to the hybridized strips, the colorimetric reaction starts taking place allowing for the visualization of the different bands on the strip. The first reaction zones to develop are those of *rpoB* **WT** 2 (no. 6) and *rpoB* **WT** 5 (no 9). The *katG* **WT** (no. 18) and *inhA* **WT** 1 (no. 22) reaction zones also exhibit this behaviour in relation to the other bands of their specific gene regions (**Figure 78**). These four bands show up as very intense bands on the H_{37}Rv baseline control strip, which is the drug sensitive control strain. It is therefore critical to carefully study the colour intensity of these **WT** bands on strips from patient specimens.

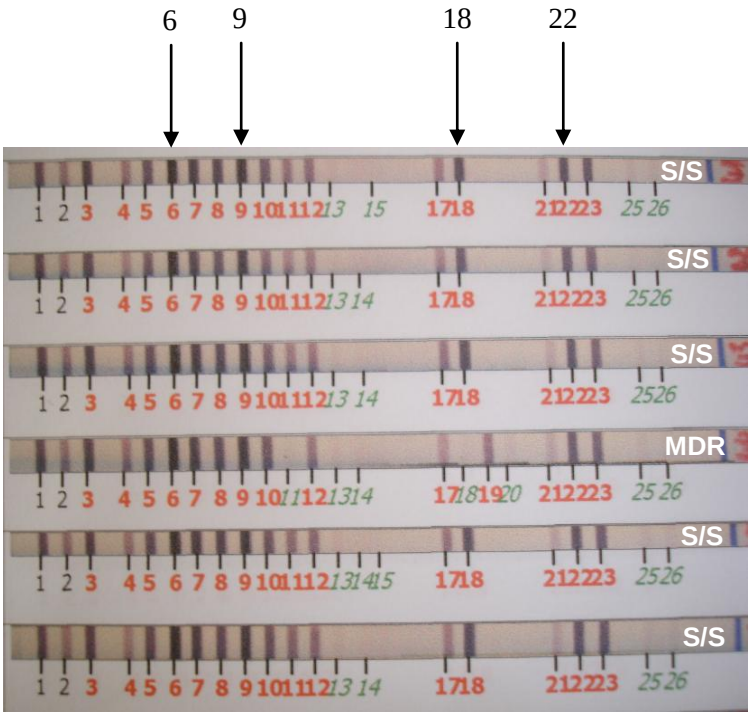


Figure 78: Arrows point to the most intense bands appearing after substrate incubation.

If one or more of these four bands (6, 9, 18, 22) is not as intense as those on the baseline pattern, the specimen should be reported as resistant to the respective drug. The intensity has also to be compared with those of the **AC** band (no 2), that on each strip functions as the baseline against which all other bands on the strip are to be interpreted/"scored" (as described previously). **Figure 79** and **Figure 80** show examples of *rpoB* **WT** 2 band (no. 6) and *katG* **WT** band (no. 18) which are far less intense than the **AC** and **WT** bands in both samples, indicating resistance to RMP and INH respectively.

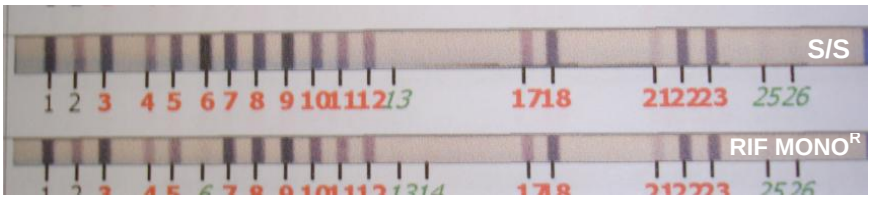


Figure 79: The *rpoB* **WT**2 band (no. 6) on the bottom strip is far less intense than that of the top strip and of the AC band (no. 2). The bottom strip must then be interpreted as indicating resistance to RMP.

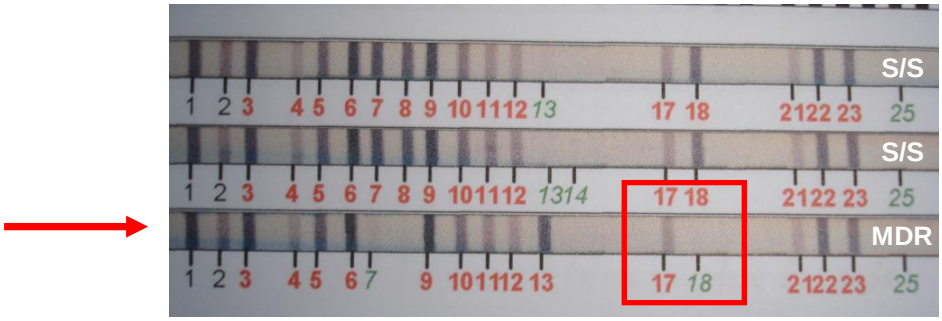


Figure 80: The banding pattern on the bottom strip (red arrow), shows the absence of the *katG* **WT** band (no. 18). In addition to being resistant to RMP, this absence indicates that the strain is resistant to INH as well, thereby identifying the strain as MDR.

Band number 11 (*rpoB* WT 7 probe)

In baseline RMP-susceptible banding patterns, the intensities of *rpoB* **WT** 7 and *rpoB* **WT** 8 bands (strip numbers 11 and 12) are equivalent. However, on strips of RMP-resistant strains with the following banding patterns, the *rpoB* **WT** 7 band is more intense as compared to the baseline bands:

- *rpoB* **WT** 8 band is absent, with or without presence of *rpoB* **MUT** 3
- **Both *rpoB* WT** 8 **and *rpoB* MUT** 3 bands are present (hetero-resistance)

Although the reasons of such associations are unknown, it is important to be attentive to more intense *rpoB* **WT** 7 bands because it can facilitate the detection of RMP-resistant banding patterns. Multiple examples of this association are shown in **Figures 81 & 82**.

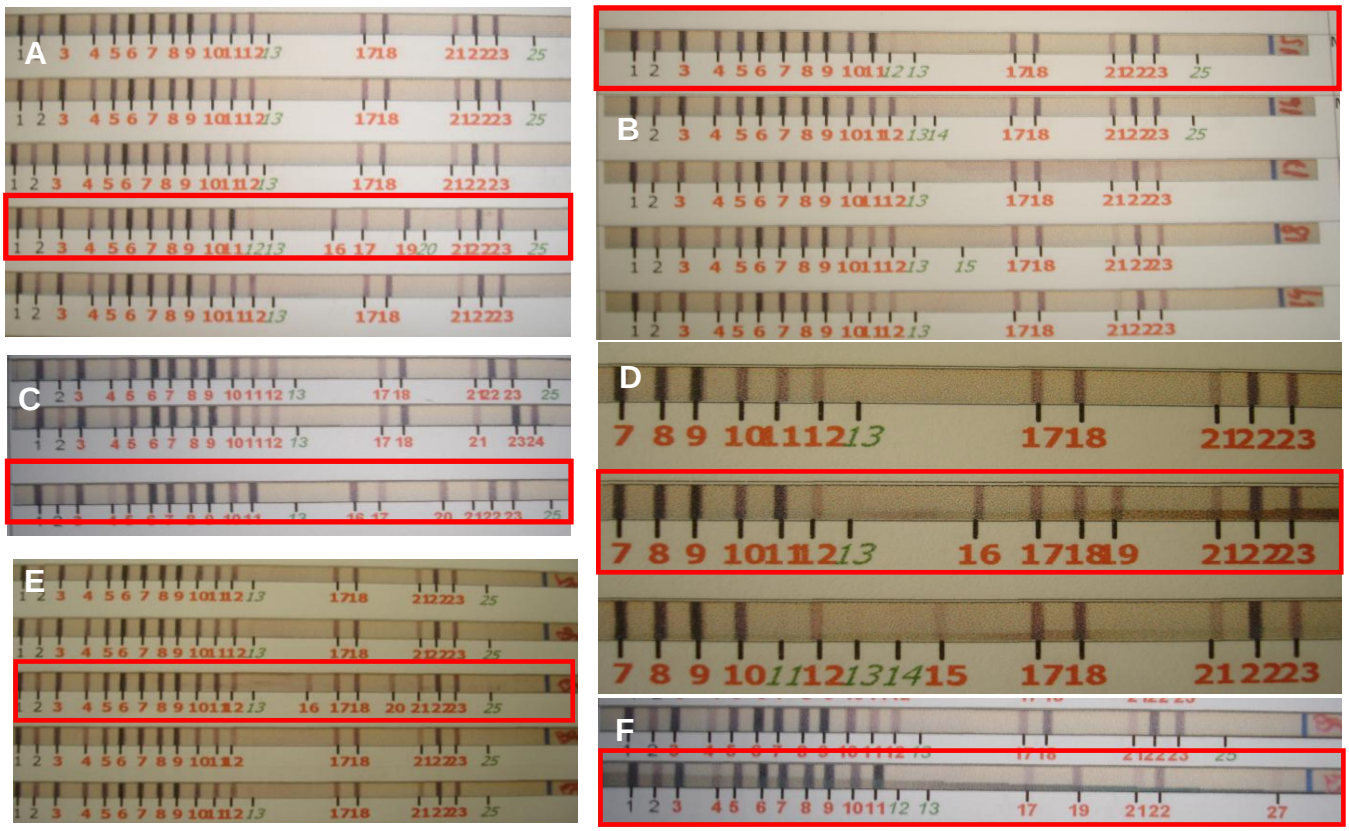


Figure 81: Examples of LPA patterns where different RMP-resistant banding patterns are accompanied by *rpoB* **WT** 7 bands (number 11) much more intense than those observed on any of the other strips in that particular run. From **A** to **F**, each one of these strips is shown into a red square to differentiate them from the other strips of the same run. (**A**) Banding pattern of a known MDR patient, where the **WT** 8 probe (number 12) is faint and the corresponding *rpoB* **MUT** 3 probe (number 16) is present. (**B**) Banding pattern where the **WT** 8 probe (number 12) is faint without the presence of the usually associated *rpoB* **MUT** 3 probe. Note that the *rpoB* **WT** 8 probe in **A** and **B** is faint and less intense than that of the **AC** band, reflecting heterogeneous populations with emerging resistance (hetero-resistant* strains). (**C**) Banding pattern where the **WT** 8 probe (number 12) is absent and the *rpoB* **MUT** 3 present. (**D**) and (**E**) Banding pattern of hetero-resistant* strains with presence of both *rpoB* **WT** 8 and *rpoB* **MUT** 3 probes. (**F**) Banding pattern of a known MDR patient with absence of both *rpoB* **WT** 8 and *rpoB* **MUT** 3 probes.

** See section on "Hetero-resistance"*

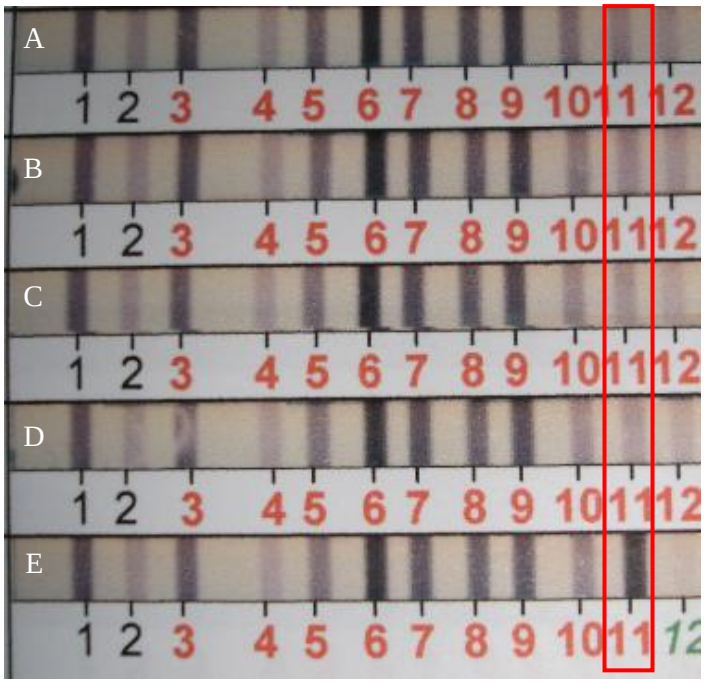


Figure 82: Example of the **increased intensity** of the *rpoB* **WT** 7 band (no. 11) on strip **E** as opposed to the intensity of **WT** 7 bands on other strips from the same run (**A** - **D**). Also note the decrease in the intensity of the *rpoB* **WT** 8 band (no. 12 in green) on strip **E**. The *rpoB* WT 8 band (no. 12) on strip **E** is also less intense than that of the **AC** band (no. 2).

RARE MUTATIONS IN *rpoB*

Considering the different causes that lead to development of resistance, it is not surprising that mutations associated with drug resistance have been found to occur at many sites within the genome of *M. tuberculosis*, specifically in mutational hot spots such as the 81 bp region of *rpoB* (e.g. within *rpoB* **WT**1 to *rpoB* **WT**8, see **Figure 71A**).

The more commonly found mutations in this region are represented by the four **MUT** probes present on the strips. As described earlier, if **MUT** 1 is present, **WT** 3 and **WT** 4 should be absent, if **MUT** 2A or 2B are present, **WT** 5 should be absent, and if **MUT** 3 is present **WT** 8 should be absent. However, specimens tested thus far have shown that exceptions to these patterns can occur. As discussed above, both **WT**

and the corresponding **MUT** bands can be present in the case of hetero-resistance, or mixed infections.

In contrast, the absence of hybridization signals at one or more of the different *rpoB* **WT** reaction zones without corresponding **MUT** bands indicates the presence of a less common or rare mutation. Strips containing such banding patterns are to be interpreted as resistant to RMP (**Figure 83**) due to the fact that even a single rare mutation in this region will likely result in an altered protein that is resistant to inactivation by RMP. However, some argue that many of the single nucleotide polymorphisms that are responsible for the absence of the **WT** bands are due to silent mutations that have no effect on the protein's interaction with RMP. This controversy may have arisen since isolates containing some rarely-occurring mutations have been found to vary in susceptibility to RMP in conventional phenotypic assays. The lack of association of some rare mutations with phenotypic resistance to RMP may be due to the specific RMP susceptibility-testing method used, such as the proportion method (LJ or Middlebrook media) or on methods that are based on the manufacturers' instructions (e.g. the BACTEC 960 MGIT SIRE).

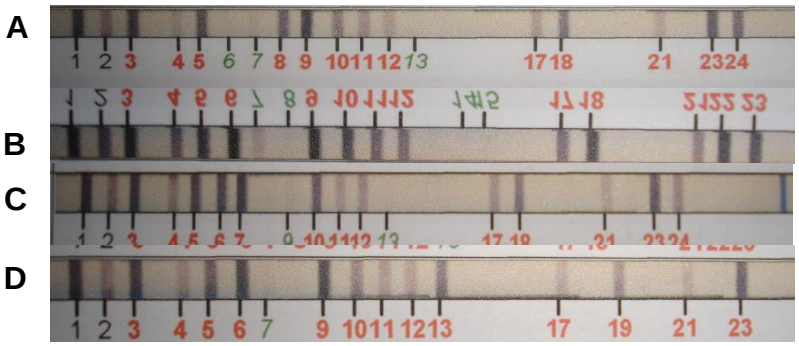


Figure 83: Examples, from clinical specimens, of rare mutations and the accompanying mutant probes in *rpoB*. (**A**) Absent **WT**2 (no. 6) and **WT**3 (no. 7) without the presence of **MUT**1. (**B**) Absent **WT**3 (no. 7) and **WT**4 (no. 8) without the presence of **MUT**1. (**C**) Absent **WT**4 (no. 8) and **WT**5 (no. 9) without the presence of **MUT**1. (**D**) Absent **WT**3 (no. 7) and **WT**4 (no. 8) with the presence of **MUT**1, note how the intensity of **MUT**1 (no. 13) has increased.

Table 8, adapted from Van Deun *et al*., shows details from a study where isolates that were found to be RMP-resistant in the LPA (missing **WT** band but no **MUT** band) were sent to several international laboratories for conventional drug susceptibility testing. It was found that the RMP susceptibility results from the different laboratories were highly discordant. These results suggest that the strains may have an MIC that is very close to the critical concentration of the drug used in the various media, and thus the strains are demonstrating low-level resistance to RMP. It was suggested that such *M. tuberculosis* strains with low-level resistance to RMP should be reported as borderline susceptible in conventional assays, and isolates with an absent **WT** band and no corresponding **MUT** band should be reported as resistant when interpreting the LPA. Clinical findings from this study also confirmed that RMP resistance can be missed by standard growth-based methods. Ohno *et al.* have observed similar findings between MIC's and mutations within *rpoB*.

GENE DELETIONS

Mutational events occur at random sites during normal DNA replication in bacteria. However, "selective pressure" will result in selection and amplification of naturally-occurring mutations that provide the organisms with an advantage to survive under adverse conditions (such as in the presence of antimicrobial drugs). This process selects for advantageous random mutations either of a single nucleotide resulting in an amino acid change that alters a gene product, or a deletion mutation of either a region of a gene or an entire gene.

Table 8: Characteristics of strains with mutations in the *rpoB* specific reaction zones (adapted from Van Deun *et al.*)

rpoB mutation	Regimen Category as per WHO[3]	Treatment Outcome	MTBDRplus® *rpoB* reaction zone
Ser531Leu	1	Failure	WT8, MUT3
Leu511Pro	2	Failure	WT2
Asp516Tyr	2	Failure	WT3/WT4
Leu533Pro	2	Cure, with relapse	WT8
His526Leu	2	Cure, with relapse	WT7
His526Ser	2	Cure, with relapse	WT7
Leu511Pro	2	Cure, with relapse	WT2

*Note: The **entire** rpoB gene could never be deleted since this is an essential protein required for survival of the organism.*)

Either type of mutation in the gene target could interfere with the killing action of the drug and result in resistance to the drug. **Figure 84** shows examples of deletions that have been observed in the regions tested for on the GenoType® MTBDR*plus* LPA.

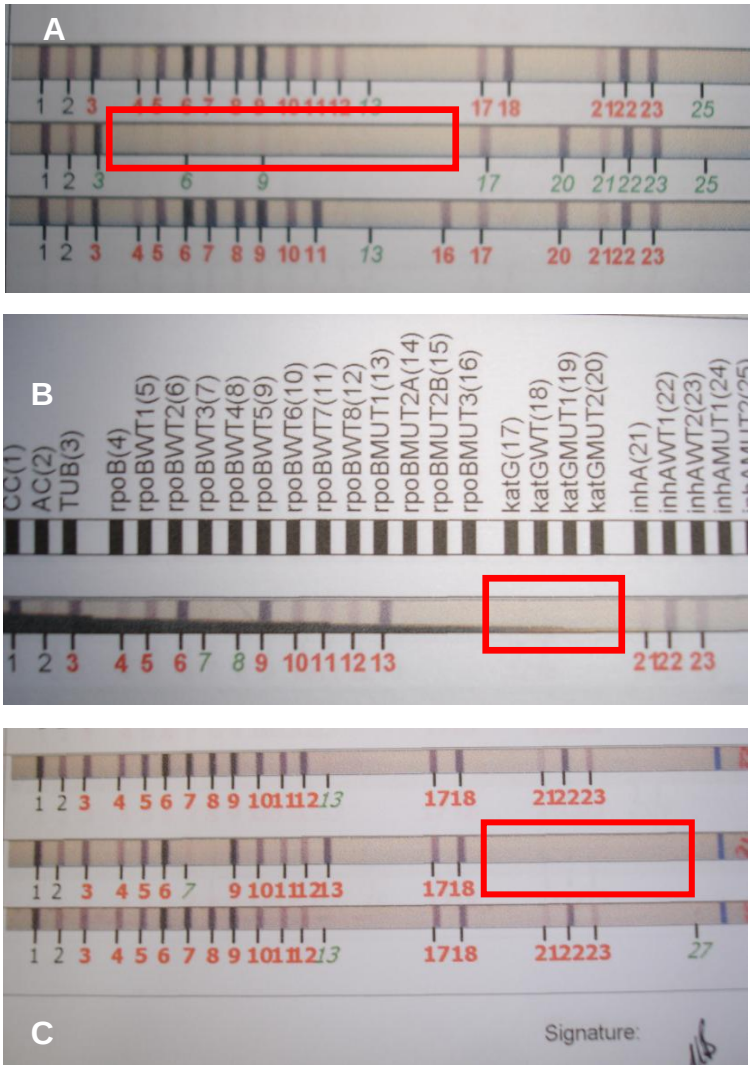


Figure 84: Examples of deletions in all three gene regions associated with MDR-TB on the LPA strips. (**A**) Deletion in *rpoB* indicative of resistance towards RMP, with additional resistance to INH as exhibited by *katG* **MUT** 2 band. (**B**) Deletion in *katG* indicating high-level resistance to INH, with the absent *rpoB* **WT**3 and **WT**4 bands. (**C**) Deletion in *inhA* indicative of low-level resistance to INH. Again the *rpoB* **WT**3 and **WT**4 bands are absent. All three examples are of MDR strains.

INTER-READER VARIABILITY

It is very important that the banding patterns observed on the LPA are interpreted correctly. Interpretation of the banding patterns is usually done by eye reading (**Figure 85**) with the aid of the scorecard, thus making this part of the procedure reader-specific, with the potential for inter-reader variability leading to misdiagnosis and treatment failure. An existing instrument has been modified by Hain LifeScience to assist in the interpretation of the LPA. The GenoScan® laser scanner (**Figure 86**), electronically interprets the banding patterns on the GenoType® MTBDR*plus* LPA strips, thereby eliminating inter-reader variability and ensuring standardized interpretation of results. However, it is strongly advised that the original way of transferring and taping the hybridized strips to the score sheet, and interpreting them with the hand-held scorecard, as seen in **Figure 85**, be followed and subsequently mastered before attempting to introduce the GenoScan® instrument. This is especially true when training new staff.

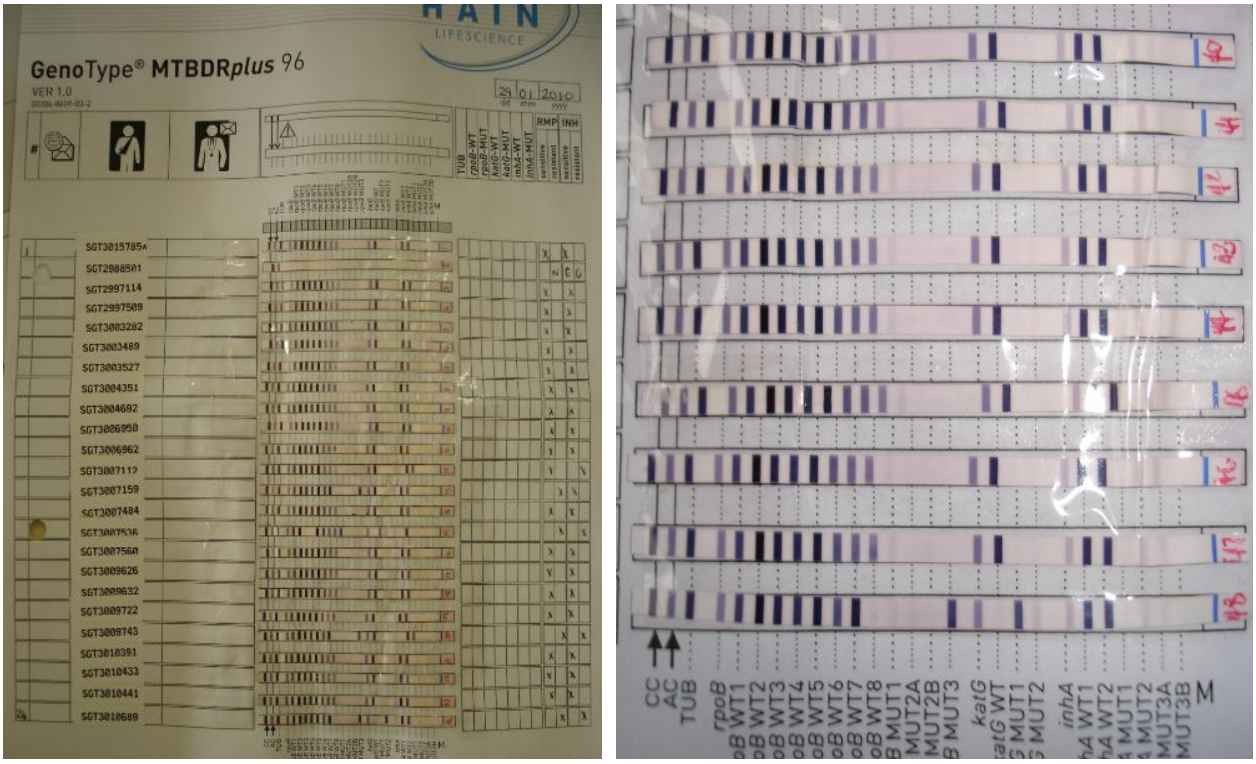


Figure 85: Examples of the alignment and taping of the completed strips to the score sheet. The results are then interpreted using the scorecard provided in the kit.

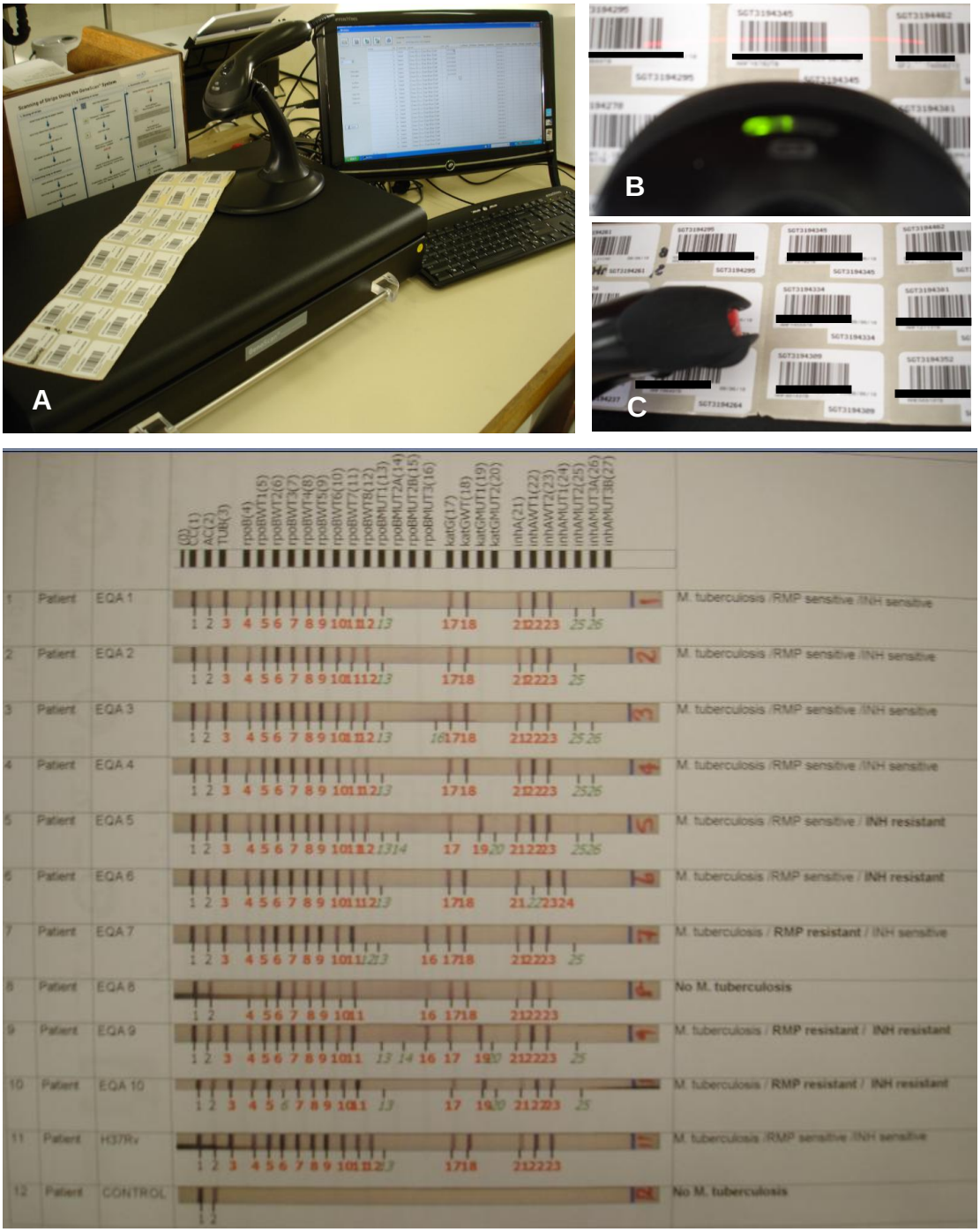


Figure 86: Use of the GenoScan® instrument to eliminate inter-reader variability in the molecular mycobacteriology laboratory. (**A**) To eliminate typing errors, the GenoScan® automated strip scanning instrument uses a barcode scanner to electronically enter patient identification numbers. Note the bar-coded stickers containing patient identification numbers as they would appear on the worklist that was created before the extraction process. (**B & C**) The printed barcodes are scanned in order to create a workload file on the GenoScan® instrument. (**D**) The interpretations need to be transcribed back onto the worksheet and double checked for transcription errors. Here the unique patient identification number, the scanned strip, and the final interpretation are shown.

SENSITIVITY AND SPECIFICITY

A pilot study from a high-volume public health laboratory evaluated the test characteristics of the GenoType® MTBDR*plus* LPA (Barnard, *et al.*). It was shown that when compared to conventional DST on solid media the sensitivity, specificity, positive and negative predictive values for the detection of resistance to RMP were 98.9%, 99.4%, 97.9, and 99.7 respectively; for detection of resistance to INH, the values were 94.2%, 99.7%, 99.1%, and 97.9% respectively; and for the detection of MDR, the values were 98.8%, 100%, 100%, and 97.7% respectively. In addition, a meta-analysis by Bwanga *et al.*, where results from five published studies on the GenoType® MTBDR*plus* were analyzed, showed that the pooled sensitivity and specificity for detection of resistance to RMP by the LPA were both 99%, and for detection of resistance to INH were 96% and 100% respectively. The meta-analyses also showed that the turn-around time for the GenoType® MTBDR*plus* assay ranged from 1 - 2 days.

TYPE OF SPECIMEN USED

The type of specimen used may vary, but the GenoType® MTBDR*plus* has been validated for use on smear-positive patient material that has been decontaminated, digested and concentrated. It has been found that the LPA works well on both pulmonary and extra-pulmonary specimens that have been assayed directly after NaOH-NALC decontamination. Contaminated MGIT cultures that are positive for AFB can also be subjected to LPA testing. Any *M. tuberculosis* DNA that is present will be detected by the TB specific primers in the PCR reaction.

Isolates from blood cultures can also be tested with the LPA, provided that the AFB-positive blood culture is first inoculated and grown on a slope of Middlebrook medium. This transfer is necessary since heme-compounds in the blood culture bottles and albumin in the egg-based media will inhibit the PCR reaction. Once grown, colonies at the top of the Middlebrook slope (the driest part) should be picked

off with a disposable loop, suspended in molecular grade water and extracted according to the kit directions. This method will take longer due to the waiting period for colonies to grow on the slope, but it will allow susceptibility results to be reported for specimens collected in Myco-F-Lytic blood culture bottles.

PREVALENCE OF TB AND ITS INFLUENCE ON THE LPA NEGATIVE PREDICTIVE VALUES

The negative predictive value (NPV) of an assay, defined as the proportion of **true** negative results among test-negative subjects, is highly dependent on the prevalence of disease within a specific setting (high or low burden). The evaluation study done by Barnard *et al*. demonstrated negative predictive values above 97% for all variables tested. This study was performed in South Africa, currently ranked by WHO as 5th in terms of number of total TB cases (0.46 million), and fourth in terms of total numbers of MDR-TB cases (16,000) in the world. Because of the differences in the TB prevalence in various countries, the meta-analyses on the LPA reports from South Africa, Italy, Spain and Germany (performed by Bwanga *et al.*) could not combine the negative-predictive values for these countries.

REGIONAL DIVERSITY OF STRAINS

Regional factors contribute to the development and diversity of drug resistance in *M. tuberculosis.* Some of these factors include settings with high TB incidence; geographical isolation of patients infected with particular *M. tuberculosis* strains; and patient-related risk factors such as the genetic make-up of individuals and their lifestyle habits. As expected, these factors differ between geographic locations and invariably lead to diversity of strains found in regional, national and international locations. It has therefore been found that strains in a particular region of the world

can exhibit resistance-associated gene mutations other than those represented on the GenoType® MTBDR*plus* LPA.

In addition, the different mutations and their combinations included on the GenoType® MTBDR*plus* LPA can also vary both locally and internationally. For example, mutations that occur within *rpoB* in the Western Cape of South Africa do not occur within the same codons in strains circulating in the neighbouring Eastern Cape province. Yet all these variants are identified by the LPA as exhibiting drug resistance to RMP and must be reported as such. Comparisons of specific mutations and banding patterns can be extremely useful when looking at the epidemiology and origins of strains of TB. Such analyses allow one to follow strains as infected patients move from rural areas to cities, from cities back to the periphery, or from region to region. Policy makers and epidemiologists within a country's National TB Program should be familiar with the local strain diversity including the most commonly found resistance-associated mutations. If there are a number of reports of phenotypic drug resistance that are not confirmed by the LPA, there may be strains of *M. tuberculosis* circulating in the population with resistance-associated mutations in genes other than those included on the LPA. If so, the National TB Program should be alerted and ensure that such strains are studied further either in-house or referred to a reference laboratory for sequence analysis.

EXTERNAL QUALITY ASSESSMENT

It is important for the laboratory to have a regularly scheduled External Quality Assessment (EQA) program in place, in order to validate that the laboratory is capable of reporting correct results. If an LPA EQA program is not available nationally, laboratories within a country could take part in an Inter-Laboratory Comparative Analysis (ILCA) program. This program involves two or more local laboratories sending each other sets of DNA specimens for LPA testing. The results obtained by the testing laboratory are sent back to the referral laboratory. Subsequent comparison of the results will aid in determining the competency level of the laboratories involved.

The ILCA also functions as a measure of the reproducibility of the test, and in the past, has shown consistency between different laboratories for the LPA with a 100% pass rate. **Figure 87** shows an example of the results obtained with an ILCA. Although this process assesses the laboratory's competence in performing the LPA, it does not test the proficiency in performing the extraction process or the use of the required equipment. It would be ideal for all laboratories performing the LPA to participate in a national and/or international program that would provide proficiency testing on the complete procedure.

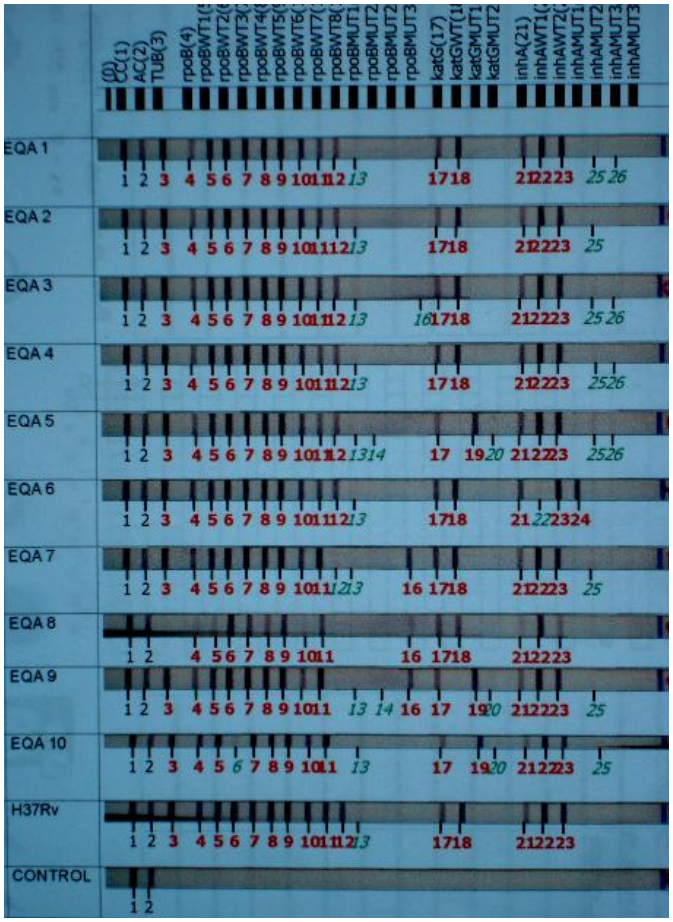
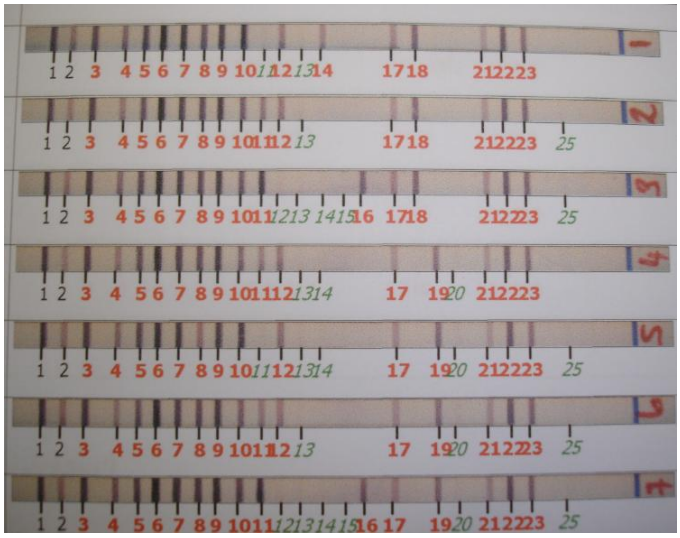


Figure 87: An example of strips from 10 DNA specimens, results unknown, sent for External Quality Assessment. It is important to always include both a positive control ($H_{37}Rv$) and a negative control in the run when performing proficiency testing.

REPORTING OF RESULTS

Similarly to the recommended double checking of smear results to ensure that they are accurate and consistent, it is recommended that the interpretation of LPA strips should be checked by more than one staff member. Such confirmation of results is an integral part of a laboratory's Quality Assurance system. If there is a difference of opinion about the interpretation of any strip, a third person can be consulted, and if consensus cannot be reached, retesting of the specimen may be required. **Figure 88** gives an example of some completed LPA strips and their interpretations.



1. MTB+, RIF MONO^{R}
2. MTB+, FULL SUSCEPTIBILITY
3. MTB+, RIF MONO^{R}
4. MTB+, INH MONO^{R}
5. MTB+, MDR
6. MTB+, INH MONO^{R}
7. MTB+, MDR

Figure 88: Examples of banding patterns on the LPA, and the corresponding interpretations to be reported. A second staff member should always double check the initial reading, before entering the results into the Laboratory Information System.

Note: The numbers 1 to 3 all exhibit susceptibility towards INH, but the presence and/or absence of the WT and MUT probes are completely different for each strip, thus eliminating the possibility of a carry-over event.

Note: It is important to be suspicious of a potential problem when two or more adjacent strips have similar resistance patterns. The majority of strains tested in each run are likely to be pan sensitive, so several resistance strains in a row should raise some suspicion that perhaps sample-to-sample (carry-over) contamination has occurred.

If possible, always compare the molecular results with other laboratory results, i.e. culture, identification, conventional drug susceptibility testing if used and, if possible, with clinical findings.

INTERPRETATION EXERCISE

Figure 89 is intended as an interpretation exercise. The results are given on the next page.

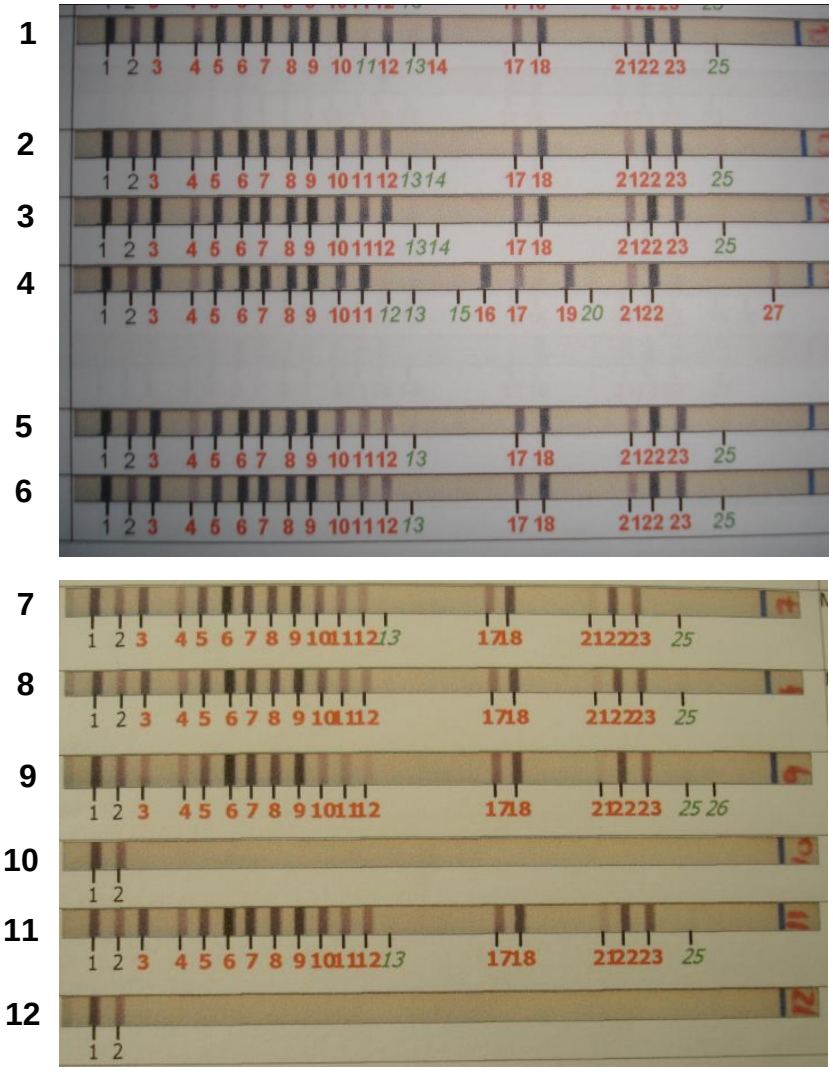


Figure 89: Interpretation exercise.

INTERPRETED RESULTS OF FIGURE 89

1.	MTB+	**RIF-RESISTANT**	INH-SUSCEPTIBLE
2.	MTB+	RIF-SUSCEPTIBLE	INH-SUSCEPTIBLE
3.	MTB+	RIF-SUSCEPTIBLE	INH-SUSCEPTIBLE
4.	MTB+	**RIF-RESISTANT**	**INH-RESISTANT**
5.	MTB+	RIF-SUSCEPTIBLE	INH-SUSCEPTIBLE
6.	MTB+	RIF-SUSCEPTIBLE	INH-SUSCEPTIBLE
7.	MTB+	RIF-SUSCEPTIBLE	INH-SUSCEPTIBLE
8.	MTB+	RIF-SUSCEPTIBLE	INH-SUSCEPTIBLE
9.	MTB+	RIF-SUSCEPTIBLE	INH-SUSCEPTIBLE
10.	MTB-		
11.	MTB+	RIF-SUSCEPTIBLE	INH-SUSCEPTIBLE
12.	MTB-		

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Bwanga *et al.* **Direct susceptibility testing for multi drug resistant tuberculosis: A meta-analysis.** *BMC Infectious Diseases*. 2009, 9:67 doi:10.1186/1471-2334-9-67

Ohno *et al.* **Relationship between Rifampin MICs and *rpoB* Mutations of *Mycobacterium tuberculosis* Strains Isolated in Japan**. *Antimicrobial Agents and Chemotherapy*, Apr. 1996, Vol. 40, No. 4, p. 1053 – 1056.

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Chapter 10: TROUBLESHOOTING

Troubleshooting must be applied when either a procedural step or the final results of a specific assay deviate from the routine, and especially if analytic failures are occurring on a regular basis. Failure of the line probe assay (LPA) may be caused by a variety of reasons: reagents and their storage; specimens or the specimen types; equipment and possible lack of maintenance; infrastructure of the facility; and personnel and their competency. The troubleshooting exercise should begin with the simplest and most probable cause. By following a systematic process of elimination, problems will be identified and ultimately lead to the cause of the failures. Effective troubleshooting and resolution of the problem will be confirmed when results are found to be valid and reliable once again.

This chapter will focus on the most common problems that require troubleshooting in the molecular tuberculosis (TB) laboratory. First, the simplest reason for analytic failure (misinterpretation of the smear result or grading) will be discussed. Secondly, the different procedures and steps to troubleshoot other potential causes will be presented in table format, listing the problem, the most probable cause, and finally a solution or preventative measure.

MISINTERPRETATION OF THE SMEAR RESULTS

The value of the LPA to rapidly detect TB and its associated susceptibility pattern - whether it is susceptible to RIF and INH, mono-resistant to either, or resistant to both (MDR) - can only be realized if acid-fast bacilli (AFB) smear microscopy, the most crucial and longstanding method for TB diagnosis, is accurately performed. For example, if the banding patterns of the TUB band and the *rpoB* WT2 and WT6 reaction zones on the LPA are present but very faint, the smear might have been misread as positive (grading ≥1+) when actually it should have been graded as scanty or perhaps negative with artefacts that were mistakenly identified as AFB.

Furthermore, DNA extraction for the LPA is performed on the residual portion of the NaOH-NALC decontaminated specimen that remains after the MGIT tube has been inoculated and the smear made. If the smear is negative or scanty, it is unlikely that there will be enough bacteria present in the residual portion to yield a sufficient amount of DNA for a successful LPA. Thus, if a negative or scanty smear is incorrectly interpreted as positive, the LPA will be unsatisfactory. If an unsatisfactory LPA result is seen, the smear (which must be kept for a month) should be re-read by an independent observer to validate the initial smear results. Usually, if a negative smear is read as positive, the MGIT culture will likely be culture negative.

In addition to errors in reading of the smear, AFB-negative specimens may become contaminated with AFB from positive specimens or with environmental mycobacteria when supplies used in the NaOH-NALC specimen processing are contaminated. This situation will also result in un-interpretable and unsatisfactory LPA results. It is recommended that the aspirator flask taps be flamed between different decontamination batches, and cleaned and sterilized often in order to limit possible contamination of specimens.

DEFINITE POSITIVE SMEAR, BUT NEGATIVE LPA

An AFB-positive smear but negative LPA can be due to the presence of nontuberculous mycobacteria (NTM). The species-specific primers used in the GenoType® MTBDR*plus* LPA will not recognize these different species, and thus the LPA will be negative. The presence of NTM can be confirmed by doing a common mycobacteria (CM) LPA on the specimen in question. Alternatively, inhibitors may be present and interfere with the PCR reaction. This possibility will be discussed below.

UNSATIFACTORY LPA RESULTS FROM CULTURES

In order to ensure that sufficient biomass is present for DNA extraction, it is recommended that the growth inits (GUs) on the BACTEC 960 be adjusted to a minimum of 300 GUs before a tube is flagged as positive. The results of the LPA are often either indeterminate or unsatisfactory when the assay is performed on growth from MGIT tubes with GUs of 75 or 150. Using MGIT tubes with a minimum of 300 GUs ensures that there is enough sample material for several assays: a smear; identification methods; the extraction of DNA for the LPA; and second line drug susceptibility testing if the LPA indicates resistance to RIF and/or INH. If the MGIT tubes are removed from the BACTEC machines before sufficient growth has occurred, a poor LPA result can be anticipated. It is therefore recommended that the threshold value of the BACTEC machine be adjusted to 300 GUs.

Care must be taken when picking colonies from solid media. Too many colonies will result in an increased amount of DNA, which may deplete the DNA polymerase early in the amplification procedure. Also, care must be taken not to scrape off any of the egg-based media, since it may inhibit the PCR reaction.

UNSATISFACTORY LPA RESULTS DUE TO INSUFFICIENT DNA POLYMERASE

Faint banding patterns could be the result of insufficient DNA polymerase in the PCR master mix. If this is suspected, the first approach would be to assess if the pipette that was used to add the DNA polymerase to the master mix is delivering appropriate volumes. The pipette must be validated in order to ensure accuracy and precision. It is also recommended that whenever the different reagents and solutions are drawn up inside a filter tip, the tip should be visually inspected to ensure that the volume looks appropriate. If the volume is less or different from what is usually seen, a switch to a new tip should be made, and if the problem persists, a switch should be made to a different clean and validated pipette that is dedicated to the same area.

RESISTANT BANDING PATTERN, BUT THE TUB BAND IS ABSENT

When the TUB band, specific for identifying *Mycobacterium tuberculosis*, is absent on a strip but bands are present that would ordinarily indicate resistance to RIF or INH, the LPA should not be reported. This result is most probably due to the presence of NTM since many mycobacteria share similar sequences with *M. tuberculosis* in the resistance-associated regions, and may have mutations associated with resistance to these drugs.

SMEAR POSITIVE, BUT THE AMPLIFICATION CONTROL BAN IS ABSENT

The absence of the amplification control band may be the result of inhibition of the PCR due to the presence of a variety of contaminants: powder from gloves; haemoglobin from a blood stained specimen; urea; and other organic compounds. The inhibition might also be due to incomplete lysis or insufficient time or

temperature for the heat kill, degradation of DNA due to prolonged storage at incorrect temperatures, or DNA polymerase inhibition. All consumables used must be DNase, RNase and Pyrogen free, since these components will also inhibit the PCR reaction.

CONTAMINATION OF THE NEGATIVE CONTROLS

If any negative control in a run is positive, none of the results from the contaminated run should be reported to the clinician. The whole batch must be re-examined from the extraction stage through hybridization and subsequent interpretation of results. This problem causes a delay in turnaround time and has a negative impact on patient treatment. A preventative measure to eliminate the chances of contamination is to use ultra-violet irradiation of the aliquoted PNM master mix, since primers are more resistant to UV-irradiation than template DNA. It is very important to ensure that the aliquots are not overexposed to UV irradiation since this will affect the sensitivity of the reaction. Chapter 6: Contamination Control contains further details as well as additional preventative measures.

NO BANDS SEEN ON THE IPA STRIP

When no bands are seen on the LPA strip, likely causes could be failure to add a reaction component during the master mix preparation, selecting the wrong thermal cycler program or hybridization program, or failure to start the thermal cycler or hybridizing instrument properly. Alternatively, these instruments should be checked to ensure that they are not faulty with inaccurate temperatures for cycling and incubation.

CONTAMINATION OF A BATCH LEADING TO DISCREPANT RESULTS IN REPEAT BATCH

When contamination occurs during the DNA extraction step, the problem will not be detected until the procedure is completed and the strips are read. At that time it will not be known where contamination occurred, so, after thorough cleaning of surfaces, the amplification, hybridization and detection steps should be repeated using the originally extracted DNA. If the strips are now clean, the results can be reported and it can be assumed that the contamination occurred at some time other than the DNA extraction step, and the cleaning likely resolved the problem. If the contamination is still present, it is likely that the contamination occurred during the DNA extraction step. In this case, DNA must be re-extracted from the original specimens or cultures before proceeding with LPA. This wastes both time and LPA kit components, and emphasizes the importance of using rigorous contamination control measures for all aspects of LPA usage.

STRIPS NOT HYBRIDIZING AT THE *inhA* REGION

The strips must be completely covered in hybridization solution. If the end part of the strip where the *inh*A reaction bands are located is “surfing” on top of the solution (**Figure 90**) it should be gently pushed down by placing a pipette tip on the part of the strip that is above the surface. The tip can be left in place until the hybridization time has elapsed.

Figure 90: Example of strips (numbers 85 and 86) that are “surfing” in the *inhA* reaction zone region of the strip.

WEAK HYBRIDIZATION SIGNALS

In the event of weak or questionable hybridization signals over most of a strip, the results should not be reported (**Figure 91**). Alternatively, if the results for the TUB band and most gene(s) are valid but the reaction bands for one particular gene are only partially developed, the result can be reported as positive for the *M. tuberculosis* complex and the results of the valid reaction zones can be reported. The LPA should then be repeated to confirm the results and fill in the information on the inconclusive gene.

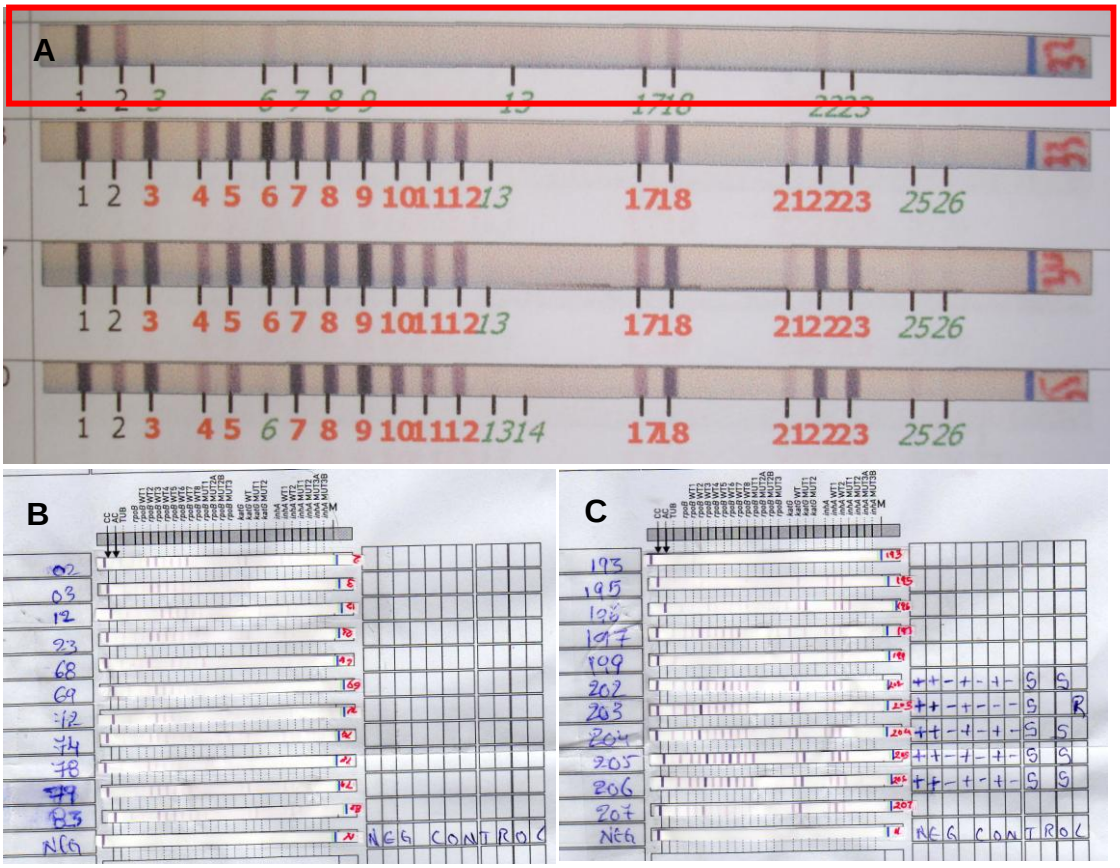


Figure 91: Example of weak hybridization signals. (**A**) The intensities of the banding patterns in the first strip, blocked in red, are very weak as opposed to those of the other strips from the same run. Note that only the bands indicated by the red numbers are as intense as those of the AC band. (**B**) The signals of all the strips are weak. This may be due to insufficient DNA polymerase added to the master mix, or improper extraction procedure. (**C**) Possible amplification of both sputum specimens and cultured isolates in one PCR run using 30 cycles for both. Note that none of the weak banding patterns were interpreted.

It is important to avoid interpreting the banding pattern of a specimen when only a few reaction zones are weak on the strip, as compared to the rest of the strips in the same run. The specimen might contain NTM as illustrated on the strip in **Figure 92**.

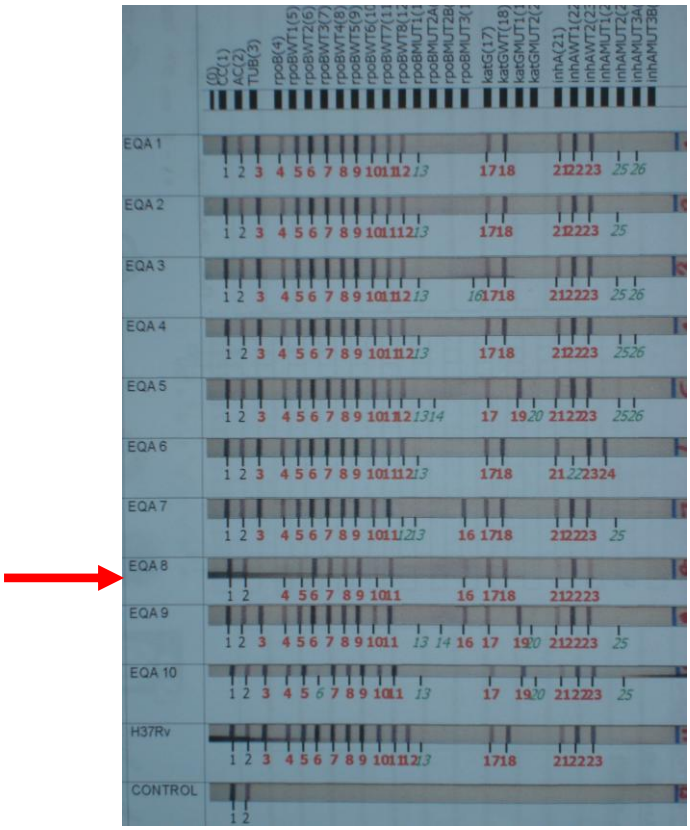


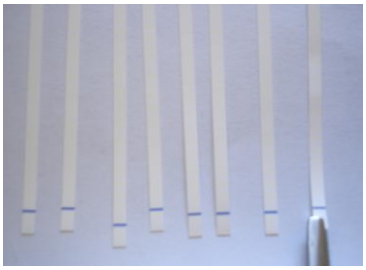
Figure 92: EQA 8 shows an example of weak signals at the TUB band, in addition to MUT3 of *rpo*B and others. When compared to the other strips, the TUB band is absent and some other bands are weakly positive due to shared homology of *rpoB*, *katG* and *inhA* genes within the mycobacterial species.

TABLE 9: TROUBLESHOOTING GUIDE

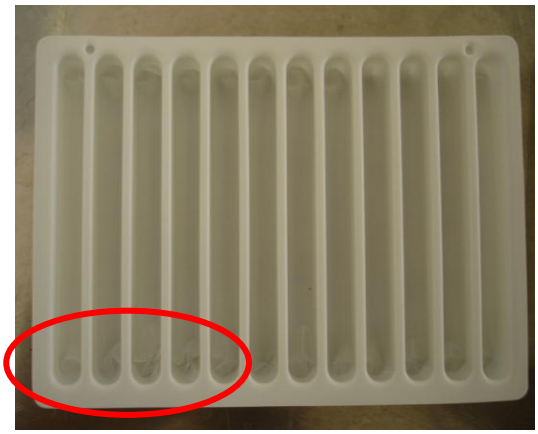
Problem	Possible Cause	Solution/Prevention
Failure to amplify	Temperature inconsistencies within the thermal cycler block that result in unequally distributed or inappropriate temperatures	Temperature validation of the thermal cycler is required. If a particular well always fails to amplify and the rest are properly amplifying, that specific well should be skipped. It is more cost-effective to skip a well than buy a new machine
	Powdered gloves were used	Powder-free gloves must be used. The powder is made from rice or corn that has become inedible, and may contain DNA polymerase inhibitors
	Impure or insufficient DNA	The purity of a processed specimen should be checked by re-reading the smear and looking for artifacts or food particles that may resemble AFB. The parallel culture should be checked as well to see if it is contaminated. If contaminated with non-AFB, an additional specimen should be requested.
	The presence of inhibitors or the quantity of extracted DNA does not allow efficient amplification	The presence of amplicons should be checked by running the PCR products on a 2% agarose gel
	Inhibition caused by heavily contaminated cultures	A new specimen should be requested from the clinician

No visible amplicons on gel	Poor extraction technique or Ethidium Bromide was not added to the gel	The DNA extraction and amplification should be repeated. Ethidium bromide must be added to the gel in order to visualize the amplicons.
The bands on the GenoType® MTBDR*plus* strips are in a fixed pattern that is not indicative of *M. tuberculosis*	The wrong PNM mix was used during reagent preparation	The drawers of the - 20ºC freezer must be organized so that the GenoType® MTBDR*plus* PNM and CM (or any other Hain Lifescience PNM mix) have dedicated drawers, thereby preventing them from being confused. Furthermore, the table-top cooler and PCR rack of the separate master mixes should be of a different colour so that one can easily identify specific reagents.
Bands on the agarose gel, but weak signals on the strips	Low probe concentration on the strips	The amount of amplicons used in the hybridization reaction should be increased to 30 µl (the same volume of denaturing buffer should be added)
	Denatured amplicons came into contact with strips	The hybridization solution must be added to the denatured amplicons before placing the strips into the wells. This also applies to the automated Hybridizer, depending on the program setting on the machine, where either the strips are added after the green hybridization buffer (HYB) has been dispensed into the wells or before loading the tray into the machine. If the strips are added to the wells before the HYB has been added, it is important to ensure that the strips and the denatured amplicon solution do not come into contact with one another.

Amplification but weak signals	AFB smear was misinterpreted as positive	The initial smear should be reread. If still positive, the extraction technique should be reviewed. If the smear is negative, the competency of the microscopist must be assessed and retraining offered if necessary.
	Insufficient amount of DNA due to poor extraction technique	If there is enough of the sample left in the tube after the first extraction, the extraction must be repeated. If there is not enough sample left or if everything has been used for the first extraction, then the amount of DNA added to the PCR master mix should be increased from 5 to 8 µl.
High background colour	Too much CON-C (streptavidin) added to the solution	The conjugate solution should be prepared correctly by using a ratio of 1:100 when diluting the concentrate into the diluent
	Use of over-concentrated CON-C and/or SUB-C	
	Washing steps were not thorough enough	The TwinCubator® or the GT-Blot should be checked to ensure that they are either shaking or oscillating properly
	The wash solutions were not warm enough due to very low ambient temperature	The ambient temperature should be maintained above 21 - 22º C

High, uneven background	The strip might be contaminated	The strip should always be handled with clean forceps on the showing the blue line (**Figure 93**). Gloves should always be worn when handling the strips. Forceps must still be used even when gloves are worn, since even a fingerprint from a glove can cause an increased background.  **Figure 93:** Correct handling of strips
	Contaminated forceps	Disposable forceps should be used if possible. If disposable forceps are not available, there must be two differently marked forceps in the hybridization area: one for the removal and numbering of the clean strips, and the other for the transfer and removal of the hybridized strips. The forceps should be subjected to UV-irradiation after each use and/or decontaminated. Residual bleach or alcohol must never be left on the strip since it could destroy or inhibit the amplicons or probes on the strip and interfere with the band intensity.
	Dirty trays	The trays must be washed properly after each use since residual salts from the hybridization buffers may cause an increased background in subsequent tests

	Insufficient pre-warming of hybridization buffers	The hybridization solution must be pre-warmed and completely free of precipitates before using. The viscosity of the solution must be low, since a gel-like buffer will only partially cover the strip and lead to an increased background.
	Insufficient hybridization buffer	When using an automated Hybridizer (e.g. the GT-Blot 48), the tray must be checked to ensure that the strips are completely covered after the addition of each of the different hybridization buffers
	Strip was upside down	The strips should be checked to ensure that they are facing up after each addition of a hybridization buffer. This can be done by ensuring that the strip numbers are visible after the new buffer has been added.
	Too large an amount of amplicons were denatured and added to the HYB	Decrease the amount of amplicons that are denatured prior to the addition of the HYB. This will reduce the background while still retaining the sensitivity.
	Strip has dried out	The strip should never be allowed to dry between pre-hybridization and hybridization

	Strip has dried out because of a puncture in the hybridization tray, allowing the buffers to seep through	The trays should be visually inspected for holes before adding the denaturing buffer and amplicons. **Figure 94** shows an example of a punctured tray. If there are holes and it goes unnoticed, this will create a contamination problem.  **Figure 94**: A puncture at the bottom of the tray allows buffer to leak into the machine.
	Inadequate rinsing of the strips after the conjugation and substrate steps	According to the SOP, proper rinsing of the strips must be done during the two different steps of the procedure
	Hybridization temperature too high	The temperature of the Hybridizer should be checked using distilled water as the medium for the reading. A high-quality calibrated thermometer should be used. If the temperature is out of range, the service technician must be contacted for repair.
Binding to all reaction zones	Non-specific binding due to too low temperature in the instrument during the hybridization step	The temperature check should be done and if out of range, the service technician contacted

Low sensitivity	Incomplete binding of single stranded DNA to the probes due prolonged contact of the strip to the denatured amplicons in the absence of hybridization buffer	This is applicable only to the automated Hybridizer. It is important to ensure that the hybridization procedure begins immediately upon contact of the unhybridized strip with the denatured amplicons. If there is a delay, the strips will dry out.
	Insufficient streptavidin	The concentration of the conjugate solution must be accurately diluted by using a ratio of 1:100 of the concentrate to the diluent
Random hybridization signals which may look like a heterogeneous infection	Contamination of neighbouring wells by spillage	The strips on either side of a strip where spillage is suspected need to be investigated. If the flanking strips are both interpreted as susceptible to INH and RIF, the strain in question is likely hetero-resistant. The strain pattern needs to be carefully investigated before ruling out a mixed infection. If neighbouring strips show resistance bands, the hybridization must be repeated.
Non-specific banding patterns	Non-specific amplification of primers	It is important to use Qiagen HotStarTaq® DNA polymerase. This enzyme prevents non-specific annealing of primers at low temperatures that lead to generation of unwanted products. This may happen during master mix preparation or in the first cycle of the PCR.
Low sensitivity on the automated reader	The intensity is set too low on the GenoScan® reader	The sensitivity needs to be increased, and the strip re-scanned. Alternatively, the bands with weak signals on the reader, but valid by an eye reading, can be altered manually by adjusting the cut-off value(s) for the band(s).

Pipetted volumes inaccurate	Faulty pipette(s)	The pipette(s) should be recalibrated or replaced if unrepairable
Over-exposing the strips or missing a hybridization step during detection	This is due to delaying the response to the signal from the TwinCubator® that a particular step in the procedure has been completed	Leaving the strip exposed to a particular buffer will have a negative impact on the outcome of the strip in various ways depending on the specific buffer. For example, if strips are exposed to the conjugate or substrate solutions for too long, an intense background will be seen. If the conjugate has not been added, no bands will develop when adding the substrate. In this case, the substrate can be removed and the strip rinsed with ddH_20, after which the conjugate can be added and the procedure completed.
Assay failure	Stability issues (nucleases)	Optimally, the template DNA should be tested the day after extraction following overnight storage at 4ºC
	Inhibitors in bloody specimens	Heme causes inhibition of the PCR. The sputum must be processed so that all traces of blood are removed from the specimen prior to the DNA extraction.
	• Technique • Contamination • Workflow • Knowledge	Incorrect performance of the assay during any step will result in failure of the LPA. The SOP must be followed precisely. The organization of the PCR facility and workflow must be well established to prevent and combat possible contamination.

Successful amplification, but no results on strips when the automated hybridizer was used	Hybridization buffers were not placed in the correct order or their corresponding aspirator tubes were misplaced in the hybridization buffer bottles	The plugs of the aspirator tubes that fit onto the bottles are colour-coded in order to avoid confusion. The corresponding buffer sequence is marked on the inside of the lid clearly indicating where each buffer goes (**Figure 95**). The green hybridization buffer aspirating tube has a green plug, and the stringent wash buffer a red one. The orange (conjugate) and yellow (substrate) plugs must not be confused. If these are switched, there would be no colour reaction, since the substrate would be added to the wells first and the conjugate last.  **Figure 95:** Colour coded aspirator tubes. The loading sequences of the buffers are indicated in colour-coded dots on the inside of the lid.
Irregular dispensing of the automated hybridizer	One of the hybridization solution dispensing tubes may become clogged due to: failure to set the machine to wash the tubes; irregular use of the machine; or solutions that were not properly pre-warmed.	When the machine is found to be dispensing into every other well, it must be stopped and the mouth of the tube should be de-clogged. An opened paper clip or other wire can be used. Once de-clogged, the machine should be restarted and allowed to dispense into the wells as normal. The specific step at which the blockage occurred can be selected on the GT-Blot 48, and the machine can be set to complete the hybridization run from that

		point forward. Every other well may now have 2 ml of the hybridization solution. The 2 ml is not a problem, and with the aspiration step, all the liquid will be removed from the well, and 1 ml of the other solutions will be added to the wells.
PCR inhibition	Failure of lysis or incomplete lysis (where whole cells are used directly as source of template DNA)	The equipment used for the extraction should be checked to ensure that it is functional, and that specimens are not removed from the sonicator before the lysis procedure has been completed
	Degradation of the template DNA	The DNA should not be left at room temperature overnight or for long periods before use. The freshly extracted DNA should be stored at 4ºC and preferably used the next day.
	Polymerase inhibition	Powder from gloves, impure DNA, or other inhibitors can hinder the PCR
False-positives and false-negatives	Contamination	When contamination is found, the areas must be thoroughly decontaminated and “fumigated” with sodium hypochlorite left in the room in an open container. All possible sources of contamination must be investigated, from the reagents to the equipment used. Amplicons must never be manipulated or stored in the proximity of the PCR reagents.

Power failures	Multi-factorial	It is advisable to connect an uninterruptible power supply (UPS) to both the thermal cycler and the hybridization machine in the event of a temporary power failure. This will prevent wastage of reagents and an increased turnaround time should a power failure occur during the use of the instruments.
Weak or no signals including the conjugate control (CC) zone	This may be due to the hybridization reagents that were not properly pre-warmed	The green and red hybridization solutions must be pre-warmed to 45ºC
	Ambient room temperature is too low	A low room temperature causes a lower reagent temperature, which in turn influences the hybridization. The central heating/cooling thermostat must be appropriately adjusted.
	Insufficient amounts of CON-C and/or SUB-C were added to their respective diluents	The volumes of the pipettes must be checked for accuracy
Weak hybridization signals except in the CC zone	Due to improper DNA extraction	Insufficient template DNA (or quality thereof) will result in weak amplification, leading to weak hybridization signals. The specimen must be properly re-extracted, and the extraction equipment checked.
	Incorrect cycler setting for a particular type of specimen	Direct patient material must always be amplified for 40X cycles, while isolates must be amplified for 30X cycles

	Insufficient DNA polymerase was added to the PNM master mix during reagent preparation	It is essential that the operator pay attention and visually check the amount of liquid in the pipette tip before adding any liquid into a tube
	The PNM and DNA polymerase (*Taq*) were frozen and thawed too many times (optimal 5-6 times)	The PNM and *Taq* should not be subjected to too many freeze-thaw cycles since this treatment will degrade the components. Also, when these solutions are in use on the bench, they should be stored in a tabletop cooler.
	The master mix components were not thawed properly, or the PNM and *Taq* were not kept at the appropriate temperatures during storage or preparation of the master mix	The components must be thoroughly thawed, and can be assisted by gentle inversion but not by vortexing. The primers may be degraded by such vigorous treatment. If there is still “ice” in any of the vials, the concentration will not be homogenous, and the PCR may fail. After thawing, the components must be kept in a tabletop cooler. The *Taq* contains glycerol so there is no need to thaw it before use. It should always be kept in the tabletop cooler when removed from the - 20^{o}C freezer.
	The ambient temperature is too low	A room temperature that is very low might cause rapid cooling of the prewarmed hybridization buffers. This may indirectly lower the temperature of incubation, and also cause the other reagents that are not prewarmed to be colder than the ideal room temperature (21ºC). It is suggested that the time for the substrate incubation should be increased if the ambient temperature is very low.

	The incubation temperature is too high or too low	An incubation temperature that is lower than required may result in nonspecific binding of the amplicons to probes on the strips. Alternatively, a higher incubation temperature may prevent binding of the amplicons to the probes. In addition, a higher temperature during the stringent wash step may result in weak intensities of the banding patterns. The temperature of the Hybridizer should be monitored if such problems are seen. If the problem persists and the thermostat on the Hybridizer gives an appropriate reading of 45ºC, and a digital thermometer reads a different temperature that is above or below 45ºC, the problem is likely to be with the machine's internal temperature probe.
	The reagents have expired	Reagents must always be checked for expiration dates and the dates must be documented as part of the quality control within the laboratory. Expired reagents must never be used since excessive precipitates may form over time. Also, care must be taken not to let the buffer containers remain open during preheating since the salt concentrations will increase due to evaporation. An increased salt concentration will result in variability in the intensity of the bands. Upon receipt of reagents, they must be dated with the day, month, and year received and initialed. Similarly, once they are placed into use, they must be dated and initialed.

	The ratio of concentrate to diluent of the conjugate and substrate buffers were incorrectly measured or insufficiently mixed, or the volume of these added to the strips was not 1 ml	The procedure for the reconstitution of the concentrates into the diluents must be done precisely as directed in the SOP, and the pipettes must be validated as accurate and precise
Heterogeneous (non-homogeneous) staining of bands	The strips were not completely immersed in reagents during the incubation steps	The strips must be checked to ensure that they are fully immersed after the addition of each reagent. If necessary, a pipette tip can be placed on top of a strip to keep it submerged. Care must be taken so that buffer from a well with a problematic strip does not spill into any of the other wells.
	Denatured amplicons were not properly mixed with the hybridization buffer before adding the strip to the well	Before adding the strip to the well, the purple solution containing the denatured amplicons should be completely diffused into the green hybridization buffer
	Tray was not shaken properly	The instruments should be checked to ensure that the TwinCubator® is shaking at 300 rpm and the GT-Blot 48 is oscillating properly
	The reusable tray had not been properly cleaned so residual amplicons remained	The trays must be thoroughly washed and rinsed after each use
Strong and fast colour development occurs after adding the substrate buffer	This phenomenon depends on the amount of amplified DNA used and on the specific reaction conditions	The incubation with substrate should be stopped as soon as signals are clearly visible. Prolonging the incubation with substrate will result in the development of cross-hybridizing bands (artifacts) that may be incorrectly interpreted as actual bands.
Intense bands and associated artifacts	Too much DNA in the extracted sample	The extracted DNA should be diluted before the performing the PCR

TABLE 9: PIPETTE TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution/Prevention
Pipette cannot aspirate liquid	If there is a filter, it may be blocked or the O-ring and seal ring may not be assembled in the correct order	The filter should be replaced and the pipette manual consulted for further instructions
Liquid leaks from the filter tip	The nozzle cylinder may be loose or worn, or the plunger may be damaged. The O-ring and seal ring may be worn, or the tip may not be attached correctly.	The pipette instruction manual should be consulted. The filter tip should be checked to ensure that it is correctly attached to the pipette.
The push button does not operate well	Liquid has probably been sucked up into the body of the pipette	The pipette instruction manual should be consulted on how to disassemble the pipette and clean all its parts
Tip cannot be ejected from the pipette	The nozzle in the cylinder might be loose	The cylinder should be tightened or screwed in place

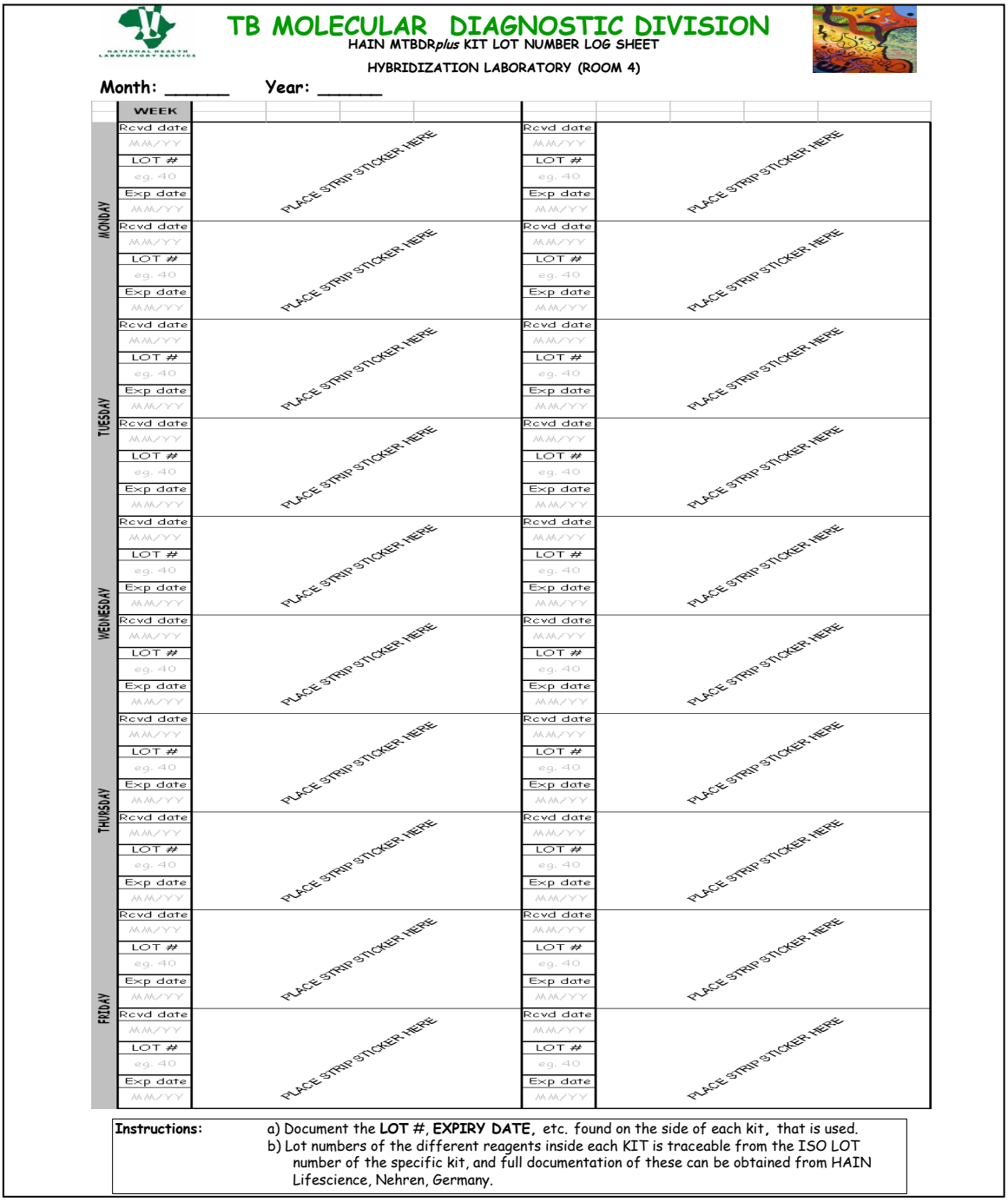
INSTRUMENT TROUBLESHOOTING

Detailed knowledge of the functioning of the instruments is key to dealing with unforeseen situations. When a problem with an instrument is suspected, the troubleshooting section of each instruction manual must be consulted. The laboratory worker must be familiar with the manuals of all the equipment and must follow and apply the procedures provided in each instrument's specific troubleshooting guide.

Appendix A

Reagent	Area			
	DNA Extraction	Pre-amplification (Master mix preparation)	Amplification (Template addition)	Post-amplification (Hybridization/Detection)
HYB Buffer (Green)				●
STR Wash (Red)				●
RIN Buffer (Opaque)				●
CON-C (Orange)				●
CON-D (Transparent)				●
SUB-C (Yellowish Brown)				●
SUB-D (Transparent)				●
DEN Buffer (Blue)				●
Strips				●
PNM Mix (Purple)		●		
HotStarTaq® (Transparent with orange lid)		●		
10X PCR Buffer (Transparent with blue lid)		●		
MgCl2 (Transparent with yellow lid)		●		
Molecular Grade Water	●	●		
Sodium Hypochlorite (1%)	●	●	●	●
Sodium Hypochlorite (5% stock solution)				●
Ethanol (70%)	●	●	●	●
Ethanol (96% stock solution)				●
Distilled Water	●			●
Contrad®	●	●	●	●

Table 1: Area specific reagent usage



TB MOLECULAR DIAGNOSTIC DIVISION

HAIN MTBDR*plus* KIT LOT NUMBER LOG SHEET

HYBRIDIZATION LABORATORY (ROOM 4)

Month: _____ Year: _____

	WEEK		
MONDAY	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE
TUESDAY	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE
WEDNESDAY	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE
THURSDAY	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE
FRIDAY	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE	Rcvd date MM/YY LOT # eg. 40 Exp date MM/YY	PLACE STRIP STICKER HERE

Instructions:

a) Document the **LOT #**, **EXPIRY DATE**, etc. found on the side of each kit, that is used.

b) Lot numbers of the different reagents inside each KIT is traceable from the ISO LOT number of the specific kit, and full documentation of these can be obtained from HAIN Lifescience, Nehren, Germany.

Figure 1A: Example of the GenoType® MTBDR*plus* reagent and strip logsheet

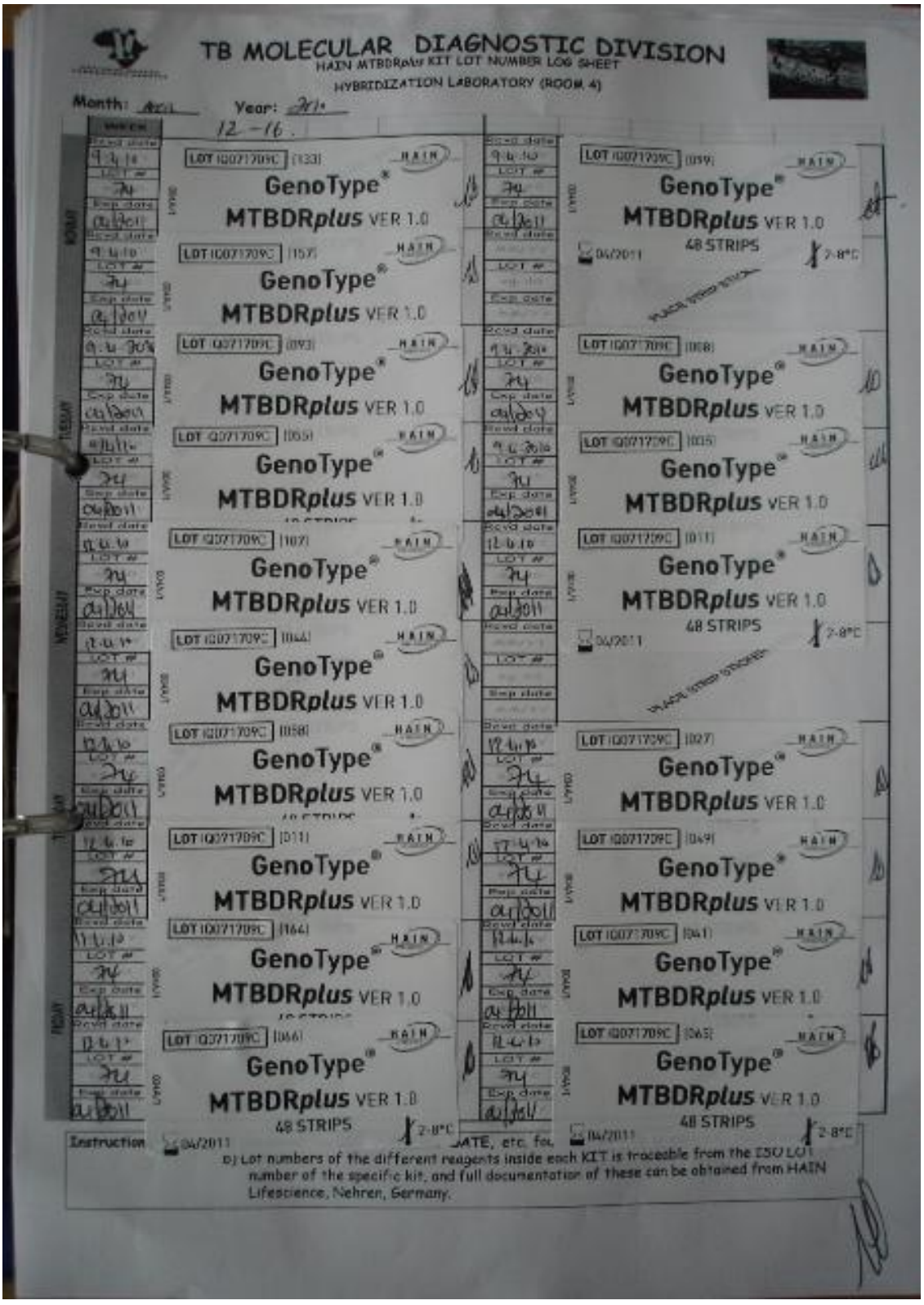
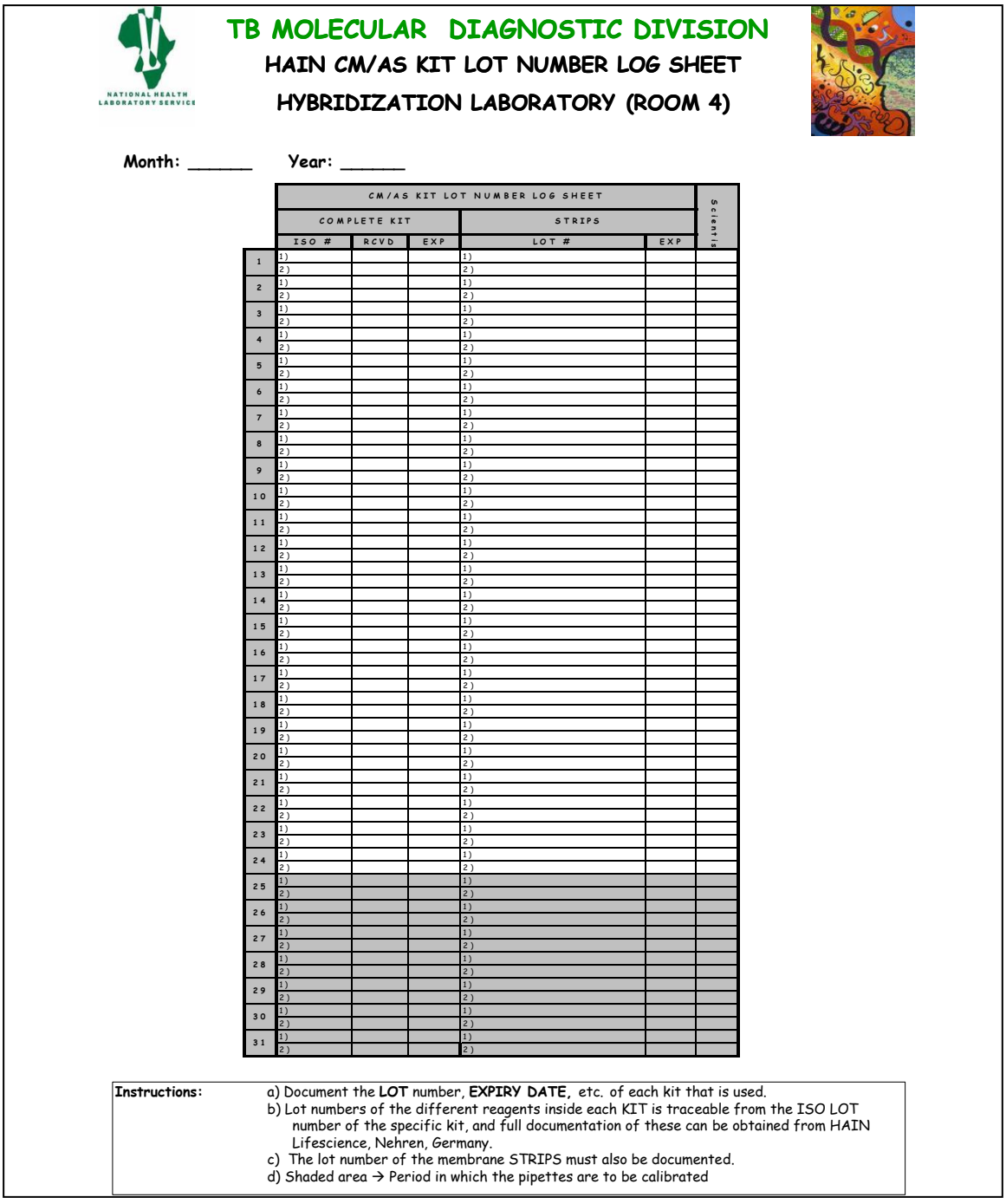


Figure 1B: Completed GenoType® MTBDR*plus* reagent log sheet for the week of 12 – 16th April 2010. The "Lot number" is that of the **kit** and the expiration date is that of the strips.

Note: The individual who did the hybridization also signs next to the strip sticker.



TB MOLECULAR DIAGNOSTIC DIVISION

HAIN CM/AS KIT LOT NUMBER LOG SHEET

HYBRIDIZATION LABORATORY (ROOM 4)

Month: _____ **Year:** _____

	CM/AS KIT LOT NUMBER LOG SHEET					
	COMPLETE KIT			STRIPS		
	ISO #	RCVD	EXP	LOT #	EXP	Scientist
1	1)			1)		
	2)			2)		
2	1)			1)		
	2)			2)		
3	1)			1)		
	2)			2)		
4	1)			1)		
	2)			2)		
5	1)			1)		
	2)			2)		
6	1)			1)		
	2)			2)		
7	1)			1)		
	2)			2)		
8	1)			1)		
	2)			2)		
9	1)			1)		
	2)			2)		
10	1)			1)		
	2)			2)		
11	1)			1)		
	2)			2)		
12	1)			1)		
	2)			2)		
13	1)			1)		
	2)			2)		
14	1)			1)		
	2)			2)		
15	1)			1)		
	2)			2)		
16	1)			1)		
	2)			2)		
17	1)			1)		
	2)			2)		
18	1)			1)		
	2)			2)		
19	1)			1)		
	2)			2)		
20	1)			1)		
	2)			2)		
21	1)			1)		
	2)			2)		
22	1)			1)		
	2)			2)		
23	1)			1)		
	2)			2)		
24	1)			1)		
	2)			2)		
25	1)			1)		
	2)			2)		
26	1)			1)		
	2)			2)		
27	1)			1)		
	2)			2)		
28	1)			1)		
	2)			2)		
29	1)			1)		
	2)			2)		
30	1)			1)		
	2)			2)		
31	1)			1)		
	2)			2)		

Instructions:

a) Document the **LOT** number, **EXPIRY DATE**, etc. of each kit that is used.
b) Lot numbers of the different reagents inside each KIT is traceable from the ISO LOT number of the specific kit, and full documentation of these can be obtained from HAIN Lifescience, Nehren, Germany.
c) The lot number of the membrane STRIPS must also be documented.
d) Shaded area → Period in which the pipettes are to be calibrated

Figure 2: Example of the CM/AS kit reagent log sheet. Since this test is used less frequently, the lot numbers of the strips are written out individually rather than the label being attached as for the GenoType® MTBDR*plus* kit.

NATIONAL HEALTH LABORATORY SERVICE

TB MOLECULAR DIAGNOSTIC DIVISION

MASTER MIX COMPONENTS LOT NUMBER LOG SHEET

PRE-AMPLIFICATION LABORATORY (ROOM 2)

Month: _____ **Year:** _____

	MASTER MIX COMPONENT LOT NUMBER & EXPIRATION DATE								Scientis
	PNM		Buffer		MgCl2		Taq		
	LOT #	EXP	LOT #	EXP	LOT #	EXP	LOT #	EXP	
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
25									
26									
27									
28									
29									
30									
31									

Instruction: a) Document the LOT number and **EXPIRY DATE** of each component that is used.
b) Shaded area → Period in which pipettes are to be calibrated.

Figure 3: Example of the PNM, 10X buffer, $MgCl_2$ and DNA polymerase reagent log sheet.

TB MOLECULAR DIAGNOSTIC DIVISION

DNA POLYMERASE & HAIN TEST KIT USAGE LOG SHEET

	TAQ / KITS IN STOCK		
WEEK	TAQ	MTBDRplus	CM/AS
MONDAY			
TUESDAY			
WEDNESDAY			
THURSDAY			
FRIDAY			
SATURDAY			

	TAQ / KITS IN STOCK		
WEEK	TAQ	MTBDRplus	CM/AS
MONDAY			
TUESDAY			
WEDNESDAY			
THURSDAY			
FRIDAY			
SATURDAY			

	TAQ / KITS IN STOCK		
WEEK	TAQ	MTBDRplus	CM/AS
MONDAY			
TUESDAY			
WEDNESDAY			
THURSDAY			
FRIDAY			
SATURDAY			

	TAQ / KITS IN STOCK		
WEEK	TAQ	MTBDRplus	CM/AS
MONDAY			
TUESDAY			
WEDNESDAY			
THURSDAY			
FRIDAY			
SATURDAY			

Instructions: a) Document, **DAILY**, the amount of stock that is left over in the fridge after the work for the day has been done. (Taq → un-used ; Kits → in un-opened boxes)

Figure 4: Example of stock control log sheet.

It is the responsibility of the individual who prepared the master mix to count how many tubes of *Taq* DNA polymerase are left. The person who performed the hybridization is required to count how many kits are left. The staff must place their initials next to the line for the particular day.

Appendix B

A. RECIPES FOR CLEANING AGENTS

1% Sodium Hypochlorite

- To prepare 1% sodium hypochlorite, add 5% sodium hypochlorite concentrate to distilled water (dH_2O) in a ratio of 1:4
- Specifically, to prepare 1 L of 1% sodium hypochlorite, add 200 ml of 5% sodium hypochlorite to 800 ml of dH_2O.

70% Ethanol

- To prepare 70% ethanol, add 100% absolute ethanol to dH_2O in a ratio of 7:3
- Specifically, to prepare 1 L of 70% ethanol, add 700 ml of absolute ethanol to 300 ml of dH_2O.

Note: Fresh batches of 1% sodium hypochlorite and 70% ethanol should be made up every one to two days.

B. PROCEDURES TO PREVENT CONTAMINATION

Area 1: DNA EXTRACTION ROOM

- **Weekly cleaning of the DNA extraction room:**
    - At the beginning of each week, the DNA extraction room must be thoroughly cleaned.
    - Cleaning should be top-to-bottom, starting with the bench tops and finishing with the floor.
    - Once the upper surfaces have been cleaned, the floor should be swept with a broom.
    - The floor should be mopped with 1% sodium hypochlorite, first using the leftover diluted bleach and then preparing more 1% sodium hypochlorite if needed.
    - Clean the mop under running water, and spray and mop the floor with 70% alcohol.

- **Biological Safety Cabinet (BSC):**

  Daily method:

    - Put on protective clothing, mask and gloves.
    - Clean the BSC thoroughly before and after each batch of specimens.
    - 1% sodium hypochlorite should be used first, followed by 70% ethanol after an interval of 30 seconds.
    - Clean the glass panel as well. Switch the hood's **light** off after cleaning.

Weekly method:

- Put on protective clothing, mask and gloves.
- Prepare sufficient paper or roller towels with 1% sodium hypochlorite and 70% ethanol.
- Remove the inner working surface of the BSC and clean it and the entire interior of the BSC thoroughly with 1% sodium hypochlorite and 70% ethanol as described above.
- The glass window of the hood should be cleaned to ensure that all smudging and smears on the glass are eliminated.
- Replace the metal inner working surface of the BSC.
- After cleaning is completed, perform a smoke test to ensure that the BSC is functioning properly, i.e. the smoke must be “sucked” into the vents and NOT escape into the outside environment.

- **Centrifuge and heating block:**

Daily method:

- After cleaning the BSC, clean the centrifuge and heating block the exact same way as the cabinet.
- Use 1% sodium hypochlorite followed by 70% ethanol, on both the interior and exterior areas of these instruments.
- The heating block should be at room temperature before beginning the cleaning.

- **Ultrasonic water bath:**

  Daily method:

  - After the BSC and centrifuge have been cleaned, clean only the exterior of the ultrasonic water bath as described above.

  Weekly method:

  - Once a week, discard the old water in the ultrasonic water bath.
  - Carefully clean the inside of the ultrasonic water bath with 1% sodium hypochlorite followed by 70% ethanol.

Area 2: PRE-AMPLIFICATION ROOM

- **Precautions:**
  - No entry after 10 AM.
  - Absolutely **no specimens or amplicons** are to be brought in or allowed to enter this room.
  - No reagents removed from this room should be brought back into this room.
  - **No cell-phones** should be brought in or used in this room.
  - Pipettes and other required instruments should always be kept in this lab and used exclusively for pre-PCR activities.
  - Each reagent should be set aside before working with the next reagent to avoid contamination of the preceding reagent with subsequent solutions.
  - All cleaning supplies and brooms for this room should be stored in this room.
  - Routine laboratory cleaning staff should not have access to this room so that contaminants from other areas are not unknowingly tracked in.

- If multiple rounds of PCR's are to be conducted on the same day, all master mixes must be prepared at the same time (in area 2) and moved to area 3 for subsequent addition of DNA.

- **Weekly cleaning of the pre-amplification room:**
  - At the beginning of each week, the pre-amplification room must be thoroughly cleaned.
  - Cleaning should be top-to-bottom, starting with the bench tops and finishing with the floor.
  - Once the upper surfaces have been cleaned, the floor should be swept with a broom.
  - The floor should be mopped with 1% sodium hypochlorite, first using the leftover diluted bleach and then preparing more 1% sodium hypochlorite if needed.
  - Clean the mop under running water, and spray and mop the floor with 70% alcohol.

- **Bench tops including pipettes & PCR equipment:**

  Daily method:

  - Put on protective clothing, mask and gloves.
  - Clean the bench tops and PCR equipment thoroughly before work is done in this room.
  - As directed above, use 1% sodium hypochlorite first, followed after an interval of 30 seconds by 70% ethanol.
  - After cleaning, the master mixes for the day should be prepared.

- Once the reagents have been made, the surfaces and PCR equipment should be cleaned again as indicated above.

Area 3: AMPLIFICATION ROOM

- **Weekly cleaning of the amplification room:**
  - At the beginning of each week, the amplification room must be thoroughly cleaned.
  - Cleaning should be top-to-bottom, starting with the bench tops and finishing with the floor.
  - Once the upper surfaces have been cleaned, the floor should be swept with a broom.
  - The floor should be mopped with 1% sodium hypochlorite, first using the leftover diluted bleach and then preparing more 1% sodium hypochlorite if needed.
  - Clean the mop under running water, and spray and mop the floor with 70% alcohol.

- **Bench tops including thermal cycler, pipettes & PCR equipment:**

  Daily method:

  - Put on protective clothing, mask and gloves.
  - Clean the bench tops and PCR equipment thoroughly before the work is done in this room.
  - As directed above, use 1% sodium hypochlorite first, followed after an interval of 30 seconds by 70% ethanol.
  - Carefully add the template DNA to each reaction tube and place the tubes in the thermal cycler.

- After starting the thermal cycler run, clean the bench tops and PCR equipment as above.

- **Thermal cycler (PCR machine):**

  Weekly method:

  - Put on protective clothing, mask and gloves.
  - Clean the exterior of the thermal cycler thoroughly.
  - As directed above, use 1% sodium hypochlorite first, followed after an interval of 30 seconds by 70% ethanol.

Area 4: HYBRIDIZATION ROOM

- **Weekly cleaning of the hybridization room:**

  - At the beginning of each week, the hybridization room must be thoroughly cleaned.
  - Cleaning should be top-to-bottom, starting with the bench tops and finishing with the floor.
  - Once the upper surfaces have been cleaned, the floor should be swept with a broom.
  - The floor should be mopped with 1% sodium hypochlorite, first using the leftover diluted bleach and then preparing more 1% sodium hypochlorite if needed.
  - Clean the mop under running water, and spray and mop the floor with 70% alcohol.

- **Bench tops including GT-Blot, pipettes and equipment:**

  Daily method:

  - Put on protective clothing and gloves.
  - Clean the bench tops and equipment first using 1% sodium hypochlorite, followed 30 seconds later by 70% ethanol.
  - After the hybridization for the day has been completed, clean the bench tops again.
  - The used tips and completed PCR tubes must be placed in appropriate waste buckets and autoclaved to destroy any contaminating amplicons.
  - If the PCR tubes are kept after the LPA has been completed (for possible re-hybridization), they must be stored in a refrigerator or freezer well separated from other reagents or kit components.

- **TwinCubator®:**

  Daily method:

  - Put on protective clothing and gloves.
  - Clean the exterior of the TwinCubator®, and then the interior of the wells and the glass plate with 1% sodium hypochlorite followed by 70% ethanol.

- **GT-Blot hybridization machine:**

  Weekly method:

  - Put on protective clothing and gloves.
  - Clean the exterior of the GT-Blot with 1% sodium hypochlorite followed by 70% Ethanol.
  - Select the washing cycle on the machine.

- Add 10 ml sodium hypochlorite to the wash solution (water)
- Run the cleaning cycle twice, consecutively.
- Add clean water and put the GT-Blot on a third washing cycle.
- Take tubes out and leave to air-dry.
- Throw out the remaining water left in the container.