Development and application of nanopore sequencing based methods for rapid, culture-free diagnosis of tuberculosis

Michael John Strinden

Submitted in partial fulfilment of the requirements of the degree of Doctor of Philosophy

University of East Anglia, Norwich, UK

Faculty of Medicine and Health Sciences

March 2022

Abstract

Tuberculosis (TB) is a condition of global health concern with an estimated 1/3 of the human population infected. A growing percentage of these infections also demonstrate resistance to antibiotics, increasing morbidity and mortality in affected populations. The gold standard for drug susceptibility testing (DST), microbial culture, is very slow (weeks-months) and can't provide the necessary information within a clinically useful timeframe. Culture DST also requires specialist equipment that is not broadly available, therefore drug-resistant TB (DR-TB) is underdiagnosed globally. These limitations mean there is an urgent need for the development and uptake of new, rapid, DST technologies. Existing molecular technologies such as Xpert MTB/RIF offer rapid TB diagnosis but are only capable of detecting Rifampicin resistance due to limitations in PCR multiplexing technology. Comparatively, the GenoType MTBDRplus and MTBDRsl assays provide broader DST testing capability but are far from comprehensive for detecting all important drug-resistance associated mutations.

Targeted next-generation sequencing (tNGS) has the potential to rapidly diagnose TB and determine drug-resistance by amplification of known mutation loci. We developed a tNGS assay for DST covering 13 anti-tuberculous drugs using known SNPs (~200) in 16 *Mycobacterium tuberculosis* genes. Genotypic and phenotypic test performance were assessed during a blinded study of 392 contrived samples provided by the Foundation for Innovative New Diagnostics (FIND). This tNGS assay was found to have an overall genotypic sensitivity of 95% and specificity of 99% when compared to Illumina. The phenotypic sensitivity was 95%-97% and specificity was 96%-100% across all targeted drugs.

Clinical metagenomics has the potential to diagnose TB, perform DST, and provide epidemiological information directly from sputum in a single assay. We developed a metagenomic sequencing based TB test and evaluated it on spiked sputum samples from collaborators at the Norfolk and Norwich University Hospital (NNUH). Analysis showed commensal bacteria were present in high numbers, accounting for the majority of reads, thereby reducing analytical sensitivity. Attempts to design a commensal depletion protocol proved unsuccessful and metagenomic development was halted.

In conclusion, two approaches for rapid DST and TB diagnosis were designed and tested using contrived clinical samples. The tNGS method showed excellent potential for clinical use and is undergoing continued evaluation by FIND and the WHO under their Seq&Treat program. Continued development of the method has led to reductions in assay complexity, cost and turnaround time and use of the new WHO mutation list and simplified analysis tool will aid implementation of the test in the future.

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Acknowledgments

Firstly, I'd like to thank my incredible wife Leah who supported me through the highest and lowest points of my studies. I am eternally grateful for your continued support and understanding while this journey consumed me.

I'd also like to thank my supervisory team, Prof. Justin O'Grady and Prof. John Wain. This work would not have been possible without their help, guidance, and support. I feel together we made something truly incredible.

To Dr. Gemma Kay, who unfailingly had time and energy to assist me through every phase of my PhD. I wish everyone could have a mentor as patient and knowledgeable as you.

Finally, to my family who gave me the encouragement to pursue my passions and continue my studies. It has been a long bumpy road but we made it!

Chapter 1 - Introduction

1.1: Mycobacterial Biology

Mycobacterium tuberculosis was discovered by Dr. Robert Koch in 1882. *M. tuberculosis* is a gram-positive, acid-fast bacilli approximately 2-4 μm long, and is transmitted primarily via aerosols (Figure 1.1). *M. tuberculosis* is strictly intracellular and lacks a known environmental reservoir outside of endemic hosts [1–4]. Lipid-rich cellular walls and layers of peptidoglycan, lipoglycan, mycolic acids, and waxes create an extremely hardy microbe [2,3]. The Genus Mycobacteria are extremely fastidious organisms and can be divided into slow-growing and rapid-growing groups [3,5–19]. The doubling time for slow-growing members of the MTBC is approximately 12-24 hours, as opposed to *E. coli* or the fast growing *Mycobacterium abscessus*, which have doubling times of approximately 20 minutes [20,21]. Slow growth rate is correlated with highly conserved genomes with high GC contents, which experience very low rates of single-nucleotide polymorphism mutations (SNPs) [4,15]. The average mutation rate for *M. tuberculosis* is only 0.3-0.5 SNPs per genome per year [2,11].

Mycobacteria can be identified through microscopy using Ziehl-Neelsen staining, as the mycolic cell wall precludes absorption of *Gram* stains [22]. Curiously, this characteristic means that mycobacteria are not truly *Gram+* or *Gram-*. However, many still characterise mycobacteria as *Gram+* since mycobacteria are phylogenetically dissimilar to other *Gram-* organisms [4].

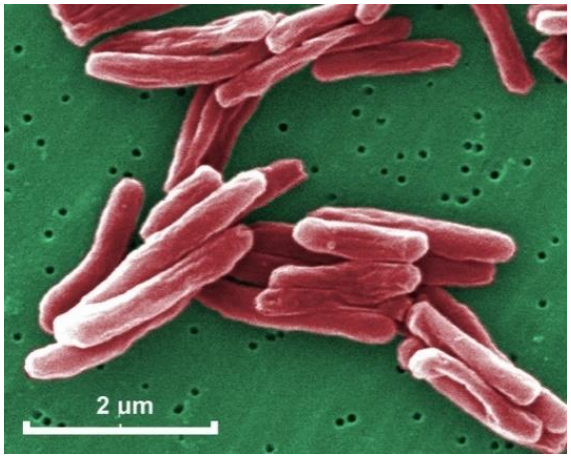

Figure 1.1: 15,549x magnification colourised scanning electron microscope image of Mycobacterium tuberculosis [23]

The mycobacterial genus is thought to have first evolved around 150 million years ago during the Jurassic period [2]. *M. tuberculosis* and related species in the *Mycobacterium tuberculosis* complex (MTBC) are theorised to have emerged at least 11,000 years ago and have been co-evolving with their hosts since [2,13]. This timeline is a matter of debate, however, and relies on measurements of modern average mutation rates. Depending on the study and the mutation rate measures, the MTBC has been calculated to have emerged anywhere between 70,000 and 6,000 years ago [11]. Less contentious, on the other hand, is the proposed geographic region for the emergence of the MTBC, as studies of gene loss and mutation consistently indicate the nearest common MTBC ancestor evolved in Africa [11].

Close co-evolution has resulted in a highly transmissible taxon of bacteria with "remarkable" longevity within hosts and advanced methods of immune system evasion [2]. Due to co-evolution, modern *M. tuberculosis* and members of the MTBC share numerous characteristics and are found in hosts in every known environment (excluding polar regions) along with non-tuberculous mycobacteria (NTM) species [2,24]. There are many hypotheses about the driving force behind emergence and co-evolution with humans, most of which revolve around the transition of human populations to larger societies. One example is that the harnessing of fire by early *Homo sapiens* created a perfect milieu for mycobacteria by increasing group population sizes while simultaneously increasing the prevalence of smoke-induced lung damage, lowering host resistance to pulmonary infection [11].

The MTBC is currently comprised of 10 mycobacterial species capable of causing TB or TB-like disease within hosts (Figure 1.2). Three species specialise in human infection: *Mycobacterium tuberculosis* sensu stricto, *Mycobacterium canettii* and *Mycobacterium africanum* [1,2,15]. Additionally, bi-directional zoonotic TB transfer is well documented from cattle (*Mycobacterium bovis*) [13], goats and sheep (*Mycobacterium caprae*) [2], seals and sea lions (*Mycobacterium pinnipedii*) [19], and rodents (*Mycobacterium microti*) [25]. Recently, three new species have been added to the

MTBC: *Mycobacterium mungi* from mongeese, *Mycobacterium suricattae* from meerkats, and *Mycobacterium orygis* from oryx [11,26,27].

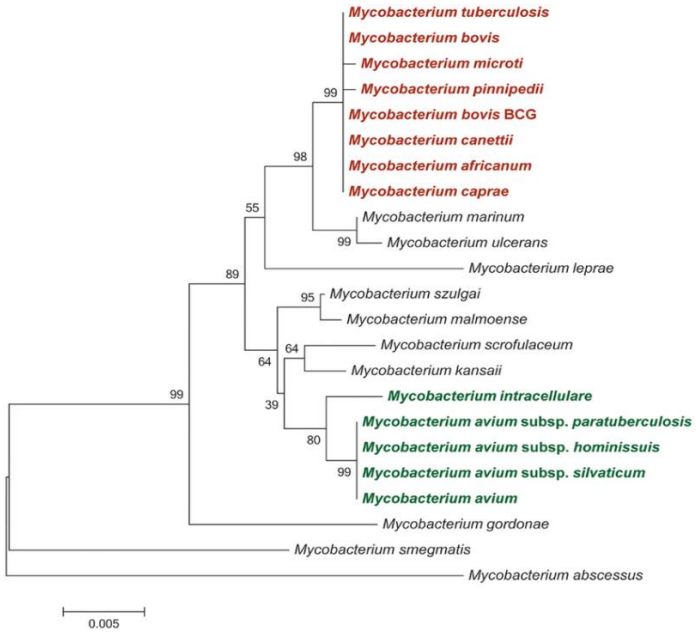

Figure 1.2: Phylogenetic tree of slow-growing mycobacteria anchored by two rapid-growing mycobacteria: Mycobacterium smegmatis and Mycobacterium abscessus. Select Mycobacterium Tuberculosis complex members are denoted in red [28].

MTBC members are highly homologous (~99.9% genetically identical) and have identical 16S rRNA sequences. This homology is evident when compared to NTMs; in the MTBC, the maximum genetic difference between members is approximately 2,000 SNPs while NTMs can vary up to 65,000 SNPs, a 32.5-fold difference [11]. MTBC members are primarily clonal with little horizontal gene transfer. This makes differentiation between species difficult at the genetic level and impossible using microscopic methods [19,29]. MTBC evolution to become obligate intracellular pathogens is a matter of much study with several hypotheses posited. However, it is generally agreed that a transition from environmental organism to intracellular pathogen likely occurred through numerous small steps, for example, a move from an environmental existence to free-living protozoa hosts such as amoebae [11]. There is also evidence that a transition to a specialised pathogenic lifestyle was aided by large deletions within the MTBC species' genomes, reducing the average genome length to two thirds of that found in non-pathogenic mycobacteria, thereby increasing fitness [11].

1.2: Tuberculosis Epidemiology

1.2.1: What is TB?

Tuberculosis (TB), caused primarily by *Mycobacterium tuberculosis*, is an infectious respiratory disease of grave importance to global health [15,19,30]. Most commonly, TB presents as a pulmonary disease (84% of cases), although extrapulmonary and disseminated disease presentations also occur (16% of cases) [1,13,31]. Extrapulmonary TB infections are generally confined to the thorax; usually the pleura, pericardium, and perihilar lymph nodes [32]. Disseminated TB infections create tubercles throughout the body, i.e., throughout the extremities or the nervous system [32]. TB infections occur globally, with regional variations in incidence, prevalence, and causative agent.

As of the early 21st century, TB has surpassed HIV/AIDS as the global leading cause of death from a single infectious agent. The most recently available reports quantified annual TB fatalities at 1.4 million globally in 2019 [33]. Reports also identify TB as the 13th highest out of all causes of death globally (Figure 1.3) [33]. Retrospective studies have shown that 70% of smear-positive cases prove fatal within 10 years if untreated, as well as 20% of smear-negative, culture-positive cases [33]. In properly treated cases, mortality rates decrease to 14% across all active infections [33].

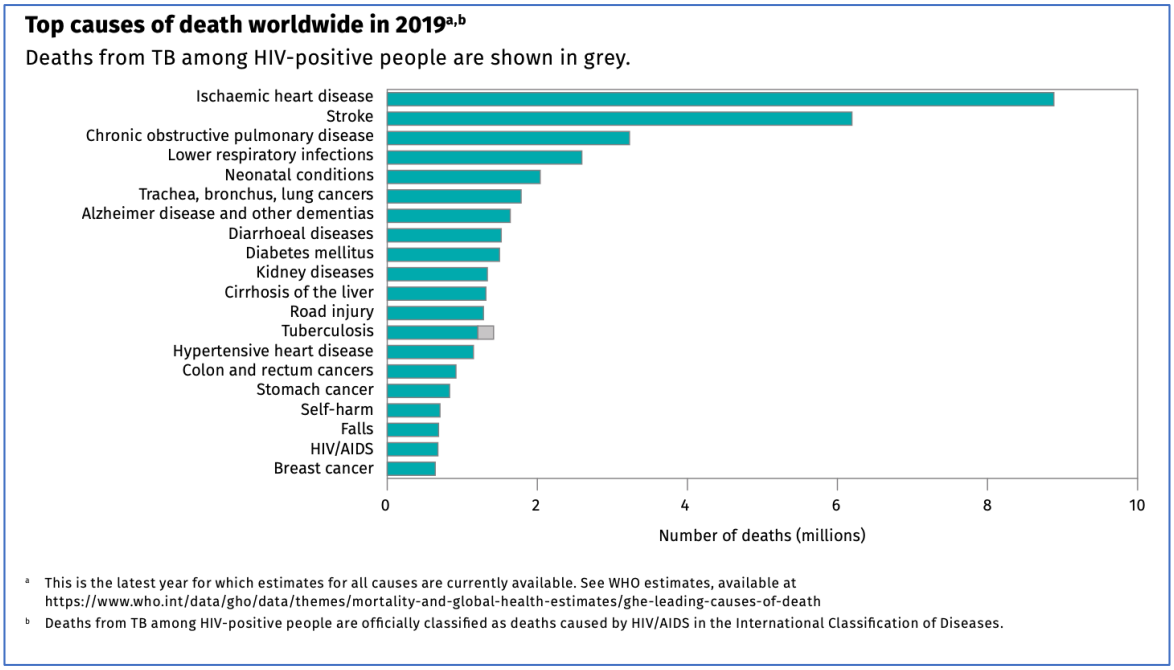

Figure 1.3: Causes of death globally in 2019 [34]

Beyond its mortality rate, latent TB infection (LTBI) contributes to global morbidity. Latent infections are defined as a state of persistent immune response to stimulation by *Mycobacterium tuberculosis* antigens with no evidence of clinically manifest active TB [35]. Approximately one-third of the global population harbour an LTBI with an incidence between nine and eleven million cases annually [2,24,31]. Exact numbers for LTBIs are difficult to discern clinically, as the primary means of diagnosis is the Mantoux tuberculin skin test. A positive Mantoux test result is as likely to be due to previous exposure to TB bacilli, or vaccination, as it is to be due to live bacilli, resulting in limited diagnostic value [36]. Approximately 1% of LTBI cases will develop active TB annually [29,37], while the WHO estimates that between 5%-10% of LTBI cases will develop into active TB during an individual's lifetime [38].

1.2.2: TB Demographics

In the 2021 annual Global Tuberculosis Report, the WHO provided the most recent epidemiological data gathered from 198 countries, which was collated from internal reports provided by governmental health surveillance systems. The report covers approximately 99% of the global

population, and its findings show that TB disproportionately infects adults aged 15 and over (90% of cases). TB incidence is also slightly elevated in males aged over 15 (56% of new cases) compared females over 15 (32% of new cases) [33].

TB infections in HIV-positive individuals are of marked epidemiological interest. HIV co-infections are separately analysed in the 2021 WHO Global TB Report and are often the focus of research studies [34,39,40]. Due to HIV's immunosuppressing nature, seroconversion of TB infection can happen earlier, resulting in symptomatic cases that may evade diagnostic detection [38]. This diagnostic evasion is correlated with paucibacillary infections, which fall below the limit of detection for smear-microscopy, and higher incidence of extrapulmonary disease among HIV+ individuals [38]. Diagnosis delay is highly correlated with adverse treatment outcomes. Aside from diagnostic difficulties, earlier seroconversion results in a 26-fold increase in the likelihood of HIV+ individuals developing active TB [38].

Co-infection with HIV is most prevalent in Africa with rates >50% in many countries. In South Africa, for example, robust MDR and HIV testing systems identify 40%-80% of MDR and XDR cases are also HIV+ [38,41]. Accurate numbers for TB mortality in HIV+ individuals are difficult to obtain, since death due to TB in HIV+ individuals is often reported only as death by HIV [33]. However, a 2015 study found HIV/TB case fatalities are primarily (92.3%) due to multi-organ involvement and failure [42]. These deaths are classified as deaths by HIV, however, they are still recorded for the WHO annual report.

Paediatric TB studies are less prevalent in the literature, but some existing studies place the incidence of TB attributable to children under 15 as 6-15% [43]. Lack of documentation is generally attributed to the paucibacillary nature of paediatric TB, increased presentation of extrapulmonary TB in paediatric patients, and difficulties in collecting diagnostic samples from paediatric patients [41]. In children and adolescents, advancement from infection to active disease is rapid and the prevalence of extra-pulmonary and disseminated TB is increased [43,44]. A WHO report from 2015, the

last time paediatric TB was explicitly described, reported 210,000 deaths in children under 15 from TB globally per year. Of these 210,000 deaths, 17% were comorbid with HIV [44].

The 2021 WHO annual report also summarised global incidence rates across all demographics (Figure 1.4). The annual incidence is estimated at 10.4 million cases globally [12,33]. Incidence rates vary by country, from 5/100,000 to 500/100,000 annually (global average 130/100,000). The incidence rate disparity is correlated with data that shows 87% of incident cases occurred in 30 high-incidence countries in 2019. Of these 30 countries, 8 account for approximately two thirds of global cases. Global incidence has declined 11% since 2015, largely driven by a 25% decrease in the WHO European region, although incidence is increasing in the WHO American region due to upward trends in Brazil [34].

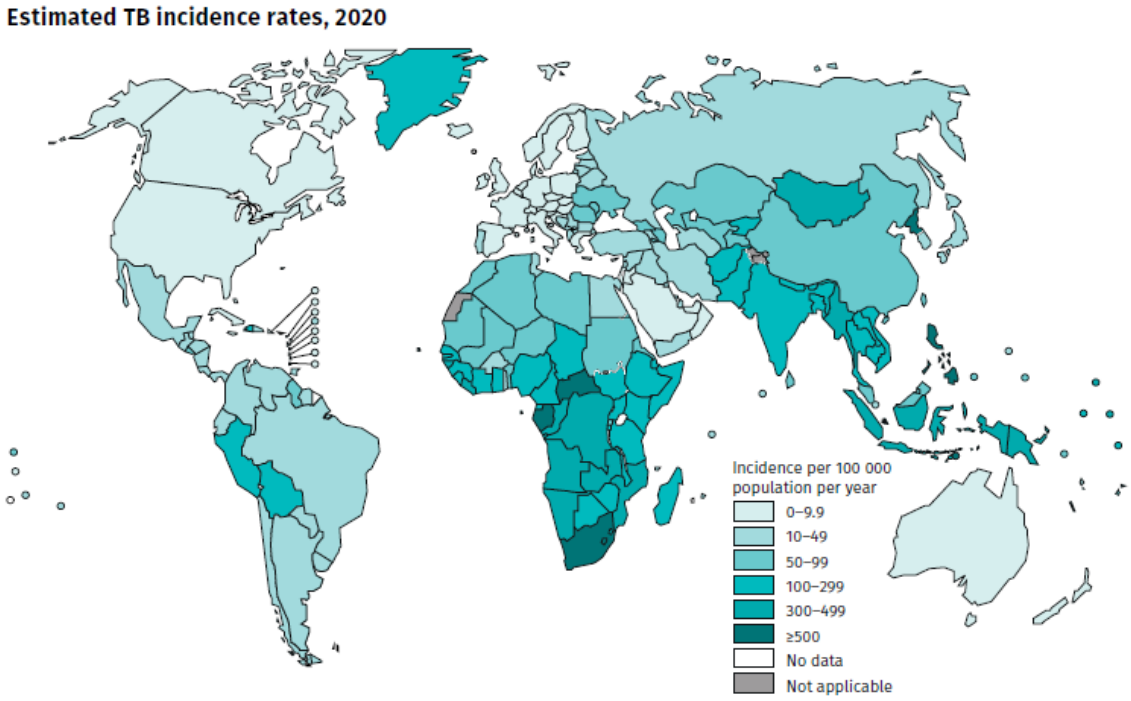

Figure 1.4: Global incidence rates of WHO reporting countries, 2021 [34]

1.2.3: Global TB Trends

The WHO have reported that over the past two decades TB incidence and mortality rates have been declining globally (Figure 1.5). Annual incidence has declined by an average of 1.7% per annum,

although the total reduction is still short of the 20% target for 2020. The decline in incidence is being monitored by increases in reporting rates in most countries, aiding in global case estimates [38]. Likewise, the 2020 WHO Global TB Report saw a 14% reduction in mortality from 2001-2019, which still falls short of the 35% 2020 target [33].

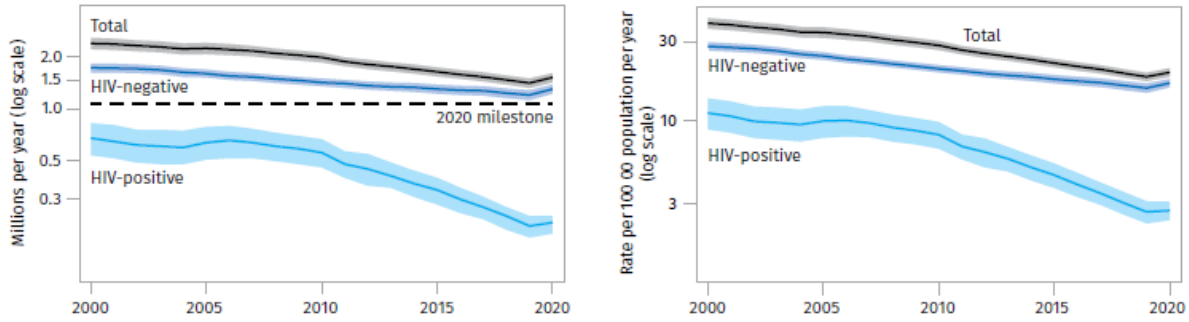

Figure 1.5: Global trends in the estimated number of TB deaths (left) and the mortality rate (right), 2000-20 [34]. Shaded areas represent uncertainty intervals. The horizontal dashed line shows the END TB Strategy 2020 milestone.

1.2.4: COVID and TB

The SARS-CoV-2 (Covid-19) pandemic has had a significant negative impact on global TB control and treatment efforts. Reports from the WHO and STOP TB Partnership predict mortality rates will likely return to either 2012 or 2015 levels (depending on the modelling system used). The STOP TB Partnership also calculates an increased incidence of 6 million cases and 1.4 million preventable deaths by 2025 as a direct result of the Covid-19 pandemic [33].

This increase in incidence and mortality is forecast for numerous reasons. For instance, detection rates are dropping by 20%-50%, in part due to the reallocation of resources and avoidance of healthcare by the public (Figure 1.6). Two examples are India and South Africa, both of which reported a drop in TB notification rates >50% over the 2019 calendar year. Major sources for TB resurgence have been cited to be the redirection of medical resources, such as molecular diagnostic tests, and the discouragement of those with either chronic conditions or mild symptoms from

seeking medical aid [33]. Moreover, loss of economic stability and major disruption to supply lines contribute to an increase in case rates [33].

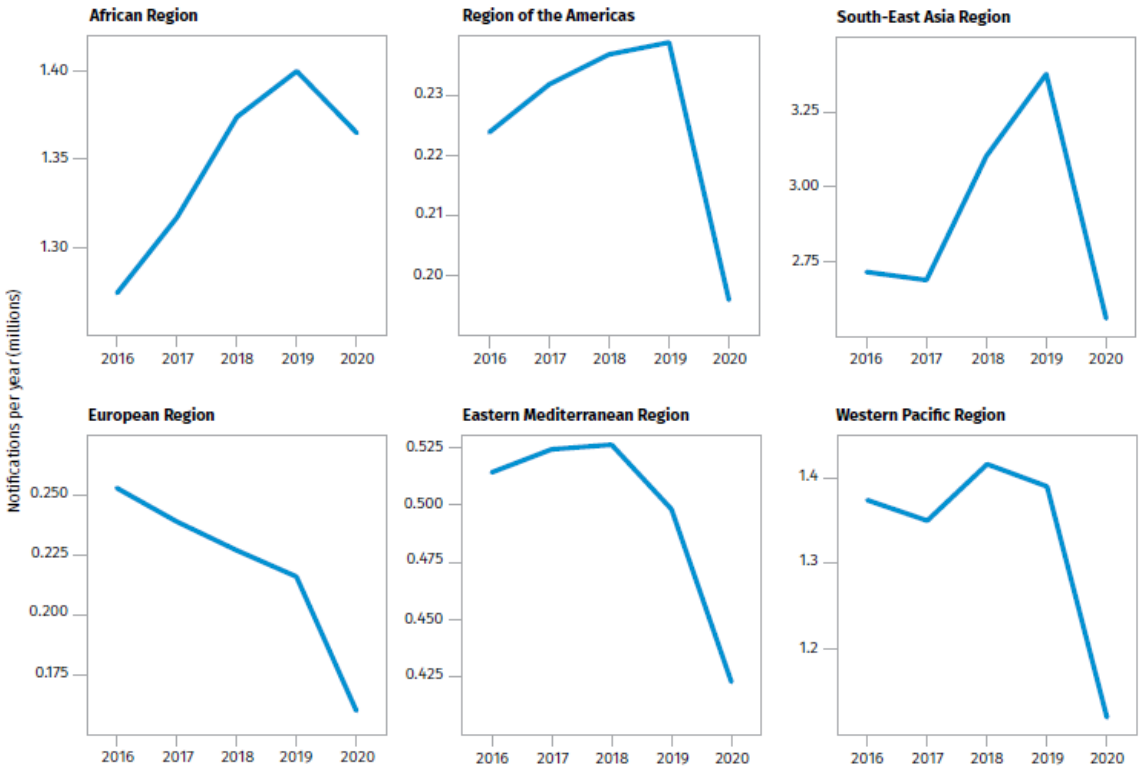

Figure 1.6: Trends in case notifications of individuals newly diagnosed with TB by WHO region, 2016-2020 [34]

1.3: Drug Resistance in TB

Drug-resistant TB is a growing issue, even when overall incidence of TB was in decline [41]. The most recent WHO reports cited 3.3% of new cases and 18% of recurring cases globally were drug-resistant (Figure 1.7) [33]. A systematic review published in 2017 indicated that approximately one fifth of globally tested isolates are resistant to at least one first- or second-line drug [41]. Rates of drug-resistance were highest in former Soviet republics (SSRs) where >25% of new cases and >50% of recurring cases demonstrated some form of drug-resistance [41,45]. Disparity in global rates can be attributed in part to historic factors, such as inconsistent supply lines and medication shortages in the former SSRs [46,47].

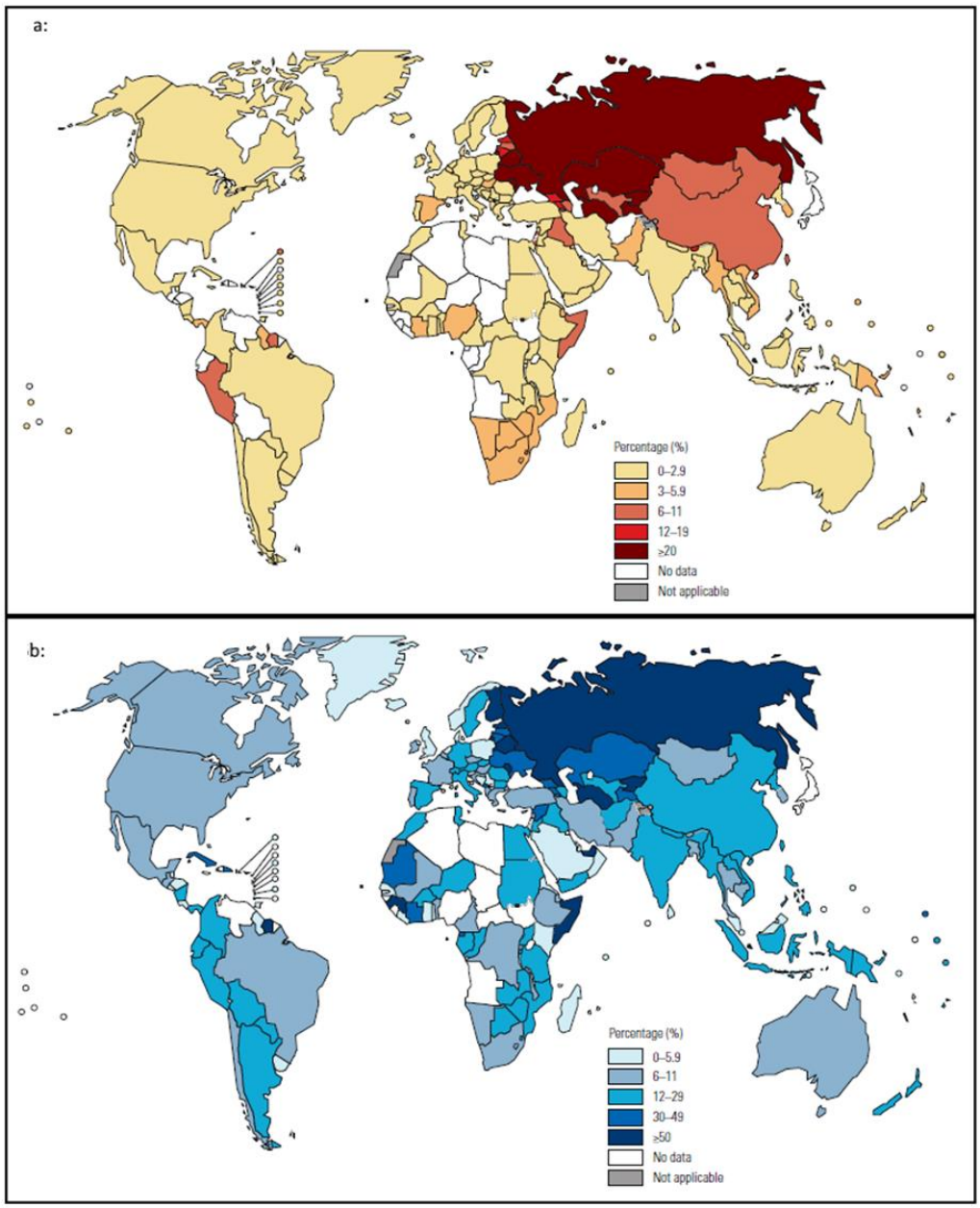

Figure 1.7: Global MDR/RR-TB rate in new (a) and previously treated (b) TB cases [33].

Drug-resistant TB can be broken down into different types (Figure 1.8); rifampicin-resistant (RR), multidrug-resistant (MDR), extensively drug-resistant (XDR), and "incurable" or totally drug-resistant (TDR) [8,16]. Increasing incidence of DR-TB is a growing issue globally [16,48,49]. All types of DR-TB are caused through genetic mutations owing to the clonal reproduction of mycobacteria [13,29].

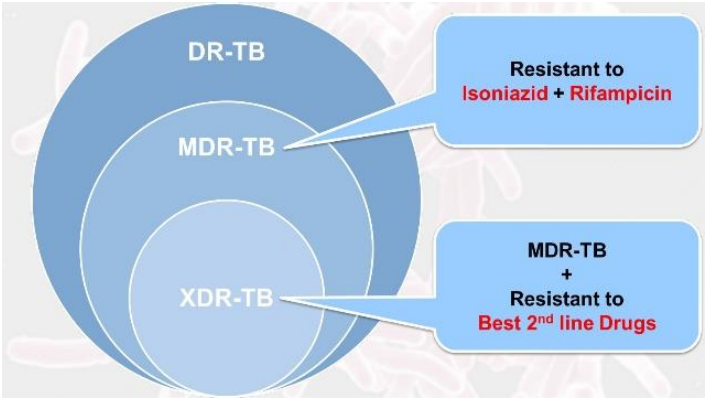

Figure 1.8: Visual representation of the relationship between the three most common types of drug-resistant TB [50].

The first recognised occurrence of drug-resistance in TB was reported in the late 1940s, shortly after the introduction of streptomycin as an anti-tuberculous agent [41,45,51]. Since then, drug-resistance has been increasingly well-documented. Investigation into the types and severity of drug-resistance has become a pressing issue, with the WHO END TB Partnership placing special emphasis on further research.

Concerns are that drug-resistant infections will reverse progress made towards the eradication of TB [41,52]. The incidence of drug resistant infections worldwide has increased over 4-fold in the past decade alone. Only 4.9% of patients demonstrated drug resistance in 2009 compared to 20% in 2019 [48]. In 2015, the last year for which complete data was available from WHO, 580,000/10.5 million TB cases worldwide were identified as RR/MDR-TB [6,45]. Of those, 9.5% were XDR or worse.

1.3.1 RR/MDR TB

Rifampicin resistant TB (RR-TB) is the most common form of DR-TB and is defined as any TB infection which exhibits resistance to rifampicin or any of its related compounds, such as rifapentine. In 2015, there were an estimated 580,000 cases of RR-TB, although only 340,000 were reported. Of the cases reported, only a fraction (36.8%) were administered second-line treatment [45]. Isoniazid resistance is thought to be more common (9.5% of all TB infections), but testing for it does not yet exist in the same way as it does for rifampicin resistance [41].

MDR-TB is the second most common type of resistance after RR-TB [8,16]. MDR-TB is defined as a TB infection that is resistant to at least isoniazid and rifampicin [41,53]. In 2019, approximately 78% of rifampicin-resistant infections were further categorised as MDR following DST [33]. Furthermore, between 2018 and 2019, the prevalence of MDR-TB increased by 10% [33].

Despite resistances, MDR-TB infections are still commonly treated with traditional WHO-endorsed DS-TB regimens in LMICs. These treatments require only a 6-month course of first-line antibiotics [14,54]. However, an augmented 24-month, or a newly designed 12-month regimen, are recommended by the WHO in places with sufficient infrastructure and resources [41].

There are several known risk factors for the development of RR/MDR-TB, the most significant being prior patient history of ≥1 month of anti-tuberculous treatment [45]. Historically, this has been considered the primary force behind the selective evolution of drug-resistant bacilli, which become prevalent during reinfection [41,45]. However, this does not account for the incidence of RR/MDR-TB in newly acquired TB infections.

Other risk factors for RR/MDR-TB include hospitalisation, incarceration, and HIV infection [45]. These external factors can impact drug-resistance in both recurrent and new TB infections while other factors specifically affect community transmission. Geodemographic variations are also known risk factors, for example, in areas with large populations of formerly incarcerated individuals or lower socio-economic status, greater community prevalence of RR/MDR-TB has been recorded [45].

Mortality and morbidity are increased in MDR-TB cases when compared to drug susceptible cases. Mortality rates are calculated to be between 40%-50% in MDR cases and treatment difficulties often lead to affected individuals suffering from chronic disease [41,53].

1.3.2 XDR/TDR TB

XDR-TB was redefined in 2020 as a strain that fulfils the definition of MDR/RR-TB and which is also resistant to any fluoroquinolone and at least one additional Group A drug

(levofloxacin/moxifloxacin, bedaquiline, and linezolid) [55]. The first recorded outbreak of XDR-TB occurred in Tugela Ferry, South Africa in 2006 [41]. The Tugela Ferry clone alerted the world to the severity of continued drug resistance in TB, and the incidence of XDR-TB has increased since this first outbreak. In 2019, 20% of reported MDR infections were also resistant to fluoroquinolones, a 2-fold increase from 2017 [33,41].

TDR-TB is less formally defined than RR-, MDR-, or XDR-TB, although some sources do describe TDR as "programmatically incurable" owing to the lack of sufficient susceptible drugs for a curative regimen [41]. The incidence of TDR-TB infections is increasing in three of the four highest incidence countries, which are China, India, and South Africa. This is particularly concerning to management programs and watch groups as these countries are generally poorly equipped to prevent onward transmission [41].

1.3.3 Factors in the Emergence of Drug Resistance

The emergence of drug resistance has been an officially recognised problem since 1994 when the WHO and the International Union Against Tuberculosis and Lung Disease (IUATL) launched the Global Project on Anti-Tuberculosis [41,51]. This project's primary focus was to establish a surveillance network for the emergence of drug resistance, and this is still active today, making it the world's oldest and largest antimicrobial resistance surveillance project [45]. This network is not without its limitations, however, as the most detailed and consistent data routinely comes from high-income countries, which are not representative of high-incidence LMICs [41,51,56]. This inconsistency in LMIC data reporting makes it difficult to accurately estimate the emergence of drug-resistance.

Primarily, drug resistance in *M. tuberculosis* and related species is attributable to SNPs [15,41]. This contrasts with many other bacterial species that acquire resistance through gene transfer systems. Because of this, development of resistant phenotypes in the MTBC is simplified while also limiting the spread of phenotypes because of the clonal nature of mycobacteria [15].

The development of DR-TB infections is primarily considered as a result of inconsistent treatment protocols, delayed treatment, lengthy treatment courses, and administration of drugs on already resistant strains; all of which lead to positive selection for drug-resistance and a higher incidence of resistant infection transfer between hosts [13,30,57]. This is especially evident where poorly funded and/or administered treatment programs with low cure rates (<50%) have increased the population of individuals with chronic disease [58]. Additionally, some species within the MTBC exhibit lineage-specific inherent resistance to pyrazinamide, like *M. bovis* and *M. canettii*, which, if misdiagnosed, can impede resistance-control methods [6,8,15].

Multiple studies from 1994 through to the present have shown previous history of TB treatment to be the most consistent risk factor for MDR-TB emergence. This is further supported by geographic distribution maps of MDR-TB which overlap areas historically known for ineffective treatment [33]. Poor treatment can result in a large population with chronic TB and, in combination with steady selective pressures, this can lead to new MDR mutations with increased stability.

However, the development of DR-TB is not solely due to ineffective treatment. DR-TB hotspots, defined as regions where the prevalence of DR-TB is >5%, have brought increased interest to community and patient-to-patient evolution and transmission [45,56].

Investigation of selective pressure leads to an interesting discussion on the development of DR-TB. Drug resistance is frequently thought of as an “end-point”, yet the development of drug resistance is a continuous biological process and is constrained by the biophysical properties of TB bacilli [56]. While external pressures may alter the relative fitness of a given bacilli, they cannot fundamentally change the underlying mutative processes which cause phenotypic variation. Thus, even constant selective pressure will not necessarily increase the rate of mutation emergence, however it will serve to fix those that do emerge more readily.

Studies into phylogeographic distribution of resistance types show significant differences in the prevalence of drug resistance between lineages and strains [41,56,59]. The most successful MTBC

lineage in prevalence and development of antimicrobial resistance is the Lineage 2 Beijing strain (Figure 1.9) [56]. The exact biological link between lineage and development of resistance is unknown and the hypothesis that an elevated basal mutation rate may be involved has not been supported in the research[56,59].

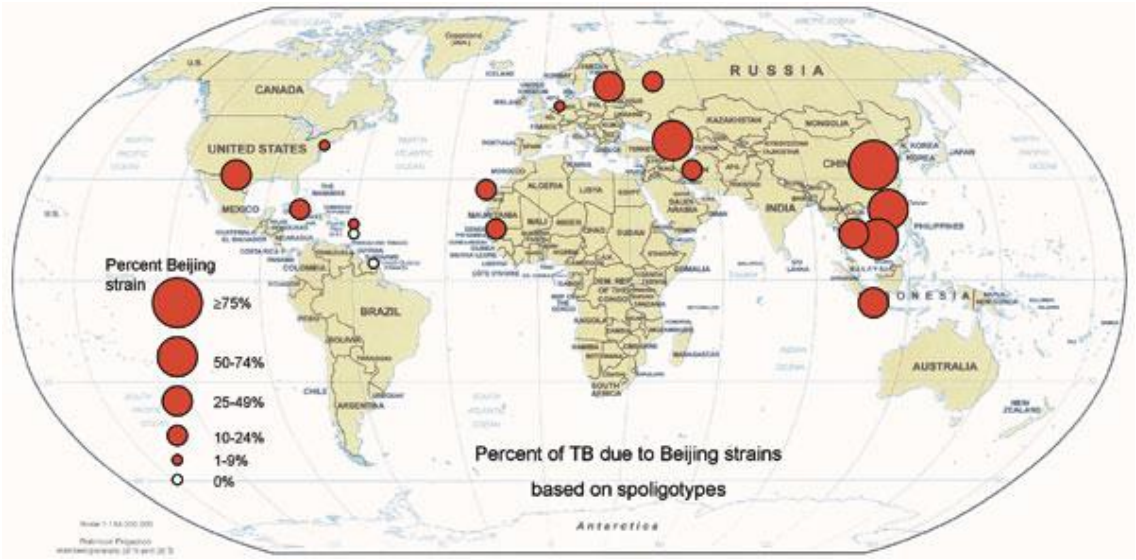

Figure 1.9: Global map showing the proportion of TB infections due to the Beijing Strain of M. tuberculosis in select geographic regions in 2002 [59].

1.4: TB Treatment

1.4.1: First-Line Medications

TB treatment primarily relies on a combination of four drugs: rifampicin, isoniazid, pyrazinamide, and ethambutol. Per WHO guidelines, all four drugs are administered for a period of two months after which point only rifampicin and isoniazid are administered for a further four months. However, in the event of drug resistance, alternative methods of treatment are recommended. First-line treatment can additionally include streptomycin, yet this is less common given a 70-year history of streptomycin resistance in TB [60,61]. These first-line medications benefit from oral administration, improving treatment compliance [61].

Compliance with this treatment protocol has a documented success rate of approximately 85% in drug-susceptible patients [62]. Despite this high success rate, more effective medications and shorter treatments are continually under research. These research avenues aim to improve patient adherence and reduce patient/health system costs [62]. In 2021 the Guideline Development Group (GDG) convened to review evidence on the safety and efficacy of a reduced 4-month treatment protocol comprised of rifapentine, isoniazid, pyrazinamide, and moxifloxacin. This method was identified to be non-inferior to the existing 6-month protocol with equivalent side effect tolerance [62]. While this regimen benefits from being shorter and all-oral, the cost of rifapentine is currently a limiting factor for short- and medium-term implementation [62].

1.4.2: Drug Resistant TB Treatment

In rifampicin-susceptible and isoniazid-resistant infections the treatment recommendation is amended to a six-month regimen of rifampicin, pyrazinamide, ethambutol, and levofloxacin. In this form of drug-resistant infection, streptomycin and other injectable medications are specifically advised against by the WHO. Similarly, in the event of rifampicin-resistant or MDR infection, an extended course of treatment is recommended, including levofloxacin or moxifloxacin to replace rifampicin. This extended course takes 24-months and relies on highly toxic injectable medications. The combination of length, administration complexity, and toxicity leads to lower treatment compliance and increased onward transmission [41].

Extended treatments come with alternative guidelines set by the WHO. For example, kanamycin and capreomycin are discouraged for an extended drug treatment regimen, while bedaquiline is strongly recommended. Likewise, clofazamine and cycloserine are approved for extended treatment regimens when necessary [31].

For MDR-TB, the goal has been to develop a consistent scalable treatment regimen, especially in LMICs, since the launch of the DOTS-Plus program by WHO in 1999. This program emphasised sustained commitment to MDR control and directly observed therapy to treat MDR positive

patients. In a trial in Bangladesh, the International Union Against Tuberculosis and Lung Diseases developed and tested an early recommendation to amend treatment protocols [63,64]. This regimen consisted of a 4-6 month intensive phase using kanamycin, moxifloxacin, prothionamide, clofazimine, pyrazinamide, isoniazid, and ethambutol, followed by 5 months of moxifloxacin, clofazimine, pyrazinamide, and ethambutol [64]. In areas with "simple" strains of MDR-TB, this regimen was found to perform consistently well; however, the efficacy fell in areas with mixed strains or a high proportion of pyrazinamide resistant strains [65–68].

A meta-analysis of medications most associated with positive MDR/XDR-TB treatment outcomes identified linezolid, levofloxacin, carbapenems, moxifloxacin, bedaquiline and clofazimine as being most effective [69]. The authors of the meta-analysis cited only modest benefits for the use of injectables and that the worst treatment outcomes occurred with the use of kanamycin and capreomycin. However, it is stressed, that the worse outcomes that arise when using injectables may be due to confounding factors, while the improved outcomes from the use of late generation medications like fluoroquinolones, bedaquiline, linezolid and clofazimine should be noted [63,69]. While this meta-analysis strongly indicates that bedaquiline should be used in the treatment of MDR/XDR-TB, it must be accompanied by a monitoring of cardiac toxicity to avoid complications.

Using new studies available at the time, and the aforementioned meta-analysis, the WHO updated their treatment guidelines in 2019 [70]. These updated guidelines addressed both long and short regimens and reclassified available pharmaceuticals into A, B, and C groups based on toxicity, efficacy, and ease of administration [63]. Further considerations for medication groupings included factors such as: reliability of DST methods, drug tolerability, population history of drug resistance, and potential interactions between drugs. In addition to ranking the medications, the WHO also stressed the need to stop the use of kanamycin and capreomycin, and to instead use amikacin if an injectable is still required for treatment [63,70].

One of the main takeaways from these reports is the continued need for research into new and optimised treatment regimens. One such avenue of research was the Nix-TB trial in South Africa, which showed promising results for treatment of XDR-TB using bedaquiline, linezolid, and pretomanid [71]. This was innovative in that it was the first completely oral and short regimen for the treatment of XDR-TB and complex MDR-TB cases [63]. A follow-up analysis identified a treatment success rate of 88.78% after 6-months of treatment and a 6-month follow-up period [72]. However, to maintain this degree of efficacy, extreme care will need to be taken to monitor for and avoid developing resistance to these newer medications, as has been the case with previously developed anti-tuberculous drugs. Likewise, increased patient monitoring is required, as linezolid is a highly toxic drug and can lead to cross-resistance with bedaquiline [63].

A second new regimen under study, the SimpliciTB trial, uses bedaquiline, pretomanid, moxifloxacin, and pyrazinamide [73]. In drug-susceptible TB (DS-TB) patients, this regimen resulted in culture negativity within a mere two months, which is a third of the time in comparison to the normal short course TB treatment for DS-TB patients [63]. At the time of writing, results on the efficacy of this regimen have not been published though hopes are high that the inclusion of pretomanid will result in improved health outcomes by 2025 [74].

These two studies provide evidence for the efficacy of reduced treatment times even for MDR/XDR-TB [63]. However, it is unlikely that either regimen will become the sole means of treatment for DR-TB cases, as strain variability and personal response to treatment will always prove to be confounding factors [63,65]. When coupled with rapid DST, through methods such as NGS, confounding factors can be mitigated and treatment can be summarily shortened. Reduction in treatment time would, in turn, promote improved health outcomes, increase treatment compliance, and reduce the financial burden of treatment for patients and health organisations.

1.5: Tuberculous Disease by Non-*M. tuberculosis* mycobacteria

1.5.1: Zoonotic TB

Zoonotic TB (zTB) transmission is also recognised as epidemiologically important, with an estimated 140,000 incident cases in 2019 [33]. This is similar to the reported incidence in 2016 (147,000 cases), indicating that as overall TB incidence decreases, zTB incidence remains stable [25]. zTB incidence rates are calculated by identifying *M. bovis* infections, and are likely to be conservative as zoonotic species beyond *M. bovis* are not systematically identified and tracked [33]. A review published in 2019 delved further into zTB transmission and infection, citing systematic and continued neglect for the condition in the WHO annual reports [25].

According to this review, published by Couto, et al., zTB is dispersed globally, though the majority of cases occur in Africa and Southeast Asia [25]. Cases are likely underreported due to requisite diagnostic techniques being largely unavailable in LMICs where zTB is most prevalent. More frequently, zTB exhibits extrapulmonary presentation, making standard diagnostic methods such as smear-microscopy largely ineffective. In cases where zTB does present as a respiratory illness, it is often reported as a TB infection, as TB and zTB infections are indistinguishable without the use of advanced molecular techniques [25].

Most commonly, transmission of zTB is through ingestion of unpasteurized animal products, especially milk and cheese. This supports findings that zTB occurs more frequently in LMICs and immigrant populations within high-income countries where food safety standards are often less stringent [25]. Due to this alternative exposure pathway, zTB primarily presents either as extrapulmonary or disseminated infection, as mentioned above. This contributes to the observed increase in mortality rate in *M. bovis* cases compared to *M. tuberculosis*, as miliary lymphatic and central nervous system infections are more common, and have inferior detection and treatment outcomes (Figure 1.10) [25].

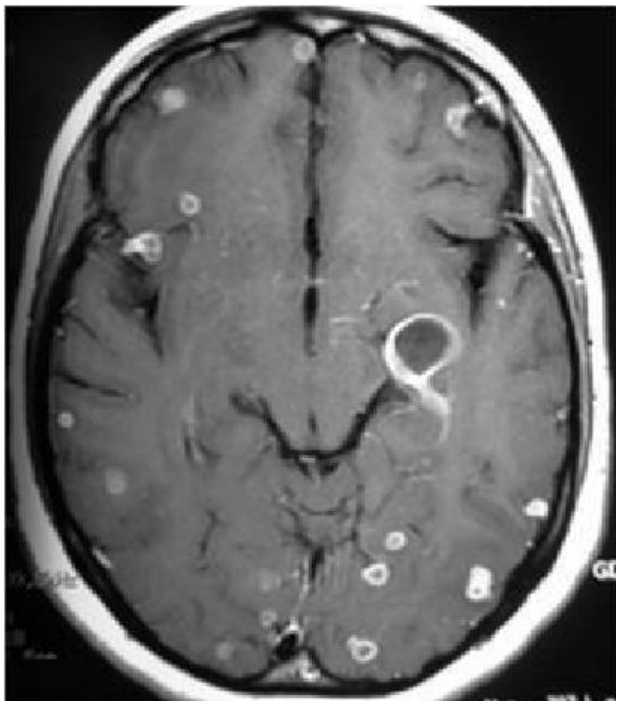

Figure 1.10: MRI of a disseminated central nervous system TB infection [75].

1.5.2: Non-Tuberculous Mycobacteria

Beyond the varied types of TB infection, there are numerous diseases which have TB-like presentation but are not caused by the *Mycobacterium tuberculosis* complex (MTBC). Primarily, these are caused by NTMs which lead to pulmonary disease (NTM-PD). NTMs are defined as mycobacterial species which are not part of the MTBC or *Mycobacterium leprae.*

Increasing global incidence rates for NTM-PD create difficulties for diagnosis [76,77]. There is evidence that as global incidence rates for TB decline, incidence rates of NTM infections are increasing even more rapidly. One longitudinal study conducted from 1995 to 2012 identified an 8 fold increase in NTM infection throughout the United Kingdom, from 0.9/100,000 people to 7.6/100,000 people [77].

NTM-PD epidemiology is hindered by the lack of reporting requirements in most countries [76,78]. These pathogens are generally ignored by health monitoring systems due to the long-held belief that patient-to-patient transmission does not occur, and thus any infections remain isolated. However, evidence from a longitudinal study conducted in cystic fibrosis centres beginning in 2012

showed genetic evidence of direct patient-to-patient transmission, either through fomites or long-lasting aerosols [77]. This makes early detection and differentiation between TB and NTM-PD increasingly important as the treatments are different and even the correct treatment course can result in negative side effects in patients such as nausea, vomiting, loss of sensation in extremities, and loss of eyesight [76,77].

1.5.3: *M. tuberculosis*-Like Pathogens

Not all *M. tuberculosis*-like infections are caused by mycobacterial species. Another common cause of TB-like disease, which can complicate diagnosis and treatment, are bacteria from the genus *Nocardia*. Nocardia species are slow growing, *Gram*-positive, acid-fast bacteria similar to many mycobacteria (Figure 1.11) [79]. A 2020 review by Duggal and Chugh discussed this often neglected disease group in clinical settings [79], focusing on 54 nocardia species known to cause disease in humans, most frequently in immunocompromised individuals, with an overall global incidence rate of approximately 1/100,000 individuals. *Nocardia* infection presentation is primarily pulmonary, with symptoms such as pneumonia, inflammation, abscess formation, and deep structural cavitation; all these serve to mimic TB in clinical diagnosis. This symptom overlap, and subsequent difficulty in clinical diagnosis, are the basis for the genus nickname, “The Great Masquerader” [79].

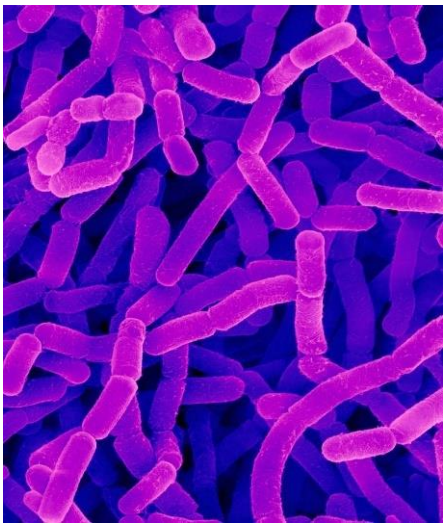

Figure 1.11: Colourised 1,600x magnification scanning electron microscope image of Nocardia asteroides structure [80].

1.6: A Brief Overview of Molecular Epidemiology

Molecular epidemiology approaches have become the standard approach to study TB epidemiology and multiple methods have been developed. These methods have the advantage of being able to differentiate the species and strain causing TB while also providing data on transmission and reinfection.

1.6.1: IS6110 Typing

IS6110 has served as the gold standard for molecular epidemiology in TB infections since 1993 [81]. This method of DNA speciation analyses the IS6110 insertion which is only found in members of the MTBC for differentiation of species. Related strains and linked cases will display extremely low levels of variability when analysed using RFLP techniques which analyse repetitive segments within the MTBC genomes. Unrelated cases will show a high degree of difference.

While useful for linking cases it ultimately displays limited utility compared to newer methods such as MIRU-VNTR due to its increased difficulty compared to this newer methodology. In further comparison, spoligotyping was developed concurrently with IS6110 and demonstrates poorer discrimination but is sufficient for many clinical and research purposes due to increased flexibility over IS6110.

1.6.2: MIRU-VNTR

Another molecular method is mycobacterial interspersed repetitive unit variable number tandem repeats (MIRU-VNTR) analysis. Mycobacterial genomes contain regions of 36bp direct repeat sequences interspersed with unique genomic segments which are PCR amplified and counted. Usually, this analysis is performed using twenty-four loci, the results of which are highly reproducible and easily compared between laboratories [82,83]. MIRU-VNTR provides a high degree of discriminatory power to assays, allowing investigation of transmission dynamics and determination of whether a case is novel, relapse, or reinfection [82,84]. However, this discriminatory power varies

by *M. tuberculosis* strain, requiring additional loci in a hypervariable region for the same diagnostic capability in the Beijing strain [82,83]. As the Beijing strain is correlated with increased drug resistance, more complex loci sets would be required for routine diagnosis. An increase in complexity summarily limits the clinical utility.

MIRU-VNTR has further benefits, including its ease of use and its relatively low cost.

1.6.3: Spoligotyping

Like MIRU-VNTR analysis, spoligotyping uses non-coding identical direct repeat (DR) regions. However, unlike MIRU-VNTR, which looks for the number of repeats, spoligotyping identifies the unique spacer segments between each DR [85]. Results are generally presented as a binary presence/absence, which allows direct comparison between isolates [82]. Identification of spacer segments also allows differentiation of *M. tuberculosis* strains; e.g., the Beijing strain lacks spacers 1 through 33, but contains spacers 34 through 43 [82].

The benefits of spoligotyping include high reproducibility and rapid turnaround times. Spoligotyping can produce a result within one day, as opposed to several weeks for IS6110 RFLP analysis [86]. However, spoligotyping struggles to discriminate between strains in regions with a high prevalence of Beijing strain infections [82]. In a test of 118 clinical *M. tuberculosis* samples, spoligotyping identified 68 different strains, compared to a *IS6110* analysis, which differentiated 88 strains. This study indicated that samples with multiple *IS6110* copies are more difficult to differentiate by spoligotyping [85].

1.6.4: Whole Genome Sequencing

Unlike MIRU-VNTR and spoligotyping, which utilise only a small fraction of the *M. tuberculosis* genome, whole genome sequencing (WGS) analyses the entirety of an isolate's genetic sequence. WGS can be performed on clinical isolates for identification of species, strain, and drug-resistant mutations within a sample [87–89].

WGS is useful for in-depth investigation of drug resistance, disease transfer, and lineage calling. However, WGS is not without its drawbacks. Firstly, WGS requires isolated cultures, thus increasing the time from suspected diagnosis of TB to obtain results [10,13]. Secondly, WGS requires extensive infrastructure and specialised staff training for accurate results and bioinformatic interpretation [16,54]. These drawbacks limit the clinical utility of WGS for drug resistance testing and diagnosis in resource limited areas outside of centralised reference laboratories.

These limitations are decreasing in severity with the development of new methodologies. For example, ONT has reduced the need for specialist skills and infrastructure, particularly with the use of the RBK kit. This kit allows library preparation from sample in 15 minutes. Likewise, new free software tools such as TB-profiler are available for sequencing analysis which decreases the need for specialist bioinformatic skills [90,91]. With these improvements WGS is becoming the new Gold Standard for TB diagnostics where resources are available.

1.7 Economics of TB

TB is extremely virulent and results in an excessive disease burden in LMICs [24,30]. The majority of global TB cases (95%) occur in countries with limited resources and health infrastructure (Figure 1.12) [24]. 43% of cases were reported in Southeast Asia, 25% in Africa, and 18% in the Western Pacific [34]. Despite this socio-economic link, the majority of TB research is conducted in high-income countries that exhibit low TB incidence [13]. Numerous financial and economic factors also arise from TB infection and prevalence, both at the governmental and individual levels.

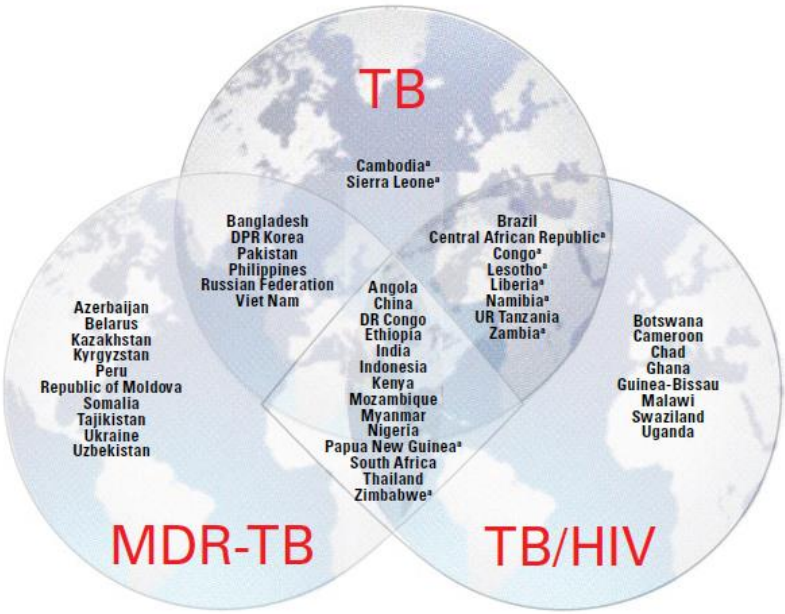

Figure 1.12: Countries in the three high-burden country lists for TB, TB/HIV, and MDR-TB between 2016 and 2020, with overlaps [33].

Treatment for TB often places a large strain on medical infrastructure and governmental funding, especially for drug-resistant infections [41]. For example, the cost of treatment for MDR-TB is approximately 100-fold that of DS-TB [51]. Furthermore, despite accounting for only 5% of TB cases in South Africa, XDR-TB treatments consume over one third of South African national TB-program resources [41]. To offset these burdens, many resources for combatting TB in high incidence countries come from extra-governmental sources, such as NGOs and foreign governments. One argument advocating for extra-national funding is that international travel and migration in the modern era compound to make drug-resistance and the spread of TB global issues [41]. Programs to improve food access, housing, and general medical access, all of which are strongly linked to TB incidence, are also important for improving global TB outcomes and reducing the impact of TB on LMICs [51].

According to the WHO, in 2019 available funding for universal TB treatment access increased from $5.6 billion to $6.5 billion [33]. 57% of this funding was provided by five countries; Brazil, Russia, India, China, and South Africa. 97% of this was allocated for domestic use in combatting TB within their own borders. However, the funding total is still short of the $13 billion goal for universal TB treatment access set by the WHO for 2022 [33].

As previously mentioned, TB research is largely funded by organisations in low-incidence/high-income countries. In 2018, 56% of the total research funding available was supplied by the US Government as well as the Bill and Melinda Gates foundation. TB research funding is primarily allocated for treatment and infrastructure research, with approximately 9% earmarked for improving diagnostic methods [33].

Aside from the burden on national healthcare and surveillance systems, at the individual level, TB diagnosis and treatment cause sizable financial burdens. The WHO calculated that, since 2015, 47% of global individuals treated for TB faced catastrophic healthcare costs (Figure 1.13), defined, in this context, as greater than or equal to 20% of annual household income. The percentage of cases facing such catastrophic costs is higher for individuals treated for drug-resistant TB infections (>87%) [34]. Costs disproportionately affect individuals in LMICs, compounding the burden seen at the national level [51].

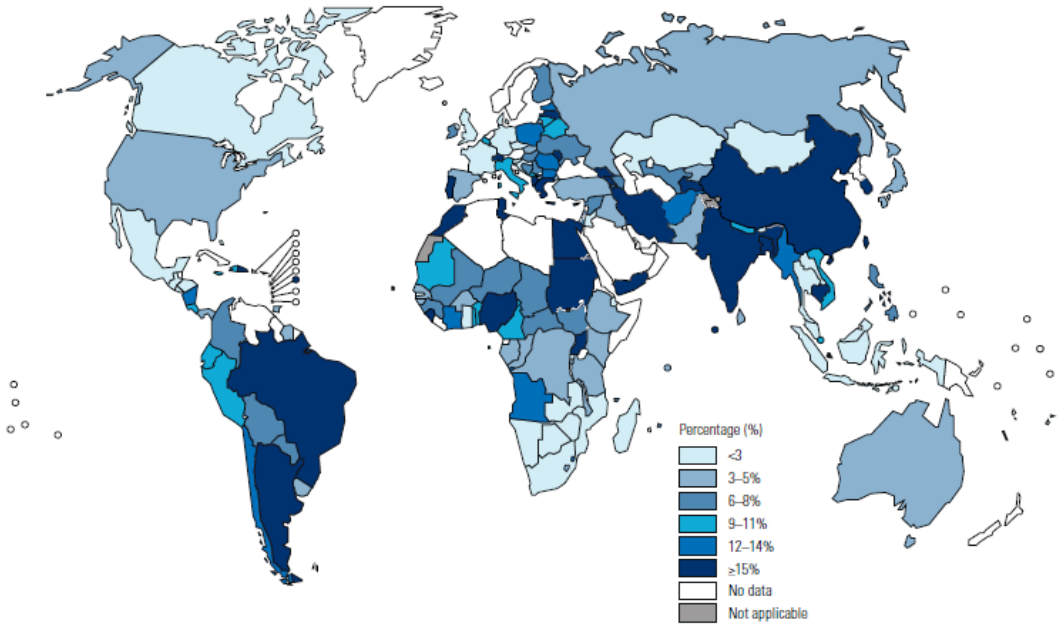

Figure 1.13: Percentage of the general population facing catastrophic health expenditure, according to the latest available data [33].

1.8: Diagnosing TB and DR-TB

1.8.1: Drug-Susceptible TB Diagnosis

Over the past century, multiple diagnostic technologies have been developed or proposed. As technologies advance, so do hopes for faster, more accurate diagnostic methodologies.

1.8.1.1 Gold Standard

Effective TB diagnostics first emerged towards the end of the 19th century with the development of acid-fast staining, microscopy, and routine culture of clinical samples [15,92]. Standard microscopy is cheap and produces results quickly, though there are two key limitations: it is burdened with a high detection threshold, approximately 10^5 colony forming units per millilitre (CFU/mL), and it is highly reliant on operator skill. Reliance on operator skill especially affects the sensitivity of microscopy diagnosis, which ranges from 20-80% for culture confirmed cases [93]. Despite these drawbacks, microscopy is still the only diagnostic tool commonly available in many low-income, high-incidence countries [3,13,14].

In contrast, culture-based diagnostics, while slower, exhibit higher sensitivity and a lower limit of detection, approximately 10 CFU/mL, which is 10,000x lower than that of visual microscopy [14,94]. Though culture-based methods are highly sensitive, they still have significant drawbacks, the most prominent being the long turn-around time due to the slow growth of MTBC [13] . For a summary of the strengths and weaknesses associated with various culture methods, see section 1.8.3.1.

These limitations demonstrate the requirement for new diagnostic and DST methods in order to improve TB control [29,95].

1.8.1.2 PCR

One such method was developed in 1985 with the invention of polymerase chain reaction (PCR) by Kary Mullis. PCR methods show a marked increase in the speed of TB diagnosis, however, they still exhibit some weaknesses. For example, an inability to inform epidemiological studies renders PCR

tests less practical for disease control and tracking programs. Similarly, PCR methods are unable to detect co-infections or mixed infections, which can complicate treatment.

Some targets used for TB diagnosis have included IS6110, *rrs*, and *rpoB* [96]. These genes are highly conserved in TB genomes and serve as consistent biomarkers for bacterial presence. Similarly, differentiation of virulent MTBC species and *M. bovis* BCG has targeted the deletion of the RD1 region [92]. These methods while sensitive and specific suffer from limited scope and breadth due to the conservative nature of PCR based diagnostics.

For a full review of the strengths and weakness of PCR techniques please see Wlodarska et al., or Schürch and van Soolingen [13,97]. A brief summary of strengths and weaknesses is presented in section 1.8.3.2.

The next logical step in TB control was to move beyond the use of amplicons and aim towards deriving information from entire genome sequences. It was at the turn of the 21st century that the potential to routinely investigate drug-resistance and molecular epidemiology in this way emerged.

1.8.2: Drug-Resistant TB Diagnosis

1.8.2.1: Gold Standard

While useful for a rapid diagnosis of TB disease, microscopy is incapable of providing a drug-resistant or -susceptible diagnosis. Culture, however, is a powerful method for drug susceptibility testing (DST). MTBC cultures are grown in the presence of known antibiotic concentrations and the results are recorded as resistant or susceptible. Mycobacteria isolates are classified as phenotypically resistant if >1% of inoculated colonies on a plate grow in the presence of critical concentrations of a given drug [41].

More recently an improved phenotypic method has come to the fore with the dissemination of the Bactec MGIT 960 manufactured by Beckton Dickenson in the USA. Using liquid medium and automated growth monitoring the reduction in oxygen attributable to the growth of aerobic

bacteria. This method reduces the phenotypic resistance calling time from several weeks to approximately one, greatly improving the turnaround time.

The use of MGIT technology has been further aided by research into broth microdilution methods. These methods have shown to increase the sensitivity and discriminatory power of MGIT diagnosis in the presence of heteroresistance, particularly for rifampicin, isoniazid, and ethambutol [98]. This is managed by using geometrically increasing concentrations of antimicrobial agents within each culture, thereby more accurately identifying the MIC for a given sample [99].

Alongside research improving sensitivity and specificity the European Committee on Antimicrobial Susceptibility Testing (EUCAST) released a universal MGIT protocol in 2019 [100]. This protocol allowed for increased transparency between laboratories and facilitated ramping up of phenotypic testing utilising MGIT.

However, both phenotypic methods (culture and MGIT) suffer from the same limitations as culture diagnosis for drug-susceptible TB, and in fact take longer, since resistance cultures are performed following isolation of MTBC from sputum. Despite these drawbacks, culture-based phenotypic testing has remained the gold standard for identifying resistance to antibiotics [12–15].

1.8.2.2: PCR

The WHO-endorsed Xpert MTB/RIF and new MTB Ultra assays are capable of diagnosing MTBC while also testing for resistance to rifampicin. WHO also endorsed the PCR based line-probe assays MTBDRplus and MTBDRsl DST which test MTBC isolates for first- and second-line drug resistance [13,54]. While these assays have lower sensitivity for smear-negative samples; MTB/RIF: 84.48%, MTB/RIF Ultra: 81.8%, MTBDRPlus: 57%, MTBDRsl: 20-37% [101–103]. Despite the decreased sensitivity, these assays are faster than gold standard methods and allow for antibiotic regimens to be implemented within clinically viable timeframes [6,52,54]. Furthermore, these assays are currently the

only molecular methods endorsed by the WHO for use in LMICs [14]. These factors demonstrate clear advantages over previous culture-only methods.

However, while rapid, these assays are only able to cover a very small percentage of the myriad SNPs associated with first- and second-line antibiotic resistance. This limitation, and low flexibility in targets, means that phenotypic resistance can be missed by omitting current and novel SNPs. In part due to this, it is common practice to utilise culture to confirm molecular DST findings, which largely negates the speed advantage of PCR methods.

1.8.3: Strength and Weakness Summary

1.8.3.1: Culture/Microscopy

Table 1.1: A summary of the primary strengths and weaknesses inherent with culture and microscopy for TB

Strengths	**Weaknesses**
Sensitive	Long processing time
Affordable	Risk of contamination and overgrowth
Able to perform simultaneous diagnosis and DST	Increased rate of false-negatives during diagnosis and DST
	Increased risk of laboratory transmitted illness
	Unable to differentiate species or strains

As mentioned previously culture serves as an extremely sensitive diagnostic procedure with a limit of detection of 1CFU/mL under ideal conditions [14,94]. Culture and microscopy also benefit from being cheap compared to molecular technologies. Further, culture can both serve as a diagnostic and DST test, although microscopy has no DST function.

Counter to these benefits culture and microscopy demonstrate numerous drawbacks. The most glaring of these is the long turnaround time for culture, approximately 8 weeks on average [15,104]. Further, the slow growth rate of TB increases the risk of overgrowth by commensal organisms [16], the risk of cross-contamination during incubation [17], and increases the likelihood of type 2 error [8]. The low bacterial load required to result in active infection in TB also increases the likelihood of laboratory acquired infection during routine TB diagnostic and DST work by 6-9 fold over the general public [105]. Finally, neither culture nor microscopy is able to routinely differentiate causative

agents or strains within the MTBc in a clinically useful manner which can result in negative patient outcomes [57].

These drawbacks often far outweigh the benefits of culture and microscopy prompting the use of more advanced diagnostic tools where available.

1.8.3.2: PCR

Table 1.2: A summary of the primary strengths and weaknesses inherent with PCR for TB

Strengths	**Weaknesses**
Rapid processing time	Reliance on highly conserved primers
Affordable	Unable to detect coinfection
Able to perform some DST	Requires culture followup for comprehensive DST
	Reduced sensitivity

PCR exhibits one major benefit over microbial culture for TB diagnosis, its speed. While culture takes weeks PCR has the potential to take mere hours to render a diagnosis [94]. This speed is aided by being only slightly more expensive than culture increasing the utility in laboratories of all sizes [106]. This speed and affordability is further enhanced by a limited ability to perform simultaneous DST during diagnosis with the proper design of primers.

However, as with culture, PCR is not with significant weaknesses. Firstly, the reliance of any PCR on highly reserved primers limits the breadth of a diagnostic assay. Similarly, this conservatism can reduce sensitivity in the presence of mutations [5,107]. The use of primers also limits the ability of an assay to determine coinfection as it is primarily a simple presence or absence of amplification which is detected [15]. This presence/absence also means that anything beyond basic DST must be performed by follow up culture, largely negating the speed advantage of PCR [52].

In summation while PCR offers a distinct advantage over gold standard culture the drawbacks still necessitate the development and implementation of improved diagnostic and DST methods.

1.9: Whole Genome Sequencing (WGS) in TB

In 1998, over a century after the advent of acid-fast microscopy and culture-based methods, the first full *Mycobacterium tuberculosis* genome, *M. tuberculosis* H37Rv, was published [108]. This isolate

became the official reference genome for *M. tuberculosis* [29]. Since the publication of this original genome sequence, sequencing methods have become faster, cheaper, and simpler, resulting in a library of thousands of complete and partially assembled MTBC genomes within the National Center for Biotechnology Information (NCBI) database [3,29]. Many sequences were constructed using Sanger methodology, which formed the basis for the original genotypic diagnostic protocols, which have been increasingly used in the research and clinical management of TB [5,7,29].

Sequencing allows for more in-depth research than previously possible through either culture or PCR. For example, the coverage of extra genome allows for molecular epidemiology and improved outbreak tracing and management. However, there are drawbacks to using WGS, preventing the widespread adoption of these techniques (section 1.9.2). Issues with WGS are driving research into quicker, cheaper, and more accurate molecular methods. Proponents for new techniques seek the advantages of sequencing without the drawbacks of culture, hoping to reduce the complexity and time required for these assays.

1.9.1: WGS DST

WGS diagnostics overcome the limitation of PCR, by targeting narrow sections of a genome. When coupled with information on resistance-conferring SNPs, this allows simultaneous DST for all anti-tuberculous medications [5,10]. However, this method still relies on the isolation of pure culture before DST can be performed and can often only be performed at larger reference laboratories [13,16,17]. Both limitations introduce delays for diagnosis, which can lead to poorer patient outcomes.

1.9.2: Strengths and Weaknesses of WGS

Table 1.3: A summary of the primary strengths and weaknesses inherent with WGS for TB

Strengths	Weaknesses
Can speciate mycobacteria	Requires culture
Can provide epidemiological data	Cannot determine transmission timing or directionality
Can simultaneously perform in-depth DST	Requires specialized infrastructure
High genomic resolution	Methods are unvalidated for use in LMICs

WGS demonstrates another large step in TB diagnostic and DST technology. This technology allows powerful and in-depth analysis of diagnostic samples which can generate multiple useful types of data. One such use of WGS is for the differentiation of mycobacteria and strains as WGS is able to supply information on the entire genome instead of the small sections previously possible [29]. This genetic information can also be used to investigate epidemiological links with benefits for both clinicians and disease control organizations [15]. Finally, this whole genome coverage allows for simultaneous DST of all known mutation conferring SNPs, providing access to a suitable bioinformatic tool [109].

However, for all its strength WGS has a few notable weaknesses. Firstly, current WGS methods require pure culture which means that it is limited by the same slow processing time as traditional culture methods [7]. Also, WGS sequencing provides a snapshot look at a genome and as such comparison of genomes cannot determine time or direction of a transmission event [106]. Further, coupled with the analytical strength of WGS is a summary increase in the expense of infrastructure required limiting the utility of these techniques outside of reference and research laboratories [9,15]. In part due to the limitations imposed by these costs WGS is not validated for diagnostic use in LMICs were the power for DST is most sorely needed [13].

As can be seen, there is currently no perfect tool for TB control. However, new assays and methodologies are continuously in development to improve the power, utility, and cost of TB diagnostics.

1.10: The Future of TB Diagnostics

Diagnostics and genomics are currently undergoing what Wlodarska et al. call a “Genomic Revolution” and what Tsalik et al. have named a “technological revolution”, phrases which indicate the rapid advancement and increasing analytical power of these methods in health related fields [13,17]. Wlodarska et al. add that “the genomic revolution is set to dramatically alter the clinical

microbiology landscape, and we expect to see the first inroads in this area in the diagnosis, treatment, and epidemiology of tuberculosis in well-resourced settings." [13].

1.10.1 Metagenomic Diagnostics

Currently, one major use of next-generation metagenomic sequencing within diagnostics is identification of pathogen genomic sequences in a timely and cost effective manner [1,7,8,15]. By investigating the large swathes of the genome, rather than smaller sections, biomarkers, or fingerprints, metagenomics allows for more detailed analysis than available through traditional sequencing methods [10,30,54,110]. Single nucleotide resolution of large genome portions, available rapidly with metagenomic sequencing, stems from the ability of an assay to cover the majority of a genome with multiple read-depth within a single sequencing run without the need for culture isolation [5,30].

Rapid and accurate detection of SNPs has been transformative for clinical diagnosis and epidemiological efforts, as most resistance in TB is attributable to SNPs [5]. This is especially vital within the MTBC, as members have extraordinarily high levels of genomic homogeneity, making speciation by phenotypic methods or first-generation sequencing methods difficult [1,12]. However, with SNP level resolution, differences are now identifiable within the MTBC, allowing for more accurate diagnosis and outbreak monitoring [10,16,54,57].

To achieve this level of resolution for pathogens the concentration of host DNA must be reduced. Samples will contain many-fold more host cells, and thereby host DNA, than bacterial cells which can overwhelm sequencing reads [111]. However, the use of targeted depletion methods can reduce the proportion of host DNA in a sample, thereby allowing the detection and multiple-read depth of bacterial contributors [111–113].

The strengths and weaknesses of metagenomics can be summarised as follows:

Table 1.4: A summary of the primary strengths and weaknesses inherent with metagenomics for TB

Strengths	**Weaknesses**
High genomic resolution [110]	Requires enrichment or host depletion [48,94]
Independent of culture [3]	Expensive [16]
Rapid turnaround time [3,49]	Requires complex infrastructure [14]
Comprehensive DST [16,30]	Complex bioinformatic analysis required [8,88]

1.10.2 Targeted Next-Generation Sequencing

Use of targeted next-generation sequencing (tNGS) methods has allowed a shift from traditional Sanger sequencing for the detection and diagnosis of disease. High-throughput methods have dramatically reduced the cost per base of sequencing and, when combined with targeted amplification, have removed the barrier from low target DNA volumes in samples [114,115]. This has in turn altered the focus of molecular diagnosis away from single-gene related illnesses such as cystic fibrosis to diseases involving multiple genes concurrently. The new limitation is then on the pace of discovery and definition of genes for a given phenotype [114].

Diagnosis and DST of TB has directly benefited from this new focus with release of new commercial technologies like the Deeplex® Myc-TB from GenoScreen [116]. The increase in defined drug-resistance associated genes allows products such as this to perform concurrent DST for more drugs than previous technologies. Because of this expanded target range the Deeplex® Myc-TB assay has seen success in Africa when compared to the GeneXpert MTB/RIF test [117,118]. One study also showed success in the detection of a drug-resistant TB strain using the Deeplex® Myc-TB kit which was undetected by WHO-endorsed methods [119]. These trials demonstrated the potential for tNGS to detect DR-TB with greater accuracy than existing WHO-endorsed methods allowed.

Due to this diagnostic power WHO, in conjunction with FIND, have called for new diagnostic and DST methods using targeted next-generation sequencing (tNGS) to combat the increase in DR-TB

worldwide[120]. Using existing PCR technology, combined with sequencing capabilities offered by systems such as those available from Illumina, ThermoFisher, and Oxford Nanopore Technologies (ONT), the aim is to create a specific and sensitive assay capable of accurately calling clinically relevant SNPs. By using a preliminary PCR step to enrich genetic regions of interest, assay specificity can be improved through careful primer design. Also, PCR amplification can improve assay sensitivity by facilitating deep, even, coverage of the target region [121].

Combined with rapid long-read sequencing this adds a new tool to the clinical genomics tool box.

1.11: Study Aims

Due to the increasing drug-resistance and prevalence of TB worldwide, new methods for diagnosis and epidemiological investigation are required. Existing methods have high sensitivity and specificity, yet are often hampered by slow turnaround times, complexity, and inability to rapidly detect MTBC and first and second line anti-TB resistance. This study seeks to address these issues by developing diagnostic tests that are rapid, user friendly, and affordable, in line with WHO/FIND aims.

Citing the need for novel techniques, this study aims to develop a standardised methodology for detection and sequencing of TB from primary sputum samples, using two NGS methods. Firstly, this study will seek to develop a tNGS assay for the detection of drug-resistant MTBC directly from clinical samples. Secondly, it seeks to develop a host depletion based rapid metagenomic sequencing test for MTBC and drug resistance detection. Both approaches will utilise nanopore sequencing and real-time analysis tools from ONT (Epi2Me) to develop sample-to-result pipelines for use directly on sputum. The tNGS approach will be developed for the FIND Seq&Treat programme – designed to generate evidence and boost in-country capacity to support the global adoption of commercial tNGS for affordable, scalable, and rapid TB DST (https://www.finddx.org/at-risk-populations/seq-treat/) – and will be evaluated as part of this programme.

Chapter 2 - Methods

2.1: Bacterial Culture Conditions for Method Development

M. bovis BCG and *M. smegmatis* were independently cultured for use in method development. The *M. bovis* BCG strain was provided by collaborators at the Norfolk and Norwich University Hospital (NNUH) Innovation Centre (section 2.2) where it was cultured in a BD BACTEC MGIT 960 until flagged positive (~10^5 CFU/mL). A freeze-dried *M. smegmatis* sample (NCTC 8159) was purchased from the Public Health England (PHE) culture collection. *M. smegmatis* was reconstituted in 1mL of LB nutrient broth before inoculating 100µL into three 10mL of LB broth cultures in 15mL falcon tubes. An uninoculated 10mL sample of LB broth was also cultured as a contamination control. *M. smegmatis* samples, and the control, underwent incubation on an orbital shaker at 37°C. Cultures were incubated for four days until suspended bacteria became visible, at which time culture tubes were transferred to a 4°C refrigerator for downstream use.

Freeze-dried culture samples of *M. kansasii* (DSM 44162)*, M. abscessus* (DSM 44196)*,* and *M. avium* (DSM 44156) were sourced from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) for use in specificity testing. These samples were each reconstituted in 1mL MiddleBrook 7H9 nutrient broth. 100 µL of reconstituted samples were then inoculated into pre-aliquoted glass culture tubes containing MiddleBrook 7H9 nutrient broth from Hardy Diagnostics in triplicate (Catalog # C32). Inoculated cultures were grown in an orbital incubator at 37°C for 12 weeks, or until suspended bacteria became visible. An uninoculated culture tube underwent the same growth conditions as contamination control.

A second strain of *M. bovis* BCG was grown by collaborators at the NNUH Innovation Centre under specific conditions to inhibit clumping and promote even distribution of bacterial cells throughout a liquid culture [122]. Freeze-dried *M. bovis* BCG was reconstituted in 1mL of MiddleBrook 7H9 nutrient broth. 10µL of the reconstituted sample was inoculated into a BACTEC MGIT liquid growth tube with PANTA (supplemental polymyxin B, amphotericin B, nalidixic acid, trimethoprim and

azlocillin) and left to grow for 30 days. After this period the MGIT tube was removed from the machine and vortexed vigorously to resuspend cells. The culture was left to sit for 10 minutes, allowing large clumps of cells to settle. After this settling period, 200µL of supernatant was removed and reinoculated into a fresh MGIT tube to which 100µL of 0.5% Tween-80 had been added. The newly inoculated MGIT tube was returned to the BACTEC MGIT machine and left to incubate for a further 18 days, after which time the tube was removed and the cultured *M. bovis* BCG was ready for further experimentation.

For solid media growth, MiddleBrook 7H10 agar plates were purchased from Trafalgar Scientific (Catalogue #7046). 100µL of each liquid BCG culture, serially diluted in phosphate buffered saline (PBS), was aliquoted into the centre of an agar plate. The liquid dilution was spread using a disposable sterile cell spreader before the plates were covered and placed into a 37°C incubator for 8-12 weeks, until bacterial growth became visible. An uninoculated agar plate was also prepared as a contamination control.

In silico sequences for *M. caprae* (GCF_001941665.1) and *M. pinnipedii* (GCF_002982275.1) were sourced from the NCBI database for use in specificity testing to avoid the necessity of culturing BSL-3 organisms. Likewise, *M. leprae* (GCF_003253775.1), *M. ulcerans* (GCF_020616615.1), and *M. marinum* (GCF_016745295.1) were also sourced *in silico* to simplify specificity testing and reduce the lead-time needed to grow new cultures from freeze-dried stock.

2.2: Clinical Sample Ethics

Excess diagnostic sputum was obtained from the NNUH Innovation Center for all contrived sample experiments. Contrived samples were created by spiking known quantities of known organisms (*M. bovis BCG, M. avium, M. abscessus,* and/or *M. kansasii*) into these sputum samples (further described in section 2.6). Sputum was pooled at the Innovation Center and no identifying information was provided. The use of excess diagnostic samples for TB diagnostics research was approved by HRA and Cambridge East Research Ethics Committee under IRAS project ID 255463.

2.3: DNA Extraction

DNA was extracted using automated systems to simplify sample preparation and minimize the risk of contamination. Two automated methods were used through the course of the study, the MagNA Pure Compact System and the Promega Maxwell Rapid Sample Concentrator (RSC) 48.

2.3.1: MagNA Pure Extraction

DNA was extracted from pure liquid cultures for use in method development and positive controls using the MagNA Pure compact system. When extracting from pure liquid culture a sample size of 200µL was used. Samples were pelleted in a benchtop centrifuge at 6,000g for 3 minutes before carefully removing the supernatant. It was standard practice to leave approximately 50µL of supernatant behind to avoid loss of sample. The pellet was then resuspended in 500µL of MagNA Pure Bacterial Lysis Buffer (BLB) available from Roche Life Science (Catalogue # 04659180001). The resuspended sample was then transferred to a Lysis Matrix E tube available from MP Biomedicals (Catalogue # 116914050-CF) for mechanical cell-lysis and sample homogenization. The samples were then bead-beaten in a Qiagen TissueLyser LT (Catalogue # 85600) at maximum speed for 15 minutes to maximize mechanical cellular lysis. Following lysis the samples were again pelleted in a benchtop centrifuge, at 21,000g for 3 minutes. After centrifugation, 230µL of supernatant was carefully removed and aliquoted into a fresh MagNA Pure tube for use in the automated extraction system. This was done by removing two volumes of 115µL to minimize the risk of transferring any of the lysis matrix which can inhibit subsequent DNA extraction. An additional 170µL BLB was added along with 20µL of Proteinase K and the mixture was incubated on an Eppendorf ThermoMixer C (Catalogue #5382000031) at 65°C, 800rpm, for 5 minutes. Finally, the sample in the MagNA Pure tube was loaded into the MagNA Pure Compact according to the manufacturer's protocol and eluted in 50µL.

NRF sputum spiked with liquid culture [*M. bovis* BCG], and contrived clinical samples received from FIND, were initially extracted using the MagNA Pure Compact. For these sample types, the initial

sample volume used was between 750µL and 1mL. After aliquoting the desired volume of sample into a clean Eppendorf it was centrifuged at 8,000g for 5 minutes to create a pellet.

The pellet was resuspended in 700µL of BLB. This resuspended sample was then transferred to a Lysis Matrix E tube [MP Biomedicals] for mechanical lysis. The sample was then bead-beaten in a MP Biomedicals FastPrep-24 5G lysis system (Catalogue # 116005500) at 6m/s for two cycles of 45 seconds each. The bead-beaten samples were then centrifuged in a benchtop centrifuge at 21,000g for 3 minutes. Following centrifugation, 400µL of the supernatant was carefully transferred to a barcoded MagNA Pure tube in two batches of 200µL each. 20µL of Proteinase K was then added to the sample and the mixture was incubated on an Eppendorf ThermoMixer C at 65°C, 800rpm, for 5 minutes. The incubated sample was then loaded into the MagNA Pure Compact following the manufacturer's protocol and eluted in 50µL for downstream use.

2.3.2: Promega Maxwell Extraction

NRF sputum spiked with liquid culture [*M. bovis* BCG], and contrived clinical samples received from FIND, were also extracted using the Maxwell RSC 48. For these sample types, the initial sample volume used was between 750µL and 1mL. After aliquoting the desired volume of sample into a clean Eppendorf it was centrifuged at 8,000g for 5 minutes to create a pellet.

The pellet was resuspended in 700µL of PBS and transferred to a MP Biomedicals Lysis Matrix E tube for mechanical lysis and homogenization. As with the samples being prepared for the MagNA Pure, these samples were processed in a MP Biomedicals FastPrep-24 5G system at 6m/s for two cycles of 45 seconds each. After lysis, the samples underwent centrifugation in a benchtop centrifuge at maximum speed for 3 minutes before 400µL of supernatant was carefully aliquoted into a fresh 1.5mL Eppendorf tube in two 200µL volumes. The sample then had 40µL of Proteinase K and 200µL of Lysis Buffer A from the Promega Maxwell RSC PureFood Pathogen Kit (Catalogue #AS1660) added and pipette mixed. This mixture was then incubated in an Eppendorf ThermoMixer C at 65°C, 800rpm, for 10 minutes. Following this incubation, 400µL of PBS and 300µL of Lysis Buffer

from the Promega Maxwell RSC PureFood Pathogen Kit were added to the sample. Finally, the entire mixed sample was loaded into the Promega Maxwell RSC following manufacturer's protocols and eluted into 50µL for downstream use.

2.4: DNA Quantification

Quantification of nucleic acid was performed using two methods; the Invitrogen Qubit 4 Fluorometer (Thermofisher Scientific Catalogue #Q33238) and the Promega GloMax Discover microplate reader (Catalogue #GM3000) using Qubit reagents.

2.4.1: Qubit

Quantification using the Qubit 4 Fluorometer was performed with 2µL of extracted elute. High sensitivity Qubit reagents from the Qubit dsDNA HS Assay Kit (Catalogue #Q33231) were mixed in a 200:1 Buffer:Dye ratio according to manufacturer specifications. For the two recommended standards in the kit, 190µL of the mixed buffer was aliquoted into two Qubit tubes. These tubes then had 10µL of the included standards 1 and 2 added, one to each. Next, 198µL of the buffer mix was aliquoted into Qubit tubes, one for each sample being quantified. 2µL of sample were then added to each of the tubes to bring all tubes to a total volume of 200µL. The samples and controls were then briefly vortexed and centrifuged before being incubated in the dark for 2 minutes. Finally, the samples were read individually on the Qubit 4 Fluorometer, standards first, according to the manufacturer instructions. The resulting DNA concentrations were recorded for analysis and quality control.

2.4.2: Promega

The Promega GloMax quantification method was used when high throughput was required as it could be performed in a 96 well plate. As with the Qubit 4 Fluorometer method, Qubit dsDNA HS Assay Kit reagents were used and mixed in the 200:1 Buffer:Dye ratio. A clear flat-bottomed 96-well CytoOne plate from StarLab [Catalogue #CC7682-7596] was used and 198µL of prepared

buffer/dye solution was added to each well for the number of samples being quantified. 2µL of sample elute was then added to each well to bring the total volume of each up to 200µL. The plate was then sealed with a foil, mixed by vortex, and spun down briefly in a benchtop centrifuge to ensure thorough mixing of each sample. Following this mixing process, the plate was incubated in the dark for 2 minutes before carefully removing the film and inserting the plate into the Promega GloMax as instructed by the manufacturer protocols. The dsDNA fluorescence protocol was selected, run, and the subsequent results were transferred to a computer for analysis using a custom excel spreadsheet created by Dave Baker of the QIB sequencing service. The resulting nucleic acid concentrations were recorded for analysis.

2.4.3: Bacterial Cell Equivalent Calculation

As the Qubit 4 Fluorometer and Promega Glomax methods yielded only DNA concentrations within a sample, a conversion formula was created to determine the number of cell equivalencies (CE) within each sample. This was possible as *M. bovis* and *M. tuberculosis* have haploid genomes, allowing the number of cell equivalents to be determined by dividing the weight of DNA detected by the weight of a single *M. bovis* or *M. tuberculosis* genome. One megabase of DNA weighs 1.09fg. The length of the *M bovis* BCG genome is 4.27 megabases. Thus, the average weight of the *M bovis* BCG genome is 4.654fg. This translates to 214,855.08 CE per ng of detected DNA. Then, as only 2µL of DNA from the 50µL elute was used for quantification, the detected concentration (in ng) was multiplied by 25. This resulted in the equation below which would yield the total number of cell equivalents extracted from a given sample.

$$(X * 25) * 214{,}855.08 = CE\ per\ Extracted\ Sample$$

2.5: DNA Quantification by qPCR

qPCR was used for quantification of host, commensal, and target DNA. Nested qPCR reactions were also used for relative quantification of target amplicons following multiplex amplification.

2.5.1: SYBR Green qPCR

Per reaction, 2µL of DNA was used as template with a working mix of SYBR Green Master Mix, forward primer, reverse primer, and molecular H_2O prepared in the concentrations detailed below (Table 2.1).

Table 2.1: SYBR Green qPCR working solution formula per sample

Reagent	**Volume per Sample (µL)**
SYBR Green Master Mix	10
20µM Forward Primer	1
20µM Reverse Primer	1
Molecular Grade H_2O	6
DNA template	2
Total volume	**20**

The master mix used for these assays was the LightCycler® 480 SYBR Green 1 Master Mix from Roche Life Science (Catalogue # 04707516001). The primers targeted the BCG RD1 region, the human RNA polymerase A gene, and the bacterial 16S gene V3-V4 fragment (Table 2.2). 18µL of the mastermix and 2µL of the template DNA were loaded into a 96-well LightCycler plate. The plates were then sealed with clear foil, vortexed, and briefly spun down prior to insertion into the LightCycler® 480 system for analysis. The reactions were then amplified using the cycling conditions detailed below (Table 2.3).

Table 2.2: SYBR Green qPCR amplification primers for the amplification of three targets of interest

Amplification Target	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**
BCG RD1 Region	AAGCGGTTGCCGCCGACCGACC	GAGGCGATCTGGCGGTTTGGGG
Human RNA polymerase A Gene	TGAAGCCGTGCGGAAGG	ACAAGAGAGCCAAGTGTCG
Bacterial 16S V3-V4 Fragment	CCTACGGGDGGCWGCA	GGACTACHVGGGTMTCTAATC

Table 2.3: Cycling conditions for SYBR Green qPCR amplification

Step	**Temperature (°C)**	**Time (mm:ss)**	**Cycles (#)**
Pre-Incubation	95	05:00	1
Amplification	95	00:30	40
	55	00:30	
	72	00:30	
Final Extension	72	05:00	1
Melt Curve	95	00:05	1
	65	01:00	
	95	Continuous	
Cooling	37	00:01	1

2.5.2: TaqMan Probe-based qPCR

Per probe reaction, 5µL of DNA was used as template with a working solution of Roche probe master mix, forward primer, reverse primer, fluorescence probe, and molecular H_2O (Table 2.4).

Table 2.4: Probe-based qPCR working solution formula per sample

Reagent	**Volume per Sample (µL)**
Roche Probe Master Mix	10
20µM Forward Primer	0.5
20µM Reverse Primer	0.5
10µM Fluorescence Probe	0.4
Molecular H_2O	3.6
DNA template	5
Total volume	**20**

The master mix used for the probe assays was the LightCycler® 480 Probe Master Mix from Roche Life Science (Catalogue #04707494001). The primers targeted the BCG RD1 region, the human RNA polymerase A gene, and the bacterial 16S gene V3-V4 fragment with Taqman FAM probes selected to fit within each amplicon (Table 2.5). 15µL of the master mix and 5µL of template were added to wells of a 96-well LightCycler plate. The plate was then covered with a clear LightCycler foil, vortexed, and briefly spun down prior to insertion into the LightCycler® 480 system for analysis. The reactions were then amplified using the cycling conditions detailed below (Table 2.6).

Table 2.5: Primer/Probe sets selected for the qPCR amplification of three DNA targets

Amplification Target	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Fluorescence Probe (5'-3')**
BCG RD1 Region	AAGCGGTTGCCGCCGACCGACC	GAGGCGATCTGGCGGTTTGGGG	[6FAM]GTGCTTCTGGTCGACGATTG[BHQ1]
Human RNA Polymerase A	TGAAGCCGTGCGGAAGG	ACAAGAGAGCCAAGTGTCG	[6FAM]TACCACGTCATCTCCTTTGATGGCTCCTAT[BHQ1]
Bacterial 16S V3-V4 Fragment	CCTACGGGDGGCWGCA	GGACTACHVGGGTMTCTAATC	[6FAM]CAGCAGCCGCGGTA[BHQ1]

Table 2.6: Probe-based qPCR Cycling Conditions

Cycle Step	Time (mm:ss)	Temperature (°C)	Cycles (#)
Initial Denaturation	05:00	95	1
Denaturation	00:30	95	40
Annealing	00:30	55	
Extension	00:30	72	
Final Extension	05:00	72	1

2.5.3: Nested qPCR Amplification

Nested qPCR was used for the relative quantification of TB multiplex PCR products. Multiplex product DNA was diluted 1:100 with molecular H_2O and used as template for SYBR Green qPCR amplification. Amplification was performed as described in section 2.5.1.

2.5.4: qPCR Amplification Analysis

The qPCR data was analysed using the Abs Quant/2nd Derivative Max analysis program in the LightCycler® software which calculated the cycle threshold (C_T) and graphed the amplification curve for each sample. This C_T was then used to compare the relative starting concentration of DNA between samples. Assuming that the PCRs were efficient, template concentration doubles every cycle, hence the relative difference in starting concentration between 2 samples could be calculated using $2^{\Delta CT}$.

2.5.5: Melt Curve Analysis

Amplification by SYBR Green included a melt curve step for the identification of primer dimers or non-specific amplification. Melt curves were assessed visually for signs of secondary or tertiary peaks (at lower or higher melting temperatures than the expected amplicon peak) which would indicate the presence of non-target amplification or primer dimers (Figure 2.1).

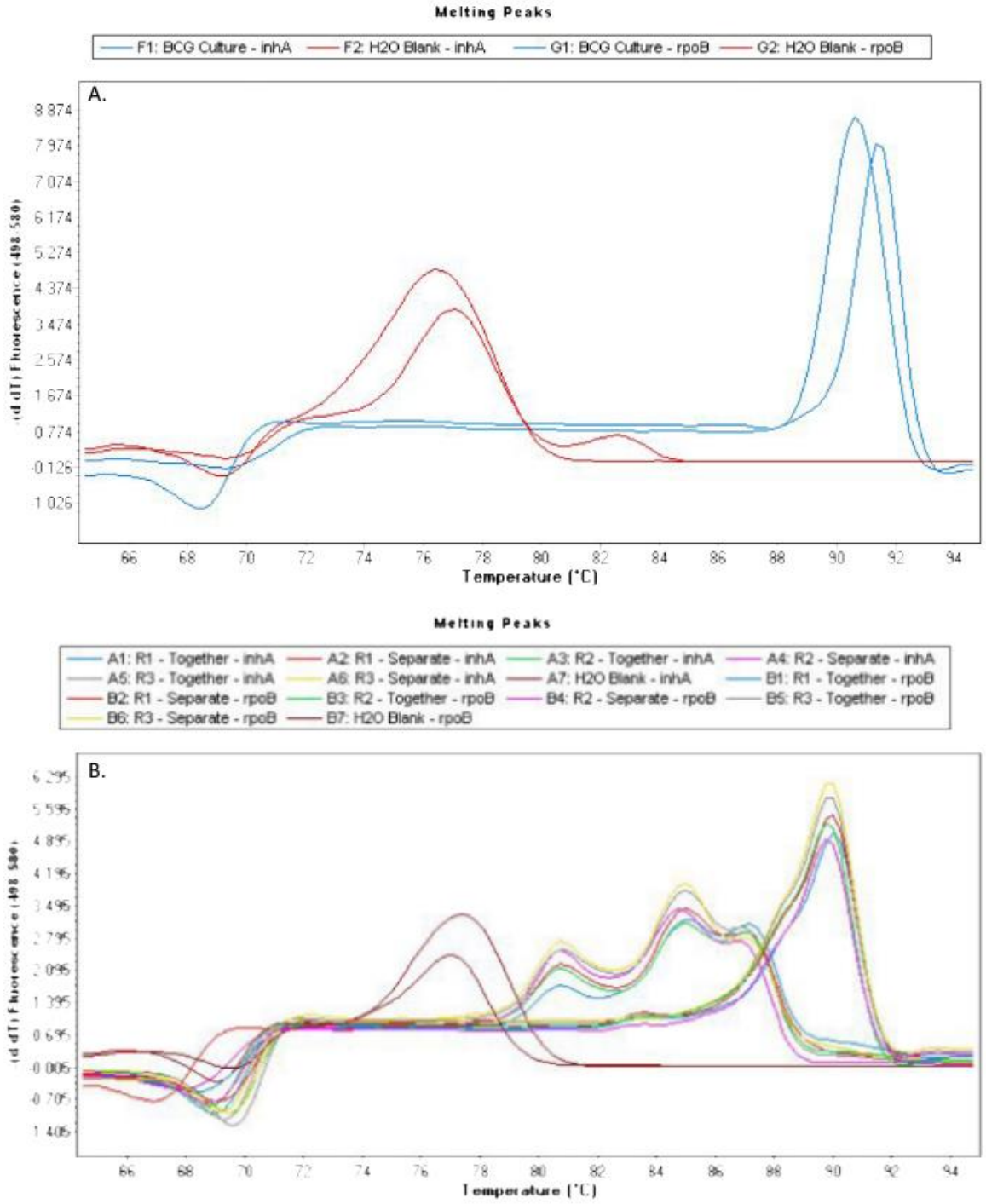

Figure 2.1: Example of qPCR melt curve analysis from two experiments. A: Clear difference between primer dimer peak in water controls and expected melt peak in BCG samples with no evidence of double peaks. B: Multiple samples exhibiting secondary and tertiary peaks

2.6: Fragment Size Analysis

Assessment of amplicon size was performed using the Tapestation 2200 platform [Agilent]. Analysis was performed using HS D5000 reagents [Catalogue #5067-5592] for improved resolution of amplicon sizes compared to Genomic DNA reagents. Electrophoresis followed manufacturer protocols and fragment size analysis was performed using the Agilent Tapestation native software.

2.7: Contrived Clinical Samples

Sputasol treated sputum samples determined to be free of respiratory pathogens (normal respiratory flora – NRF) at the NNUH Innovation Centre microbiology laboratory were used for all spiking experiments. Prior to use, all NRF sputum samples received on the same date were pooled and homogenized by vortexing for 5 minutes at maximum speed to thoroughly combine and homogenise the samples.

For spiking of samples, 900µL of homogenized NRF sputum was aliquoted into a clean 1.5mL Eppendorf tube. To this aliquot, 100µL of liquid culture of the desired microorganism and concentration was added to bring the total volume to 1mL. Spiked samples were then mixed thoroughly by vortexing for 30 seconds at medium speed before use in downstream experimentation. In the event a spiked sample would be used over multiple days it was stored at 4°C between uses.

2.8: Design of tNGS PCR Primers

PCR primers for tNGS were designed using Primer-BLAST software available from a collaboration between NCBI and Primer3 developers. Results on the specifics of primer design for assay targets is presented in Results section 3.2.2

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2.9: Nucleic Acid Host Depletion

Saponin-based host depletion methods were used to remove host DNA from contrived clinical samples prior to metagenomic sequencing. Initially, the depletion method described by Charalampous, et al [111] was used, followed by a further optimized one-pot saponin depletion method.

Both methods use Saponin as the active chemical agent, which works by creating pores in cellular membranes thereby exposing the DNA to enzymatic degradation. Saponin must be made fresh weekly and protected from light to prevent denaturation. For a 5% working solution of saponin, as required in the Charalampous method, 500mg of saponin was suspended in 10mL of PBS. This solution was mixed by vortexing and filtered through 0.22µM syringe filter prior to experimental use and storage. The optimized one-pot method required a 1% saponin working solution, made by suspending 100mg of saponin in 10mL of PBS. This working solution was then vortexed, however, no subsequent filtration was used before use or storage according to protocol.

In the depletion method published by Charalampous, et al., 200µL primary samples were centrifuged at 8,000g for 5 minutes to create a pellet. The supernatant was then carefully removed, leaving approximately 50µL around the pellet, before resuspending the pellet in 250µL of PBS. 200µL of 5% saponin was then added and the sample was briefly vortexed before being left to incubate at room temperature for 10 minutes. After incubation, 350µL of molecular grade H_2O was added, followed 30 seconds later by 12µL of a 5M NaCl solution. The sample was then vortexed and pelleted at 8,000g for 5 minutes. After pelleting, the supernatant was carefully removed, again leaving approximately 50µL around the pellet, and the sample was resuspended in 100µL PBS. 100µL of HL-SAN 5.5M buffer solution [5.5M NaCL and 100mM $MgCl_2$ in molecular H_2O], and 10µL of HL-SAN DNase were then added and the mixture was incubated on an Eppendorf ThermoMixer C at 37°C, 800rpm for 15 minutes. After incubation, 800µL of PBS was added and the sample was

pelleted in a centrifuge at 6,000g for 3 minutes. The pellet was then processed for DNA extraction as per section 2.2.

In the one-pot host depletion method 200µL of primary sample, 40µL of a 1% saponin solution, 200µL of a 5Mol HL-SAN buffer (5M NaCL and 100mM $MgCl_2$ in molecular H_2O), and 10µL of HL-SAN DNase were combined in a clean 1.5mL Eppendorf. This mixture was then incubated on an Eppendorf ThermoMixer C at 37°C and 1,000rpm for 10 minutes. Following incubation, 1mL of PBS was added to the sample and it was pelleted in a centrifuge at 12,000g for 3 minutes. The supernatant was then carefully removed from the pellet, leaving approximately 50µL around the pellet. As above, the pellet was then processed for DNA extraction as per section 2.3.

2.10: Sputum NaOH/NALC-Na Decontamination and Sedimentation

Sedimented sputum samples are commonly used in diagnostics laboratories for the investigation of TB infection. Spiked sputum samples underwent a decontamination protocol to prove the TB tNGS test would work on this sample type. The method chosen was published in the Stop TB Partnership Mycobacteriology Laboratory Manual [123].

This method used a combination of Sodium Hydroxide (NaOH), Sodium Citrate Dihydrate ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$), and NALC ($C_5H_9NO_3S$) solutions to render non-mycobacterial cells nonviable. A 6% stock solution of NaOH was made by suspending 30g of NaOH in 500mL of molecular grade H_2O. A 2.9% Na Citrate Dihydrate stock solution was also made by suspending 14.5g Na Citrate Dihydrate in 500mL of molecular grade H_2O. The working NaOH/NALC-Na Citrate digestant solution was mixed daily by combining equal volumes of 6% NaOH and 2.9% Na Citrate Dihydrate with a specific amount of NALC (ranging from 0.25-5g) according to Table 2.7.

Table 2.7: Preparation of NaOH/NALC-Na Citrate Digestant Solution

Volume of Digestant Needed (mL)	6% NaOH (mL)	2.9% Na Citrate Dihydrate (mL)	Amount of NALC to Add (g)
50	25	25	0.25
100	50	50	0.50
200	100	100	1.00
250	125	125	1.25
500	250	250	2.50
1,000	500	500	5.00

A neutralizing phosphate buffer solution was used to stop the decontamination reaction. This buffer was made by combining 7.1g Disodium Phosphate (Na_2HPO_4) and 6.8g Monopotassium Phosphate (KH_2PO_4) in 1.5L of molecular grade H_2O. This suspension was then autoclaved to ensure sterility and aliquoted into 50mL falcon tubes.

For decontamination, 250µL of spiked sputum and 250µL of digestant solution were combined in a clean 1.5mL Eppendorf. The samples were then vortexed at medium speed for 30 seconds before incubating for 15 minutes at room temperature on an orbital shaker at 500rpm. In the absence of a shaker plate for incubation, samples were vortexed at low speed for 10 seconds every two minutes to ensure thorough mixture. After incubation, the digestant reaction was neutralized by adding 1mL of phosphate buffer solution. The samples were then pelleted in a refrigerated centrifuge at 14,000g and 4°C for 5 minutes. The supernatant was carefully removed, leaving approximately 50µL around the pellet to prevent loss of sample. Finally, the pellet was resuspended in 700µL of PBS for immediate DNA extraction (as described in section 2.3).

2.11: Determining Analytical Limit-of-Detection

Analytical limit-of-detection (LoD) for metagenomic and targeted sequencing methods was determined using two methods, qPCR and MinION sequencing.

2.11.1: qPCR Determination of the Metagenomic LoD

For qPCR LoD experiments, a series of 10-fold serial dilutions were made from liquid *M. bovis* BCG culture stock. Serial dilutions from $1x10^6$ CFU/mL to $1x10^1$ CFU/mL were used to spike NRF sputum to create contrived clinical samples as described in section 2.6. After either host and/or commensal depletion the samples underwent SYBR Green qPCR amplification using the BCG RD1 primer pair described in section 2.4.

Using the Abs Quant/2^{nd} Derivative Max analysis software, the C_T of each sample was calculated. Any sample which failed to amplify prior to the included negative H_2O control was classified as a failure to detect. Also, a melt curve analysis was performed to identify amplification and detection due to primer dimers or non-specific amplification, determined by the existence of a secondary peak. Using these two analysis methods the lower bound of the LoD was identified for further investigation and optimization.

2.11.2: MinION Sequencing for Determination of the tNGS Drug Resistance Assay LoD

LoD determination by MinION sequencing used duplicate *M. tuberculosis* dilutions of 1000, 500, 100, 50 and 1 CFU/mL. Contrived samples underwent the tNGS multiplex protocol and were prepared for ligation-based MinION sequencing (section 2.13). Sequencing was performed for 3 hours with live-basecalling, after which all reads were uploaded to the ONT Epi2Me FASTQ TB Resistance Profile for analysis and interpretation. Successful detection was classified as a read depth of at least 50x for all gene targets as determined by the Epi2Me software.

2.12: Multiplex PCR Using Qiagen Kit

Multiplex groups for the tNGS assay were amplified using an Applied Biosystems™ MiniAmp™ thermal cycler (Catalogue #15856152). Reactions were prepared in the QIB Extra laboratory as a separate clean environment where no Mycobacterium DNA had been used. The multiplex reactions were prepared in 0.2mL thin-walled PCR tubes or plates, depending on the number of samples, as described in Table 2.8. Template DNA was added in a separate room (the QIB Tissue Processing

laboratory) before immediately sealing the samples. Sealed samples were then mixed by vortex before being briefly spun down in a benchtop centrifuge. The mixed reactions were then placed into a thermal cycler for amplification under the cycling conditions in Table 2.9.

Table 2.8: Multiplex group amplification reaction formula per sample

Reagent	**Volume per Sample (µL)**
2x Qiagen Multiplex Master Mix	25
10x Primer Mix (0.2µM per primer)	5
5x Qiagen Q-Solution	10
Nuclease-Free H_2O	5
Template DNA	5
Total volume	**50**

Table 2.9: Multiplex group amplification cycling conditions

Step	**Time (mm:ss)**	**Temperature (°C)**	**# of Cycles**
Heat Activation	20:00	95	1
Denaturation	00:30	94	35
Annealing	01:30	60	
Extension	01:30	72	
Final Extension	10:00	72	1
Hold	∞	4	1

2.13: DNA Purification and Concentration with AMPure XP Beads

During sequencing library preparation samples underwent repeated Ampure XP bead washes in different concentrations (Table 2.10).

Table 2.10: Example table of AMPure XP Beads Used for Different Bead Wash Concentrations

Bead Wash Concentration	**Volume of Beads to Add (µL/100µL of Sample)**
1x	100
0.8x	80
0.6x	60
0.4x	40

The required volume of resuspended AMPure XP beads at room temperature were added to the sample. The samples were then incubated at room temperature for 5 minutes before being placed onto a magnetic rack for 2 minutes. Following magnetic pelleting, the supernatant was then carefully removed, and the bead pellet was washed with 500µL of 70% ethanol. After 30 seconds the ethanol was carefully removed, and the pellet was washed with a further 500µL of 70% ethanol. Again, the supernatant was carefully removed, and any residual supernatant was removed using a p10 pipette. The pellet was left to air dry for approximately 30 seconds, being careful not to let the pellet dry too much and crack, before being removed from the magnetic rack and resuspended in the desired volume. This suspension was then incubated at room temperature for 5 minutes before being returned to the magnetic rack and left to pellet for a further 2 minutes.

DNA was resuspended in either molecular H_2O or elution buffer (EB) during the final library preparation step. Likewise, elution volume was variable dependent on requirements.

2.14: MinION Library Preparation

For sequencing of tNGS assay amplicons, multiplex groups were pooled after amplification to a total of ~1ug in 100ul (10ng/ul). MinION sequencing was then performed with the library preparation method depending on the application (metagenomic or tNGS). The release of a new sequencing kit by ONT before validation of the tNGS assay also led to a change in the library prep kit used (Figure 2.2). Metagenomic sequencing libraries were barcoded using the ONT PCR barcoding kit [Catalogue

#SQK-RPB004]. Meanwhile, tNGS sequencing libraries were originally barcoded using the ONT PCR 96-Expansion barcoding kit [Catalogue #EXP-PBC096] and later the ONT Native barcoding kit after the release of the native barcoding 96-expansion [Catalogue #EXP-NBD196]. All sequencing libraries were made using the ONT Ligation Sequencing Kit [Catalogue #SQK-LSK109].

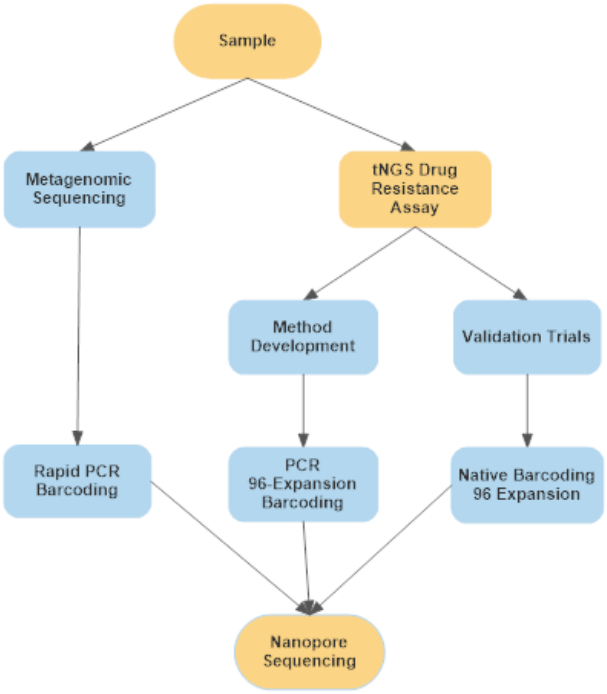

Figure 2.2: Flowchart illustrating the usage of three nanopore sequencing library barcoding methods depending on the sequencing purpose

2.14.1: Rapid PCR Barcoding Kit Library Preparation for Metagenomics

The PCR barcoding kit protocol used to prepare metagenomic samples was altered from manufacturer protocols to improve performance with respiratory samples. To start, 30µL of eluted DNA from the automated extraction was aliquoted into a fresh 1.5mL LoBind Eppendorf tube. A 1.2x bead wash (as per section 2.12) was then performed and the sample resuspended in 16µL molecular H2O for elution. Following magnetic pelleting, 15µL of elute was carefully removed and retained for library preparation.

To prepare samples for sequencing using the PCR barcoding kit, 7.5µL of template DNA and 2.5µL of fragmentation mix (FRM) were combined in 0.2mL thin-walled PCR tubes. The tubes were then

gently mixed and spun down to remove air bubbles prior to incubating for 1 minute at 30°C and 1 minute at 80°C. Following this incubation, samples were cooled at 4°C in the PCR machine for approximately 1 minute. Next, 38µL of nuclease-free H_2O, 2µL of PCR barcode (RLB), and 50µL of LongAmp Taq 2x Master Mix (NEB) [Catalogue #M0287L] were added to each sample bringing the total volume of each to 100µL. The samples were then briefly vortexed and spun down before amplifying under the following conditions (Table 2.11).

Table 2.11: Cycling conditions for ONT PCR Barcoding

Step	**Time (mm:ss)**	**Temperature (°C)**	**# of Cycles**
Initial Denaturation	03:00	95	1
Denaturation	00:15	95	25
Annealing	00:15	56	
Extension	04:00	65	
Final Extension	06:00	65	1
Hold	∞	4	1

After tagmentation of adapters and barcodes followed by PCR amplification of the library, all samples were quantified using the qubit, as described in section 2.3, and pooled equimolar in a fresh 1.5mL Eppendorf. The pooled sample then underwent a 0.6x bead wash with elution in 14µL MinION Buffer (10mM Tris-HCl pH 8.0 with 50mM NaCl). 13µL of this elute was transferred to a fresh 1.5mL Eppendorf for QC and flow cell loading (section 2.14).

2.14.2: LSK109 Ligation with PCR Barcoding Expansion

Library preparation of tNGS samples initially used the ONT LSK109 ligation kit with PCR Barcoding Expansion 1-96 for higher throughput. To begin, 45µL of the pooled 100µL template DNA for each sample was combined in a 0.2mL thin-walled PCR tube with 7µL of Ultra II End-Prep Buffer [NEB], 3µL Ultra II End-Prep Enzyme Mix [NEB] (Catalogue #E7546L), and 5µL of molecular grade H_2O for a

total reaction volume of 60µL. These samples were pipette mixed prior to being briefly spun down and incubated for 5 minutes at 20°C followed by 5 minutes at 65°C. Each sample was then transferred to its own 1.5mL Eppendorf for a 1x bead wash. Samples were resuspended in 31µL molecular H_2O before retaining 30µL of the elute for barcode adapter ligation.

Following on, samples underwent barcode adapter ligation by combining 30µL of end-prepped elute from the previous step with 20µL of barcode adapter (BCA) [ONT] and 50µL of Blunt/TA Ligase Master Mix (NEB) [Catalogue #M0367L]. Samples were mixed by pipetting and briefly spun down prior to a 10-minute incubation at room temperature. After incubation, a 0.8x bead wash was performed and samples were eluted in 25µL molecular H_2O. The elute was carefully removed after magnetic pelleting and 1µL of each was quantified by Qubit to allow dilution of samples to 10ng/µL prior to the barcoding PCR.

To attach the PCR barcodes to each sample, reactions were prepared in 0.2mL thin-walled PCR tubes in the following concentrations (Table 2.12). The samples were briefly mixed by vortexing and pulse centrifuged followed by amplification under the cycling conditions in Table 2.13.

Table 2.12: PCR Barcoding 96-Expansion reaction reagent concentrations

Reagent	**Volume (µL)**
10µM PCR Barcode	1
10ng/µL Adapter Ligated Template DNA	2
LongAmp Taq 2x Master Mix	25
Nuclease-Free H_2O	22
Total volume	**50**

Table 2.13: PCR Barcoding 96-Expansion cycling conditions

Step	**Time (mm:ss)**	**Temperature (°C)**	**# of Cycles**
Initial Denaturation	03:00	95	1
Denaturation	00:15	95	15
Annealing	00:15	62	
Extension	01:30	65	
Final Extension	05:00	65	1
Hold	∞	4	1

After the barcoding amplification was completed, samples were quantified by Qubit before being pooled equimolar in a clean 1.5mL Eppendorf. A 0.8x wash was then performed on the pooled, barcoded, samples with the washed product being resuspended in 48µL molecular grade H_2O.

45µL of elute was transferred to a new 0.2mL Eppendorf for End-Prep.7µL Ultra II End-Prep Buffer, 3µL Ultra II End-Prep Enzyme Mix, and 5µL molecular grade H_2O were then added for a total reaction volume of 60µL. The sample was then briefly mixed by vortexing before being pulse centrifuged. Following this, the sample was incubated in a thermal cycler at 20°C for 5 minutes followed by 65°C for 5 minutes. The end-prepped sample was then transferred to a clean 1.5mL Eppendorf where it underwent a 0.8x bead wash after which the sample was eluted into 61µL of molecular H_2O.

For ligation of the sequencing adapter Quick T4 Ligase [NEB #M2200L], adapter mix (AMX) [ONT], ligation buffer (LNB) [ONT], elution buffer (EB) [ONT], and short fragment buffer (SFB) [ONT] were all thawed, vortexed, spun down, and stored on ice. For the ligation reaction, 60µL of end-prepped DNA had 25µL LNB, 10µL quick T4 DNA ligase, and 5µL AMX added to it creating a total reaction volume of 100µL. This reaction was gently mixed by flicking and then pulse centrifuged prior to incubating at room temperature for 10 minutes. Following incubation, a 0.4x bead wash was performed with the use of 125µL SFB resuspension washes instead of 70% Ethanol. The sample was

finally resuspended in 15µL EB for elution, all of which was carefully retained for use in flow cell loading (section 2.14).

2.14.3: Native Barcoding 96 Expansion Kit Library Preparation

On the 21st of May, 2020, ONT released the Native Barcoding 96-Expansion [ONT #EXP-NBD196] removing the need for PCR barcoding. This method was then used as the barcoding method of choice for tNGS samples.

To begin, 12.5µL (approximately 125ng) of DNA was aliquoted from each sample into thin-walled PCR plate wells. An end-prep solution was then mixed using 1.75µL Ultra II End-Prep Buffer and 0.75µL Ultra II End-Prep Enzyme Mix per sample. 2.5µL of this mix was then aliquoted to each sample well before sealing the plate, vortexing it, and briefly spinning the plate down in a benchtop centrifuge. The mixed plate was then incubated in a thermal mixer at 20°C for 5 minutes followed by 65°C for 5 minutes.

The end-prepped samples then had a barcode ligated directly on. 0.75µL of end-prepped DNA from each sample was transferred to a clean thin-walled 96-well PCR plate. Each sample then had 3µL molecular grade H_2O, 1.25µL native barcode [ONT], and 5µL Blunt/TA Ligase master mix [NEB] added, in order. The plate was then sealed with foil, vortexed, spun down in a benchtop centrifuge, and was incubated in a thermal cycler at 20°C for 20 minutes followed by 65°C for 10 minutes. After this incubation, all samples were pooled in a clean 1.5mL Eppendorf tube and, when running 96 samples, 480µL of the pooled, barcoded, DNA was aliquoted into another 1.5mL Eppendorf. This aliquot then underwent a 0.4x bead wash with two resuspension washes in 700µL of SFB. The sample was then eluted into 35µL of molecular H_2O once the elute was again clear and colourless, 35µL was removed and retained in a fresh 1.5mL Eppendorf for adapter ligation.

The final step prior to loading is ligation of the sequencing adapter to the template DNA. To perform this step Quick Ligation Reaction Buffer [NEB], Quick T4 DNA Ligase [NEB], adapter mix (AMII)

[ONT], EB [ONT], and SFB [ONT] were thawed, mixed, briefly spun down, and stored on ice. The reaction was then prepared in a 1.5mL Eppendorf by adding the following reagents in order; 30µL pooled, barcoded, template DNA, 10µL Quick Ligation Reaction Buffer, 5µL Quick T4 DNA ligase, and 5µL AMII. The reaction was mixed by vortexing and pulse centrifuged before incubating at room temperature for 20 minutes. Following incubation, a 0.6x bead wash was performed with resuspension washes in 125µL SFB. After washes, the pellet was resuspended in 15µL EB and the clear elute was carefully retained for flow cell loading (section 2.15).

2.15: MinION Loading

The MinION flow cell was loaded according to manufacturer protocols using the Flow Cell Priming Kit (ONT) [Catalogue #EXP-FLP002]. The prepared library was quantified by Qubit using the method detailed in section 2.4 and the number of fmols/µL was calculated for loading using the online tool available at https://www.bioline.com/media/calculator/01_07.html. Optimally, between 100 and 200 fmols were loaded.

2.16: Sequencing Analysis

Drug resistance in samples was identified through the ONT Epi2Me FastQ TB Resistance Profile pipeline. Sequencing fastQ reads were uploaded using the ONT desktop agent available at http://epi2me.nanoporetech.com. Using fastQ data, the pipeline identifies genes and SNPs responsible for drug resistance against a curated database. After automated analysis through this pipeline, results were obtained from the ONT Epi2Me website and visual examination of wild-type and mutant reads was conducted to determine resistance to drugs and the existence of hetero-resistant samples (Figure 2.3). This pipeline is now discontinued; however, a similar method is currently available in Epi2Me Labs.

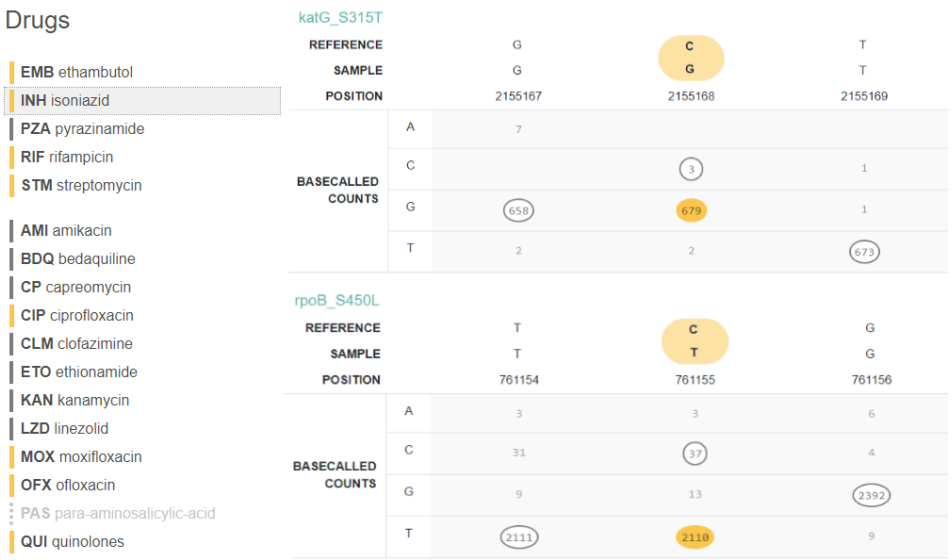

Figure 2.3: Example Epi2Me TB Resistance Pipeline Output for Resistant Samples

For QC, reads were also mapped to a concatenated fastA reference of assay target sequences using MiniMap2 and Qualimap (Figure 2.4). Visual examination for equal coverage of targets, dropouts, and coverage greater than 50x was performed using this mapping method.

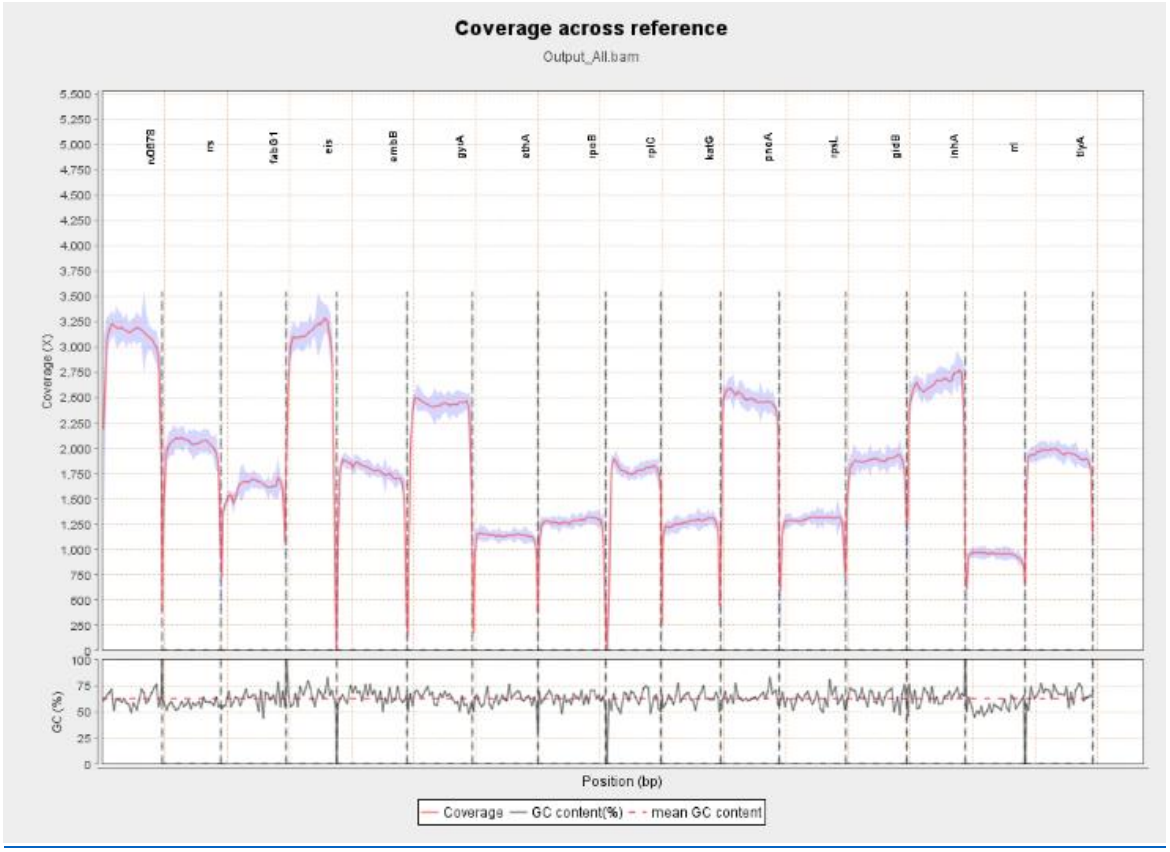

Figure 2.4: Example output of sequencing reads mapped to a concatenated assay reference for visualization of coverage in samples.

Chapter 3 – Results and Discussion

With the global incidence of drug-resistant TB increasing (from 5% in 2008 to ~12.5% in 2020) the need for rapid and accurate DST methods is increasing in urgency [34,124]. Molecular diagnostic technologies provide the speed, accuracy, and cost-effectiveness to meet this need. Earlier molecular diagnostic tools such as Line Probe Assays (MTBDRplus and MTBDRsl) have narrow foci and limited flexibility inhibiting long-term viability. While PCR based tests like Xpert MTB/RIF and MTB/RIF Ultra also have narrow DST foci their performance as fast and accurate first-line tests will make them important in TB control for the foreseeable future.

To compensate for limitations in DST coverage the use of NGS has increased the breadth of investigation and the flexibility to detect new SNPs. Specifically, three avenues of sequencing and analysis for DST have come to the fore; whole genome sequencing (which requires cultured TB), targeted sequencing (which utilizes PCR amplification of target regions) and metagenomic sequencing (which sequences whole genomes directly from sample extractions). While WGS is a powerful tool for epidemiology, the reliance on *M. tuberculosis* culture makes it too slow to be useful clinically. The remaining two avenues were investigated throughout this research with the most viable being identified as tNGS due to the ability to specifically amplify MTBC DNA even in the presence of commensal bacterial DNA and host DNA.

The aim of the study was to develop rapid and sensitive diagnostic tests for TB. The key to this is efficient DNA extraction from primary samples. This took multiple steps from optimisation of DNA extraction to design of targeted primers and troubleshooting the bioinformatic pipeline.

3.1: Comparison of Extraction and Purification Methods for Optimization of Mycobacterial DNA Yields

Firstly, molecular methods require effective sample preparation to isolate pathogen nucleic acid in replacement of culture for successful utilization. Consequently, metagenomic and tNGS methods are reliant on efficient extraction of mycobacterial DNA. Metagenomic assays are more sensitive to

issues of extraction than tNGS, as they sequence extracted DNA directly without enrichment. Thus, if extraction is inefficient, clinical applications are limited by a decreased limit of detection. However, tNGS assays amplify target DNA prior to sequencing, reducing the impact of extraction inefficiency and competing commensal and human nucleic acid on the clinical utility of an assay.

Due to the correlation of DNA extraction to LoD, optimising extraction is essential. Generally, extraction efficiency of mycobacteria using standard methods is estimated at 30-50%, due to the hardiness of the cells [125,126]. However, recent studies have demonstrated extraction efficiencies of 75-90% are possible under optimised conditions [127].

3.1.1: DNA Purification Method Comparison

The MagNA Pure Compact automated extraction system was compared with the MagMAX manual extraction method for purifying bead-beaten samples. Testing was performed on *M. smegmatis* (*M. smegmatis* was used for extraction optimization as it is non-pathogenic, fast growing, and structurally similar to *M. tb*[128]) liquid culture aliquots. Dual sets of triplicate 175µL samples of overnight *M. smegmatis* culture were aliquoted for each method. Following mechanical lysis (Methods section 2.3) samples were extracted following manufacturer specifications for each method. Extractions were quantified by high sensitivity Qubit (section 2.4.1), showing a concentration ~2-fold higher from MagNA Pure compared to MagMAX (Table 3.1).

Table 3.1: Qubit quantification comparing nucleic acid extraction methods using duplicate sample sets, MagMax and MagNA Pure Compact

Extraction Method	**Replicate 1 Mean Concentration (ng/µL)**	**Replicate 2 Mean Concentration (ng/µL)**	**Replicate 3 Mean Concentration (ng/µL)**
MagMax	0.162	0.136	0.146
MagNA Pure Compact	0.234	0.230	0.230

A second test compared the MagNA Pure Compact system against the Promega Maxwell RSC system. Two triplicate 500µL *M. smegmatis* liquid culture aliquots were prepared and bead beaten

(Methods section 2.3). Analysis indicated the MagNA Pure system yielded a statistically significant (Paired T-Test: $p<0.0001$) average of 5.4 fold more nucleic acid than the Promega Maxwell (Table 3.2).

Table 3.2: Qubit quantification comparing nucleic acid extraction methods for triplicate samples, MagNA Pure Compact and Promega Maxwell RSC

Extraction Method	**Mean Qubit DNA Concentration (ng/µL)**
MagNA Pure Compact	8.58
Promega Maxwell RSC	1.58

MagNA Pure was subsequently tested with increasing bead-beating times to ascertain which yielded the highest concentration of nucleic acids. Triplicate liquid *M. smegmatis* culture samples were bead beaten for 5 minutes, 10 minutes, and 15 minutes at maximum Qiagen TissueLyser LT speed (~6m/s). A linear increase in nucleic acid yield was observed with increasing mechanical lysis time (Figure 3.1).

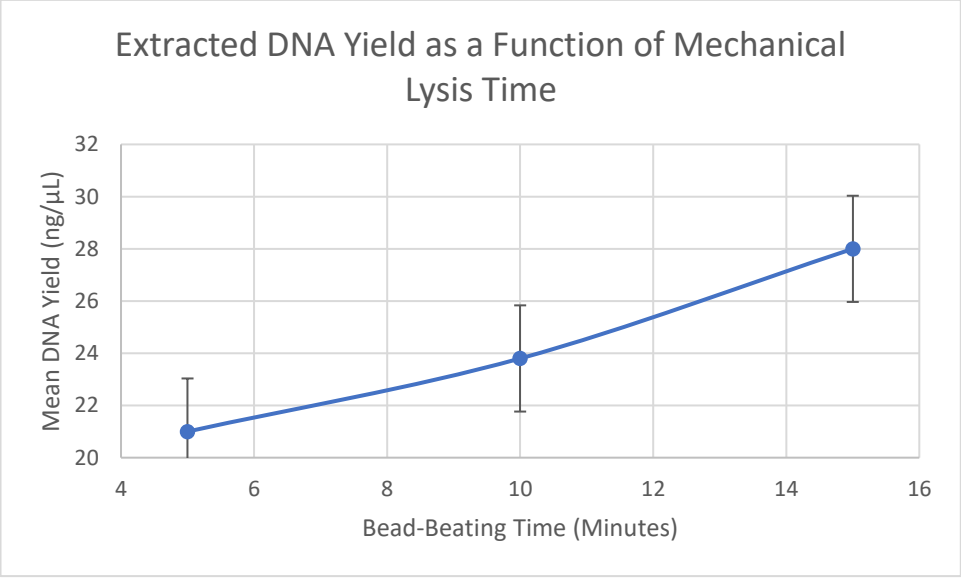

Figure 3.1: Line chart showing the increase in mean DNA yield by MagNA Pure extraction as a function of mechanical lysis time using triplicate samples. Error bars indicate one standard deviation

Different bead beaters were then assessed, in conjunction with MagNA Pure extraction. Two 1mL sets of liquid overnight *M. smegmatis* culture were prepared - one set underwent 15 minutes full-speed bead-beating in a Qiagen TissueLyser and the other underwent two 45-second full-speed cycles in an MP Biomedicals Fast-Prep 24, before extraction by MagNA Pure Compact. Results

showed 15-minute lysis in the Qiagen machine yielded a mean 24.6ng/µL DNA and two 45-second cycles in the MP Biomedicals machine yielded a mean 29.4ng/µL DNA (Table 3.3). This was an increase of 4.8ng/µL using the MP Biomedicals device with a shorter protocol. The MP Biomedicals FastPrep-24 method was chosen for subsequent extractions.

Table 3.3: Post-extraction DNA concentrations after using two mechanical cell disruption protocols.

	Extracted DNA Concentraiton (ng/µL)
Qiagen TissueLyser	24.6
MP Biomedicals Fast-Prep 24	29.4

3.1.2: Importance of Automated Extraction

For a test to be adopted for routine use in a clinical setting an automated extraction system is optimal; as automated sample preparation increases throughput while reducing the risk of contamination or human error. Thus, the optimal preparation for extraction in automated systems needed to be identified as automated systems allow little internal optimization. Emphasis was placed on mechanical lysis pre-extraction, as the primary impediment to efficient automated mycobacterial DNA extraction is the toughness of the cells [129,130].

The automated methods tested were selected based on availability and use in previous studies of respiratory and systemic infections. The MagNA Pure Compact was released in the early 2000s and was adopted as a rapid and easy means of nucleic acid isolation [131]. Preliminary results were inconsistent and one early study cited a reduction in PCR sensitivity when using nucleic acids isolated by this means [132]. Further, the MagNA Pure Compact system can only extract 8 samples per run. Despite these limitations MagNA Pure Compact automated kits have been used for numerous studies of respiratory diseases using both targeted and metagenomic methodologies [111,133,134] and so was the extraction machine of choice at the beginning of the study. Future use of this system will be limited, however, due to discontinuation of the line and support by Roche.

Comparatively, the Maxwell RSC 48 system is capable of processing 48 samples concurrently, increasing utility in high-throughput studies. There are numerous automated kits, optimized by the manufacturer for extraction of DNA and RNA from different sample types [135]. During the ongoing SARS-CoV-2 pandemic, the Maxwell RSC has been heavily used for RNA extraction from patient samples and waste water for diagnostics and monitoring in multiple laboratories [136–139]. The discontinuation of the MagNA Pure forced a switch in extraction technology during the study.

3.1.3: Comparison of Bead Beating Matrices for DNA Extraction

We compared bead-beating matrices from MP Biomedical for the mechanical disruption of the *M. tb* cell wall. Matrices were selected based on manufacturer's descriptions of organisms/sample types they are optimized for. This selection process identified seven matrices for comparison to Matrix E, the standard used in the Justin O'Grady (JOG) laboratory (Table 3.4).

Table 3.4: Bead-beating matrices chosen for comparison of mycobacterial cell lysis efficiency and their material compositions

Bead-Beating Matrix	Matrix Components
A	Garnet matrix with ¼" Ceramic Sphere
B	0.1mm Silica Spheres
C	1mm Silica Spheres
E*	1.4mm Ceramic Spheres, 0.1mm Silica Spheres, 4mm Glass Bead
G	1.6mm Silicon Carbide Particles and 2mm Glass Beads
K	0.8mm Zirconium Silicate Beads
Y	0.5mm Yttria-Stabilized Zirconium Oxide Beads

*The bead-beating matrix regularly used in the JOG laboratory group

Two triplicate (~10^4 CFU/mL) sets were prepared and processed in two different bead-beating machines, the Qiagen TissueLyser and the MP Biomedical FastPrep. Aliquoted samples were subjected to mechanical lysis for either 15 minutes in the TissueLyser or two cycles of 45 seconds in the FastPrep prior to further extraction and clean-up on the MagNA Pure Compact. Qubit quantification results indicated matrix Y was superior to matrix E by a mean of 14ng/µL (Table 3.5).

However, assessment by *M. smegmatis* Sybr green qPCR showed that matrix E and Y were equally efficient in extracting *M. smegmatis* DNA.

Table 3.5: DNA quantifications by Qubit and qPCR from various bead-beating matrices on two homogenizers, the MP Biomedicals FastPrep-24 and the Qiagen TissueLyser.

Bead-Beating Matrix	**FastPrep Mean DNA Concentration (ng/µL)**	**TissueLyser Mean DNA Concentration (ng/µL)**	**FastPrep SYBR Green qPCR ($\bar{x}$ CT)**	**TissueLyser SYBR Green qPCR ($\bar{x}$ CT)**
A	23.6	5.58	16.36	18.19
B	22.6	4.84	17.29	19.91
C	25.6	19.1	16.92	17.82
E	32.6	19.8	16.58	17.55
G	17.7	15.5	16.57	17.77
K	28.2	23.6	16.79	17.59
Y	38.8	41.6	16.82	17.33

A follow-up experiment retested matrix E and matrix Y. Triplicate 500µL overnight *M. smegmatis* culture samples (~10^5 CFU/mL) were prepared and bead beaten on the two machines as described above. Quantifications indicated the difference between Matrix Y and Matrix E was not consistent (Table 3.6) And that the high concentrations observed in Qubit were not DNA, but perhaps RNA or protein, and these may have inhibited the qPCR results in the FastPrep sample.

Table 3.6: DNA quantifications by Qubit and qPCR of two MP Biomedicals bead-beating matrices on two homogenizers, the MP Biomedicals FastPrep-24 and the Qiagen TissueLyser using triplicate samples.

Bead-Beating Matrix	**FastPrep Mean DNA Concentration (ng/µL)**	**TissueLyser Mean DNA Concentration (ng/µL)**	**FastPrep SYBR Green qPCR ($\bar{x}$ CT)**	**TissueLyser SYBR Green qPCR ($\bar{x}$ CT)**
E	80.0	23.6	18.89	17.27
Y	37.0	25.4	16.41	17.34

One further experiment was performed using samples containing human and commensal bacterial cells. Normal respiratory flora (NRF) samples were spiked with *M. smegmatis* DNA (Section 2.7) to create contrived clinical samples. Matrices E, K, and Y were tested in triplicate, using the FastPrep-24 method, to determine relative lysing and extraction efficiencies in the presence of non-mycobacterial cells and sputum matrix. Matrix E was superior for DNA yield (≥0.14ng/µL improvement) and comparative concentration (mean ΔCT = ≥0.48, 1.48-fold increase) (Table 3.7). On this evidence, lysis matrix E was chosen for subsequent extractions.

Table 3.7: DNA quantifications by Qubit and qPCR of spiked NRF sputum lysed in three MP Biomedicals bead-beating matrices using triplicate samples

Bead-Beating Matrix	**Qubit Mean DNA Concentration (ng/µL)**	**SYBR Green *M. smegmatis* Assay ($\bar{x}$ C_T)**
E	8.30	19.63
K	6.50	20.51
Y	8.16	20.11
Negative Control	Too Low	35.00

3.1.4: Mechanical Disruption Optimisation Summary

As seen in section 3.1.1, without pretreatment neither automated method performed efficiently on mycobacterial cells. Incorporating bead-beating prior to automated extraction significantly increases nucleic acid yield [140,141]. A previous study run in the O'Grady laboratory, INHALE, indicated that MP Biomedicals lysis matrix E performed optimally for respiratory samples [113]. However, as the INHALE study focused on lysis and identification of pneumonia pathogens, efficiency for MTBC extraction could not be assumed.

The proprietary oscillating technology of MP Biomedicals FastPrep proved more efficient for cellular disruption than technology used by other bead beating devices (section 3.1.1). When paired with lysing matrix optimisation (section 3.1.3) this proved an effective method for rupturing the lipid-

rich cell wall of mycobacterial cells for DNA extraction. Reviewing the literature also showed bead-beating is consistently the most efficient means of mycobacterial lysis, over chemical lysis or enzymatic extraction [125,127,140]. Lysing matrix E, designed for environmental samples, performed optimally for mycobacterial lysis over matrices explicitly designed for other hardy organisms such as yeast and fungi.

3.2: Targeted Next-Generation Sequencing

Design and testing of the tNGS assay began with selection of target genes and concluded with validation and troubleshooting using a set of 392 blinded samples (Figure 3.2). Redesign of gene target primer pairs and formulation of multiplex groups occurred in parallel with redesign of one frequently dictating a redesign of the other. Despite the complex nature of this design process the end product resulted in a highly sensitive and specific assay with the potential for implementation in clinical conditions.

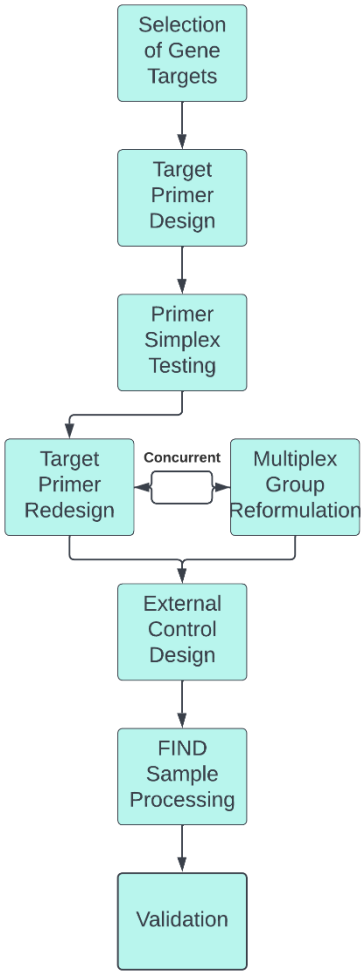

Figure 3.2: A flowchart illustrating the general progression and timeline of development for the tNGS assay. As indicated target primer redesign and multiplex group reformulation were an iterative process occurring concurrently. When both were performing to specifications development moved on and incorporated external controls.

3.2.1: Selection of Resistance Associated Mutations for Development of a tNGS Test for Drug-Resistant TB

Selection of gene targets for a tNGS drug resistance assay was predicated on a publication available from the WHO, as well as a systematic review by Miotto, *et al* [142,143]. These reports identified high, medium, and low confidence mutations in 12 genes when calculating odds-ratios (OR) of phenotypic resistance, accounting for resistance to 9 drugs (Table 3.8). High confidence mutations were defined by Miotto, *et al* as having an OR higher than 10, medium confidence mutations had

an OR 5< … ≤10, and low confidence mutations had an OR 1< … ≤5. Additionally, 4 gene targets for 4 more anti-tuberculosis drugs were identified (Table 3.9) from the Deeplex MYC-TB (GenoScreen, France) test and a study by Zhao, et al. and included in the assay design [116,144].

Table 3.8: Anti-tuberculosis drugs and the genes which are known to harbor resistance mutations as informed by two international studies

Drug	**Gene Target**	**Source**
Isoniazid	*inhA*	WHO & Miotto, et al.
	katG	WHO & Miotto, et al.
	fabG1	WHO
Rifampicin	*rpoB*	WHO & Miotto, et al.
Fluoroquinolones	*gyrA*	WHO & Miotto, et al.
Pyrazinamide	*pncA*	WHO & Miotto, et al.
Amikacin	*rrs*	WHO & Miotto, et al.
	eis	WHO
Kanamycin	*rrs*	WHO & Miotto, et al.
	eis	WHO & Miotto, et al.
Capreomycin	*rrs*	WHO & Miotto, et al.
	tlyA	WHO & Miotto, et al.
Ethionamide	*ethA*	Miotto, et al.
Streptomycin	*rpsL*	Miotto, et al.
	rrs	Miotto, et al.
	gidB	Miotto, et al.

Table 3.9: Anti-tuberculosis drugs and the genes which are known to harbour resistance mutations as informed by existing literature

Drug	**Gene Target**	**Source**
Ethambutol	*embB*	Zhao, et al.
Bedaquiline	*rv0678*	Villellas, *et al.,* Andries, *et al.*, & Ismail, *et al.*
Clofazamine	*rv0678*	Villellas, *et al.,* Andries, *et al.*, & Ismail, *et al.*
Linezolid	*rrl*	Wasserman, *et al.*
	rplC	Beckert, *et al.,* & Wasserman, *et al.*

Using this list of genes, the regions required to cover the mutations of interest were identified. To this end, a comprehensive list of high and medium confidence mutations within each gene was created. On review, it was decided to focus assay design to capture high confidence mutations and include medium confidence mutations where possible. This list included both amino acid mutations within genes and single nucleotide mutations outside genes and totaled 448 individual mutations (Appendix I). The largest proportion of mutations occurred within the *pncA* gene (37.3%), the second highest proportion within the *rpoB* gene (10%), and the lowest proportion within the *rplC* gene (0.002%).

3.2.1.1: Assay Target Selection Sources

As referenced in section 3.2.1, the primary source for selection of assay targets was a systematic review conducted by the WHO [142]. This was primarily augmented by a second systematic review by Miotto, *et al.* recommended by collaborators at FIND [143]. In addition, several non-WHO reviewed targets were included in assay formulation for newer drugs.

The primary WHO source provided gene targets for resistance to rifampicin, isoniazid, fluoroquinolones, pyrazinamide, amikacin, capreomycin, and kanamycin. The Miotto report

reviewed gene targets for resistance to ethionamide and streptomycin in addition to supporting the findings in the WHO report. In total these two reports provided confidence estimates for SNPs in 12 genes with strong correlations to drug resistance. It is of interest to note that despite its role as a first-line anti-tuberculous medication there were no SNPs or genes for ethambutol covered in either the WHO or Miotto reports.

Selection of ethambutol resistance conferring gene targets was instead supported by a 2015 study by Zhao, *et al*. investigating the embCAB genes [144]. This report found little correlation between ethambutol resistance and mutations in *embA* or *embC* genes, however, it did find a strong correlation between ethambutol resistance and SNPs within *embB*.

Finally, coverage of genes correlated with resistance to bedaquiline and clofazimine were informed by studies by Andries, *et al,* Villellas, *et al,* and Ismail, *et al* [145–147] . Genes correlated with linezolid resistance were informed by two studies; one by Beckert, *et al.* in 2012 and one by Wasserman, *et al.* in 2019 [148,149]. The assay covers three genes associated with resistance to these antibiotics, one for bedaquiline and clofazimine and two for linezolid. With these drugs included, gene targets for the assay were set and the SNPs requiring coverage were catalogued.

3.2.1.2: Target SNP Selection

The principal document used for SNP identification, as with gene selection, was the 2018 WHO/FIND report on sequencing technology for detection of drug resistance [142]. This systematic review of MTBC mutation publications included analysis utilizing the global ReSeqTB Data Sharing Platform. This analysis used a consensus approach to grade drug-resistance associated SNPs into high, medium, and low confidence using likelihood and odds ratios. Similarly, the systematic review of 52 MTBC sequencing and DST studies by Miotto, *et al.* was further used to identify resistance conferring SNPs. As with the WHO/FIND report, these SNPS were graded into high, medium, and low confidence.

Resistance associated SNPs in *embB* were catalogued by Sreevatsan, *et al*. in a 1997 study , Plinke, *et al.* in a 2006 study , and Zhao, *et al*., in a 2015 study [144,150,151]. Each study performed phenotypic DST testing before sequencing to identify SNPs in *embB* correlated with ethambutol resistance. Resistance conferring SNPs for bedaquiline and clofazimine associated gene *rv0678* were identified using an analysis of 359 clinical isolates by Villellas, *et al* [145]. This analysis identified minimum inhibition concentrations (MICs) above clinical dosages, indicating drug resistance, occurred in 2.3% of isolates. In these resistant isolates the only mutations detected occurred within *rv0678* at codon 63 (serine to arginine), supporting the gene's role in drug resistance. Bedaquiline and clofazimine resistance was further supported by the studies from Andries, *et al.* and Ismail, *et al* [146,147].

Finally, resistance associated SNPs for linezolid were identified in *rplC* using studies by Beckert, *et al.,* Wasserman, *et al,* Locke, *et al.,* and Locke, *et al* [148,149,152,153]. Phenotypic DST and WGS identified a change in codon 154 (cysteine to arginine) was the sole *rplC* mutation correlated with resistance to linezolid. The two studies performed by Locke, *et al.* also indicated codons 152, 155, 157, 159, and 169 were correlated with resistance to Linezolid in *Staphylococcus* strains and were covered to ensure full coverage of potential linezolid resistance SNPs [152,153].

This suite of identified SNPs associated with drug resistance were used as the backbone of the assay. As research continues and new treatments are developed, new target genes and SNPs can, and must, be incorporated into the assay and analysis pipeline. For example, the recent success of a regimen in the Nix-TB trial using bedaquiline, linezolid, and pretomanid for XDR-TB will require careful monitoring for the emergence of resistance-conferring SNPs. This trial showed successful treatment for XDR-TB in 88.78% of patients after 6-months of treatment and a 6-month follow-up period [71,72]. Pretomanid is a new drug and the resistance mechanisms and mutations leading to resistance are not yet fully understood. Early research has linked at least 6 genes to potential resistance to pretomanid which will need to be monitored in tNGS assays to prevent treatment failure (*fgd1, ddn, fbiA, fbiB, fbiC,* and *fbiD*) [154,155].

The recent release of an official mutation target list from the WHO will help to standardise the mutations reported by tNGS assays – this list will evolve with more sequence data and new TB drugs [156].

3.2.2: Design and Optimization of PCR Primers for target resistance genes

3.2.2.1: Design of PCR Primers

Using the list of mutations, outlined in section 3.2.1, a map was created for each target gene highlighting the location of every high-confidence resistance conferring mutation site (Figure 3.3). Each gene map used the annotated *M. tuberculosis* H37Rv reference genome (NC_000962.3) available from the NCBI database. Primers were designed using these maps and an amplicon size ranging from 900 to 1,100 base pairs, a size chosen to allow efficient amplification, be suitable for nanopore sequencing, and cover all the necessary SNPs in a single amplicon. Size range was kept consistent across all amplicons to promote consistent amplification efficiency across targets.

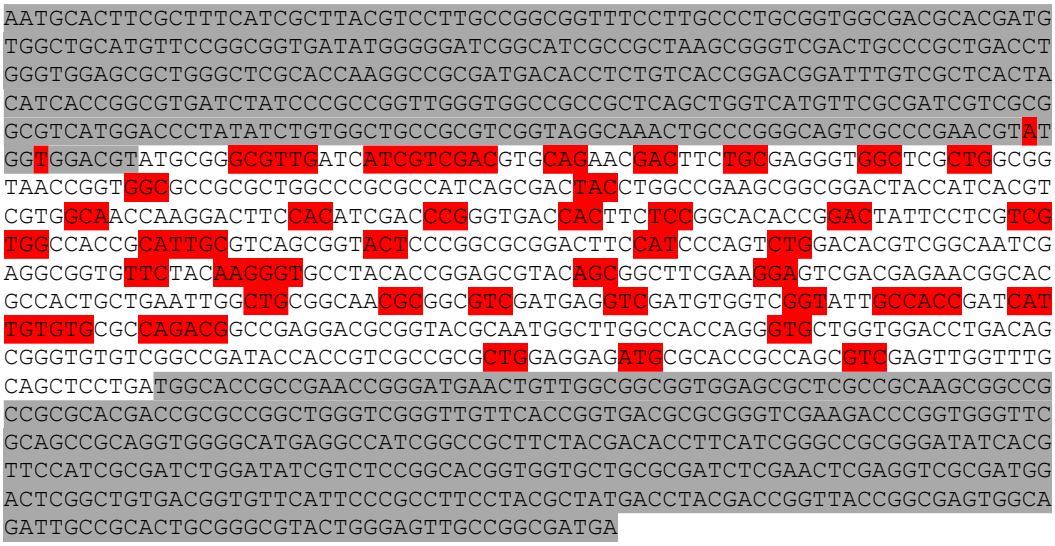

Figure 3.3: Example of a gene map showing the locations of known high-confidence resistance mutations in the pncA gene. Areas highlighted in grey are extragenic regions included to make the amplicon >900bp long.

Target gene primer pairs were designed using Primer-BLAST, a web-based software tool created collaboratively between the NCBI and the Primer3 developers. Strict parameters were set to increase the likelihood of primers amplifying with equivalent efficiency when in a multiplex (Table

3.10). The FASTA sequence maps were used to design PCR primers. In genes less than 900bp, 500bp extragenic regions were included on both the 5' and 3' ends to allow design of amplicons of a similar length.

Table 3.10: Primer design parameters for use in designing target gene primer pairs in Primer-BLAST

Parameter	**Minimum Value**	**Optimum Value**	**Maximum Value**
Primer Size (bp)	15	18	20
Primer Melting Temperature (°C)	59	60	61
Primer GC%	40	50	60
Product Size (bp)	900	1000	1100
Consecutive GC Clamp Length	1	3	N/A

All primer pairs were QC tested in simplex format using SYBR Green qPCR on *M. bovis* BCG DNA (Table 3.11). As equal amount of BCG DNA was used in all reactions, PCR assay efficiency could be assessed comparatively. The results below are from the first primer design iteration, there were 73 more (described in section 3.2.2.2).

Table 3.11: tNGS Target Gene Primer Simplex QC Test for Original Primers Using Triplicate Samples

Gene Target	**qPCR Amplification ($\overline{X}$ C_T)**
eis	24.47
pncA	25.80
fabG1 & *inhA*	19.49
rv0678	19.15
tlyA	21.89
ethA	35.00
gyrA	19.97
rrs	8.80
rplC	15.91
rpsL	20.19
embB	12.72
rpoB	16.46
gidB	18.93
katG	16.06
rrl	19.66

3.2.2.2: Gene Target Primer Pair Redesign

Primers were redesigned multiple times during multiplex group optimization (section 3.2.3) Redesigns resulted in a total of 90 primer pairs (176 individual redesigned primers).. For assessment of redesigned primers all amplifications were performed on triplicate samples and mean C_Ts were used to quantify amplification efficiency. Likewise, when sequencing was performed it was in duplicate for each amplification resulting in a total n of six for each sample in analysis.

Design of gene target primer pairs was highly iterative (section 3.2.2) and primer performance was assessed both in simplex and in multiplex. However, multiple targets were suitable following initial design or with a single redesign for improved target coverage (*gyrA, gidB, inhA, pncA,* and *rpoB*).

Conversely, several targets required extensive optimization before they performed well in multiplex format.

Amplicons were designed to be approximately 1kb to capture all the necessary SNPs in some genes and to keep a consistent length for multiplexing – 1kb is also an optimal length for nanopore sequencing. Use of 1kb targets allowed total gene coverage of 8 targets reducing the need to redesign if new drug resistant SNPs are identified in these genes.

The tNGS assay is limited by reliance on conserved primers which can lower sensitivity if a mutation occurs within the primer site or outside the targeted section of the genome [5,19]. However, the risk is reduced due to the highly conserved clonal genomes of mycobacteria, unlike many other bacterial pathogens.

However, during sequencing of contrived samples a mutation within the *katG* forward primer site was identified, resulting in a loss of sensitivity and requiring primer redesign (section 3.2.11). This was a known mutation site, but it is a mutation not associated with resistance and therefore was overlooked – it is important to consider all known mutation sites when designing tNGS primers to avoid this issue. Primer site mutations can prove especially problematic in the event of mixed infection, where primer competition will result in only one strain being detected.

3.2.2.3: Redesign of *inhA* primer pair

Primers for gene target *inhA* were redesigned once. Redesign was necessary as the original primer set combined coverage of *inhA* and *fabG1* but didn't cover all necessary target SNPs. Splitting into two separate primer pairs enabled greater coverage of target mutations for both genes (Table 3.12). New primers covered 96% of the *inhA* gene as well as the 5' gene promoter region to cover all high confidence mutations listed in appendix I.

Table 3.12: Redesign history for inhA primers

***inhA* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	GGGCGCTGCAATTTATCCC	GGCGTAGATGATGTCACCCG	941
Redesign 1*	GGCGTAGATGATGTCACCCGT	GGGCGCTGCAATTTATCCC	942

*Redesign version selected for use

3.2.2.4: Redesign of *pncA* primer pair

Primers for *pncA* were also redesigned once during optimization. The primers were moved to encompass the entire *pncA* gene and cover all known high confidence resistance-conferring mutations (Table 3.13).

Table 3.13: Redesign history for pncA primers

***pncA* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	TCAGCTGGTCATGTTCGCG	ATGAACACCGTCACAGCCG	960
Redesign 1*	TCCAGATCGCGATGGAACG	TCACCGGACGGATTTGTCG	953

*Redesign version selected for use

3.2.2.5: Redesign of *rpoB* primer pair

To ensure coverage of the rifampicin resistance determining region (RRDR), a FASTA copy of the gene was used to map the location of each known mutation (Appendix II). This map was used to identify potential primer sites which centered the amplicon on the *rpoB* variable region, while also increasing coverage from 27% to 31% of the *rpoB* gene (Table 3.14).

Table 3.14: Redesign history for rpoB primers

***rpoB* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3)**	**Amplicon Length (bp)**
Original	TAGTCCTAGTCCGAGTCGCC	ACGTCTTCTTCGGTCAGCG	963
Redesign 1*	TCATCATCAACGGGACCGAG	ACACGATCTCGTCGCTAACC	1092

*Redesign version selected for use

3.2.2.6: Redesign of *rrl* primer pair

Optimisation of primers for *rrl* (23S rRNA) required two rounds of redesign. The first redesign was to position the high confidence mutation sites more centrally within the amplicon. This alteration reduced the risk of having low coverage for mutations of clinical interest. Specificity testing with DNA template extracted from NRF sputum demonstrated this primer pair cross-reacted with human or commensal bacterial 28S or 23S rRNA genes ($\bar{X}$ C_T = 13.02). Using Primer-BLAST (Section 2.8), *rrl* primers were redesigned to increase specificity using *M. tuberculosis* H37Rv as the reference

genome. This redesign resulted in an amplicon which covered all known high-confidence mutation locations and an improved 33% coverage of the 3,138bp *rrl* gene. Specificity testing of the new primer pair demonstrated no cross-reactivity with human/commensal DNA and was selected for continued use (Table 3.15).

Table 3.15: Redesign history for rrl primers

***rrl* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	TGAGAGGTGACGCATAGCC	GATCAGCCTGTTATCCCCGG	948
Redesign 1	AACACAGGTCCGTGCGAA	TATCCTGACCGAACGTGGC	959
Redesign 2*	GGTCCGTGCGAAGTCGC	TGAACCCGTGTTCTGCGG	1044

*Redesign version selected for use

3.2.2.7: Redesign of *rplC* primer pair

rplC primers underwent two redesigns. The first redesign reduced amplicon size from 1088bp to 902bp to improve amplification speed and efficiency, while still covering the full 654bp *rplC* gene. This redesign version was used for multiplex configurations 1 through 5 until testing by nested qPCR highlighted that *rplC* was amplifying >2CTs later than other primer pairs in the group. The *rplC* gene was then redesigned using Primer-BLAST.

The redesign covered the entire *rplC* gene as well as a 153bp buffer on the 5' end and a 171bp buffer on the 3' end. This improved efficiency in line with other targets and no cross-reactivity with human or commensal bacterial DNA was detected (Table 3.16).

Table 3.16: Redesign history for rplC primers

***rplC* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	ACATCATCGATCCCACGCC	CATCTTCTTGGGTGTGCGC	1088
Redesign 1	CCGCTACCGACTGAGAAGAA	GGCGTCTTGACGTCGATTTT	902
Redesign 2*	AGTACAAGGACTCGCGGGA	TCGAGTGGGTACCCTGGC	978

*Redesign version selected for use with increased working concentration of 3µM

3.2.2.8: Redesign of *tlyA* primer pair

tlyA required four rounds of redesign. Redesign of *tlyA* was initially performed to preferentially detect high-confidence mutation sites over medium-confidence mutation sites, except as convenient. This redesign encompassed the full 807bp gene but cross-reacted with human/commensal DNA in NRF sputum when assessed by qPCR ($\bar{X}$ C_T = 17.42). Therefore, a second redesign was performed using Primer-BLAST. Testing was conducted with both *M. bovis* BCG culture and NRF sputum spiked with *M. bovis* BCG. This assay was less efficient than others in its group and needed further optimisation.

Increased concentrations of *tlyA* primers were tested to improve efficiency. Three samples with varying concentrations of *tlyA* were tested and assessed by qPCR (Table 3.17). Increasing *tlyA* primer concentration from 2µM to 3µM improved the uniformity of the multiplex group amplification, from a mean range of 3.33 to 1.59 C_Ts, hence 3uM *tlyA* primers were used for subsequent experiments.

Table 3.17: Nested qPCR mean CT results for comparison of multiplex amplification efficiency in multiplex with various tlyA concentrations

Gene Target	**2.5µM *tlyA* Stock Concentration qPCR ($\bar{X}$ C_T)**	**3µM *tlyA* Stock Concentration qPCR ($\bar{X}$ C_T)**	**4µM *tlyA* Stock Concentration qPCR ($\bar{X}$ C_T)**	**Control Stock Concentration qPCR ($\bar{X}$ C_T)**
gidB	11.52	7.39	8.13	7.79
inhA	8.21	8.09	7.98	9.83
rrl	9.35	8.54	10.56	9.45
pncA	12.53	8.80	10.85	9.18
rpsL	8.11	8.56	7.99	8.11
tlyA	30.13	8.98	8.93	11.12

Two further primer redesigns were tested (Table 3.18) but neither performed better than redesign 2 at 3uM concentration.

Table 3.18: Redesign history for tlyA primers

***tlyA* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	TGTGGGTTTCCTTCCTTGGG	AGCAGTACTTCGGTGAACCC	1041
Redesign 1	CATCGCACGTCGTCTTTCC	GTGTGGACGACCAGCAGAA	921
Redesign 2 *	CGTTGATGCGCAGCGATC	GGTCTCGGTGGCTTCGTC	1096
Redesign 3	ATCGACGCCCTACTTGCTT	CTCCAATCCCTTGGCCGAC	922
Redesign 4	TCCGGTGACTAGCGTAGGAA	ACCGCATCCTCCAATCCCT	987

*Redesign version selected for use with increased working concentration of 3µM

3.2.2.9: Redesign of *rv0678* gene primer pair

Four *rv0678* primer pairs were designed. Low coverage for the *rv0678* mutation sites were observed when performing sensitivity testing (Figure 3.4). Primer-BLAST was used to design 4 primer pairs as described previously as the original forward primer was proximal to the high confidence mutation site. Four new primer pairs were designed to encompass the entire 498bp *rv0678* gene, keeping primers sufficient distance from the important mutations.

Rv0678_S63R

REFERENCE		A	G	C
SAMPLE		A	G	C
POSITION		779176	779177	779178
BASECALLED COUNTS	A	44		1
	C			20
	G	1	27	
	T			3

katG_S315*

REFERENCE		G	C	T
SAMPLE		G	C	T
POSITION		2155167	2155168	2155169
BASECALLED COUNTS	A	10	2	5
	C		1907	5
	G	3176	1244	5
	T	4	5	3209

Figure 3.4: Example output from the Epi2Me TB Resistance Profile pipeline for the mutation in Rv0678 associated with resistance to bedaquiline and clofazimine compared to that for katG associated with resistance to isoniazid.

Primers were compared in multiplex mixes using *M. bovis* BCG spiked sputum. Analysis by qPCR showed that redesign versions 2 and 3 were most efficient ($\bar{X}$ C_T < 5). Mean C_T results for all multiplex targets showed that version 2 was best but some of the PCRs were still inhibited ($\bar{X}$ Ct range = 9.66CTs). This indicated a need for further optimisation of the multiplex as a whole. However, the redesigned *rv0678* primers were within tolerances and version 2 primers were used for subsequent experiments (Table 3.19)

Table 3.19: Redesign history for rv0678 primers

***rv0678* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	CGGAACCAAAGAAAGTGCGG	GGTGACATGCTGACCTACGG	1010
Redesign 1	CGTGGTCTTCAAGGTGAGCG	ACAAGGAGTGACCACAGGC	933
Redesign 2 *	GCTCGTCCTTCACTTCGCC	ATCAGTCGTCCTCTCCGGT	959
Redesign 3	ATCGACGGTGATTCGGCAG	CCACCTCGGTCAGATTGCG	968
Redesign 4	CGGAGCCGGAAACTTCGTA	AAGTCACTGAACGTGGCCG	1037

*Redesign version selected for use

3.2.2.10 Redesign of *fabG1* primer pair

Redesign of *fabG1* primers was initially required to separate the combined *inhA/fabG1* primer set. The amplicon was shifted to completely cover the 744bp *fabG1* gene along with short buffer regions on both the 5' and 3' ends. Further redesigns were required to improve amplification efficiency in different multiplex configurations. As with other primers, PrimerBLAST was used to design 4 primer pairs as described previously.

Multiplex mixes were tested using *M. bovis* BCG spiked sputum. Redesign version 3 was most efficient with a $\bar{X}$ C_T <5. Results indicated further optimization of multiplex group 1 was required ($\bar{X}$ C_T range = 9.66 C_Ts). However, *fabG1* redesign version 3 was within tolerance and used for subsequent experimentation (Table 3.20).

Table 3.20: Redesign history for fabG1 primers

***fabG1* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	GGGCGCTGCAATTTATCCC	GGCGTAGATGATGTCACCCG	941
Redesign 1	ACCTTCAAATCGGTGGCCT	AATCACTCCGGCCTTGGAG	1060
Redesign 2	TACGCTCGTGGACATACCG	GGTGCTCCTCGTTTTGCAC	1030
Redesign 3 *	CTTTTGCACGCAATTGCGC	AGCAGTCCTGTCATGTGCG	1058
Redesign 4	CGACAAACGTCACGAGCG	GTGCTCCTCGTTTTGCACG	1089
Redesign 5	TAGCGCGACATACCTGCTG	GTGGCCCATACCCATGCC	1066

*Redesign version selected for use

3.2.2.11: Redesign of *ethA* primer pair

Redesign of *ethA* primers was performed to improve identification of mutations and primer specificity. Primer-BLAST was used to design 5 primer pairs as described above. Primer mixes were used to amplify *M. bovis* BCG spiked sputum and *ethA* redesigns were tested in multiplex PCR (Table 3.21). Redesign version 3 was selected for subsequent experimentation as it had the narrowest $\bar{X}$ C_T range (Table 3.22).

Table 3.21: Nested mean qPCR C_Ts for five multiplexes testing redesigned ethA primer pairs using triplicate samples

***ethA* Redesign Version**	***gyrA* Nested qPCR ($\bar{X}$ C_T)**	***rpoB* Nested qPCR ($\bar{X}$ C_T)**	***ethA* Nested qPCR ($\bar{X}$ C_T)**	***rplC* Nested qPCR ($\bar{X}$ C_T)**	***katG* Nested qPCR ($\bar{X}$ C_T)**	***hsp65* Nested qPCR ($\bar{X}$ C_T)**	**Nested qPCR Range ($\bar{X}$ C_T)**
1	12.94	14.02	9.91	10.66	11.62	11.27	4.11
2	13.36	11.19	13.61	12.64	11.85	11.54	2.42
3	11.96	12.11	12.62	11.53	11.47	11.78	1.15
4	12.00	12.09	10.84	11.96	10.83	11.13	1.26
5	12.02	12.76	12.29	29.60	11.27	11.29	18.33

Table 3.22: Redesign history for ethA primers

***ethA* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	TCGGCTTGATTGACCACCC	ACGATGTAGGTGGGTGAGC	964
Redesign 1	GTCCAGGAGGCATTGGTGT	CGGAATTCGCTCCGACTCC	1023
Redesign 2	GTCCAGGAGGCATTGGTGT	TGACGGCCTCGACATTACG	1191
Redesign 3 *	GTCCAGGAGGCATTGGTGT	TGGATCCATGACCGAGCAC	1163
Redesign 4	TCAACCCCGTTGCGGTAAT	TGGATCCATGACCGAGCAC	1040
Redesign 5	ACCCCGTTGCGGTAATGAT	GAGCTACGCCATCCTGGAA	941

*Redesign version selected for use

3.2.2.12: Redesign of *rrs* (16S rRNA gene) primer pair

Initial redesign of *rrs* primer pairs was performed to improve coverage of a mutation proximal to the reverse primer. Redesigned primers included a 3' buffer region. Specificity testing identified cross-reactivity with human/commensal DNA when amplifying unspiked NRF sputum ($\bar{X}$ C_T = 23.77).

Using Primer-BLAST a new primer pair was designed covering 60% of the *rrs* gene and retaining a 3' buffer region. This primer pair negatively impacted amplification of *eis* and *embB* targets in the multiplex.

New primers were designed for all multiplex group 1 targets concurrently. During these redesigns 4 new *rrs* primer pairs were designed. Design used 2 manually selected forward primers and 2 manually selected reverse primers. Primers included a minimum 3bp GC clamp on the 3' end. *rrs* gene coverage ranged from 60% to 68% and included a 3' buffer.

Primers were tested using *M. bovis* BCG spiked sputum and analyzed by nested qPCR (amplicons diluted 1:100 using nuclease-free H_2O). qPCR results indicated that redesign version 4 amplified most efficiently ($\bar{X}$ C_T<5) (Table 3.23).

Table 3.23: Nested mean qPCR C_Ts for four multiplexes testing redesigned rrs primer pairs

***rrs* Redesign Version**	***eis* Nested qPCR ($\overline{X}$ C_T)**	***embB* Nested qPCR ($\overline{X}$ C_T)**	***rrs* Nested qPCR ($\overline{X}$ C_T)**	***rv0678* Nested qPCR ($\overline{X}$ C_T)**	***fabG1* Nested qPCR ($\overline{X}$ C_T)**
3	13.51	6.00	8.20	9.90	7.48
4	14.77	5.00	5.00	5.00	5.00
5	13.67	40.00	5.00	5.00	7.66
6	40.00	19.81	40.00	8.84	6.00

Inclusion of this *rrs* primer pair also improved the efficiency of 4/5 of the remaining multiplex group targets. The multiplex group required further optimisation as a whole, but primer redesign version 4 was selected for subsequent experimentation (Table 3.24).

Table 3.24: Redesign history for rrs primers

***rrs* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	GCTTAACTGTGAGCGTGCG	CTTTGTTGTCATGCACCCGG	1023
Redesign 1	TTCCCTTGTGGCCTGTGTG	ATGTTTCACTTCCCCGCGT	998
Redesign 2	CGTTCCCTTGTGGCCTGT	GCACGACATCACTCGTGC	947
Redesign 3	AATACGTAGGGTGCGAGCG	AAAGGAGGTGATCCAGCCG	1013
Redesign 4*	CTCTGGGCAGTAACTGACGC	GAGTGTTGCCTCAGGACCC	942
Redesign 5	TTGTCCGGAATTACTGGGCG	GACAAGAACCCCTCACGGC	1054
Redesign 6	TGGAATTCCTGGTGTAGCGG	AGTGTTGCCTCAGGACCCA	1006

* Redesign version selected for use

3.2.2.13: Redesign of *rpsL* primer pair

Initial redesign of *rpsL* primers was performed to address cross-reactivity with human/commensal DNA. Primer redesign was performed using Primer-BLAST. A primer pair covering the full 375bp *rpsL* gene and 5' and 3' buffer regions was designed.

Specificity testing exhibited no cross-reactivity when assessed by qPCR. However, sensitivity testing in mixed samples identified low sequencing coverage of *rpsL*. Analysis using the Epi2Me TB Resistance Profile pipeline showed an average coverage depth of 84x in *rpsL* while *gidB* and *rrs* showed a mean coverage of 684x for the same sample. To improve coverage depth 5 primer pairs were designed ranging in size from 933bp to 1077bp. All primer pairs covered the full *rpsL* gene and included buffer regions on 5' and 3' ends.

Analysis showed variability in *rpsL,* and other multiplex target, amplification efficiency (Table 3.25). Redesign version 3 minimized disruption to the multiplex group and had the lowest mean *rpsL* C_T. Further multiplex group optimisation was required but redesign version 3 was selected for use in subsequent experimentation (Table 3.26).

Table 3.25: Nested mean qPCR amplification C_Ts of five multiplexes testing redesigned rpsL primer pairs using triplicate samples

***rpsL* Redesign Version**	***gidB* Nested qPCR ($\bar{X}$ C_T)**	***inhA* Nested qPCR ($\bar{X}$ C_T)**	***rrl* Nested qPCR ($\bar{X}$ C_T)**	***pncA* Nested qPCR ($\bar{X}$ C_T)**	***rpsL* Nested qPCR ($\bar{X}$ C_T)**	***tlyA* Nested qPCR ($\bar{X}$ C_T)**	**Nested qPCR Range ($\bar{X}$ C_T)**
2	13.11	9.17	11.76	11.65	29.26	16.09	20.09
3	10.35	9.64	13.14	12.53	11.84	18.54	8.90
4	10.34	9.60	11.73	11.47	29.11	14.63	19.51
5	11.44	9.29	13.53	12.88	13.31	17.33	8.04
6	11.69	9.92	12.84	14.01	28.95	18.26	19.03

Table 3.26: Redesign history for rpsL primers

***rpsL* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	GAGTTTTGGTCGGCACTGC	GGGCGGGTTTGACATTGTC	992
Redesign 1	GCGGCGGGTATTGTGGTT	TAACCGGCGCTTCTCACC	1063
Redesign 2	AGGCAAGCTATGCGACACA	GTTGCGGACCCTACTCAGG	1064
Redesign 3*	CGCTTTGACCTGCCAGACT	GCGCTTCTCACCAGCGATA	1077
Redesign 4	CGATGCCTCGGATGAGACG	TCAGCACGTCCTTCTGTGC	1071
Redesign 5	TACGCTTGATGTAGGGGCG	TAATGCGCAAAGGCTCGGT	1005
Redesign 6	GGCAAGCTATGCGACACAC	AGCGATAATGCGCAAAGGC	933

* Redesign version selected for use

3.2.2.14: Redesign of *embB* primer pair

embB underwent seven redesigns for optimising performance in multiplex. The first redesign was performed to prioritise all high-confidence SNPs over medium confidence SNPs. Multiplex amplifications using *M. bovis* BCG culture were assessed post-sequencing by Qualimap visualization. Analysis showed *embB* had 7-fold lower mean coverage depth relative to other group targets (Figure 3.5).

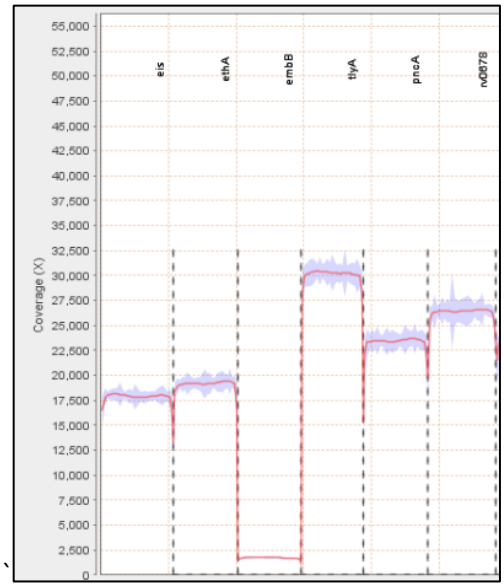

Figure 3.5: Qualimap coverage map of genes targeted by primers within multiplex group 1 after tNGS amplification and sequencing with embB primer iteration 1

A new primer pair was designed using Primer-BLAST to address this low coverage. Redesigned primers covered all known high-confidence SNP sites and 28.9% of the full gene. Redesigned primers were incorporated into multiplex group 1 and tested using *M. bovis* BCG DNA. Product was sequenced by Flongle and FASTQ files were mapped (see Methods section 2.16) to visualize relative coverage within the multiplex (Figure 3.6). Mapping showed this redesign resulted in an *embB* drop out. This redesign version also correlated with lower sequencing coverage of *rrs* and increased coverage of *hsp65*.

Figure 3.6: Qualimap coverage map of genes targeted by primers in the group 1 multiplex after tNGS amplification and sequencing with embB redesign iteration 2 primers

A third primer pair was designed to improve target and multiplex performance. Redesign version 3 reduced amplicon size in an attempt to improve amplification efficiency. Testing using *M. bovis* BCG spiked sputum showed poor *embB* coverage in the multiplex by PCR and sequencing.

A further 4 primer pairs were designed using Primer-BLAST. Multiplex amplification using *M. bovis* BCG spiked NRF sputum DNA were assessed by nested qPCR. *embB* redesign version 5 yielded the most efficient amplification ($\bar{X}$ C_T≤5) (Table 3.27) and the smallest mean C_T range with 4/5 of primer pairs demonstrating a mean C_T <5. Hence, *embB* redesign version 5 was selected for inclusion in the final multiplex and use subsequent experimentation (Table 3.28).

Table 3.27: Mean nested qPCR C_Ts for four multiplexes testing redesigned embB primer pairs using triplicate samples

***embB* Redesign Version**	***eis* Nested qPCR ($\bar{X}$ C_T)**	***embB* Nested qPCR ($\bar{X}$ C_T)**	***rrs* Nested qPCR ($\bar{X}$ C_T)**	***rv0678* Nested qPCR ($\bar{X}$ C_T)**	***fabG1* Nested qPCR ($\bar{X}$ C_T)**
4	13.51	6.00	8.20	9.90	7.48
5	14.77	5.00	5.00	5.00	5.00
6	13.67	40.00	5.00	5.00	7.66
7	40.00	19.81	40.00	8.84	6.00

Table 3.28: Redesign history for embB primers

***embB* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Amplicon Length (bp)**
Original	TGGTGATCTTGTCCGTGCC	ACCAAGATCCGCAGCATCG	1012
Redesign 1	GGGCTGATTGGCTTTGTGTT	GTCGCTGACATGGGTCATCA	936
Redesign 2	CTCAATTGCCCAGCTCCTCC	TGGGCGTGAACATCAGGAA	1031
Redesign 3	GGGGTGTTCACCGACCTG	GGTCAGGATGACGGTGCC	915
Redesign 4	CGCCGTGGTGATATTCGGC	GGGATACCAACACCGTCGT	1007
Redesign 5*	CGCCGTGGTGATATTCGGC	GCACACCGTAGCTGGAGAC	1124
Redesign 6	CTCTGGCATGTCATCGGCG	GGAGACATACCACCAGCCG	1086
Redesign 7	CCCAGCTCCTCCTCAGGC	TGGTGGGCGTGAACATCAG	1026

* Redesign version selected for use

3.2.2.15: Redesign of *katG* primer pair

A FASTA *katG* gene sequence was annotated to identify positions of the high-confidence mutation sites within the 2,223bp gene. Five new primer pairs were designed using Primer-BLAST to prioritize the high confidence mutations over medium and low confidence mutations.

Primers were compared to determine the most efficient amplification and least disruption to existing multiplex reactions. Redesign version 3 had the best amplification efficiency ($\bar{X}$ C_T = 10.3) and redesign version 4 had the least impact on the other targets in the multiplex ($\bar{X}$ C_t range = 5.16 C_Ts) (Table 3.29). However, inconsistent assay performance required further redesign of the multiplex.

Table 3.29: Mean nested qPCR C_Ts for five multiplexes testing redesigned katG primer pairs using triplicate samples

katG **Redesign Version**	***gyrA*** **Nested qPCR ($\bar{X}$ C_T)**	***rpoB*** **Nested qPCR ($\bar{X}$ C_T)**	***ethA*** **Nested qPCR ($\bar{X}$ C_T)**	***rplC*** **Nested qPCR ($\bar{X}$ C_T)**	***katG*** **Nested qPCR ($\bar{X}$ C_T)**	***hsp65*** **Nested qPCR ($\bar{X}$ C_T)**	**Nested qPCR Range ($\bar{X}$ C_Ts)**
2	13.76	11.92	8.43	9.76	17.77	10.45	9.34
3	11.33	12.27	9.60	18.26	10.30	11.53	8.66
4	10.91	9.57	9.34	8.49	13.65	9.69	5.16
5	12.11	10.69	8.74	10.23	18.95	10.99	10.21
6	11.77	11.61	9.04	11.66	15.08	11.60	6.04

The *hsp65* target was included to help speciate non-tuberculous mycobacteria if present. Other targets in the multiples (such as *gyrA, rrs,* and *rrl*) were also capable of providing that information, so the decision was made to remove the *hsp65* target from the multiplex to ease optimisation. Redesign versions 2-6 were amplified without *hsp65* using spiked NRF sputum. Nested qPCR analysis showed removal of *hsp65* primers resulted in failure to amplify in 4/5 of multiplex options (Table 3.30). However, redesign version 6 without hsp65 primers showed consistent amplification for all targets ($\bar{X}$ C_T range = 1.45 C_Ts).

Table 3.30: Mean nested qPCR C_Ts for five multiplexes testing redesigned katG primer pairs with the removal of hsp65 primers using triplicate samples

katG **Redesign Version**	***gyrA*** **Nested qPCR ($\overline{X}$ C_T)**	***rpoB*** **Nested qPCR ($\overline{X}$ C_T)**	***ethA*** **Nested qPCR ($\overline{X}$ C_T)**	***rplC*** **Nested qPCR ($\overline{X}$ C_T)**	***katG*** **Nested qPCR ($\overline{X}$ C_T)**
2	40.00	40.00	40.00	40.00	40.00
3	40.00	40.00	40.00	40.00	40.00
4	40.00	40.00	40.00	40.00	40.00
5	40.00	40.00	40.00	40.00	40.00
6	15.06	15.32	14.62	13.87	14.60

3.2.2.16: Redesign of *eis* primer pair

The first *eis* primers were redesigned to correct poor sensitivity for high-confidence mutations in the gene promoter region. Primer-BLAST was used to design a new primer pair containing the promoter region SNP sites. The new primers resulted in an amplicon covering 59% of the *eis* gene in addition to the 5' promoter region.

An NRF sputum DNA sample was amplified and sequenced by Flongle and analysed using the Epi2Me TB Resistance Profile pipeline (section 2.16). Epi2Me results showed low coverage of *eis* targets compared to other group 1 multiplex targets, indicating a need for further redesign. Four additional primer pairs were designed and compared using nested qPCR. Version 3 performed best overall but the *eis* target was amplifying inefficiently and required further redesign (Table 3.31).

Table 3.31: Mean nested qPCR C_Ts testing redesigned eis primer pairs using triplicate samples

***eis* Redesign Version**	***eis* Nested qPCR ($\overline{X}$ C_T)**	***embB* Nested qPCR ($\overline{X}$ C_T)**	***rrs* Nested qPCR ($\overline{X}$ C_T)**	***rv0678* Nested qPCR ($\overline{X}$ C_T)**	***fabG1* Nested qPCR ($\overline{X}$ C_T)**
2	13.51	6.00	8.20	9.90	7.48
3	14.77	5.00	5.00	5.00	5.00
4	13.67	40.00	5.00	5.00	7.66
5	40.00	19.81	40.00	8.84	6.00

Ten primer pairs were designed by Primer-BLAST, 7 as normal and 3 were designed with reduced amplicon size (400-600bp) for improved amplification efficiency. Redesigns generated amplicons ranging in gene coverage from 11.7% to 73.5%. Primer performance was tested in multiplex qPCR (Table 3.32). Redesign version 8 was judged to be the best primer pair overall, with a low mean *eis* C_T and early mean C_Ts for all other targets.

Table 3.32: Mean nested qPCR C_Ts testing redesigned eis primer pairs using triplicate samples

***eis* Redesign Version**	***eis* Nested qPCR ($\overline{X}$ C_T)**	***embB* Nested qPCR ($\overline{X}$ C_T)**	***rrs* Nested qPCR ($\overline{X}$ C_T)**	***fabG1* Nested qPCR ($\overline{X}$ C_T)**	***rv0678* Nested qPCR ($\overline{X}$ C_T)**
6	12.62	8.87	6.84	8.76	7.45
7	12.01	35.00	7.02	9.10	7.77
8	12.33	9.58	6.40	9.67	7.91
9	11.06	9.48	7.51	9.66	7.15
10	12.60	9.53	7.80	10.14	8.02
11	13.87	8.83	6.72	8.58	7.05
12	11.46	9.43	7.64	9.77	8.03
13	11.26	9.61	7.46	9.73	7.65
14	10.67	9.51	7.38	9.14	7.63
15	12.07	12.70	6.99	8.98	7.97

Sequencing by MinION was used to corroborate these results. The *eis* target had the highest coverage within the multiplex group despite poor nested qPCR performance (Figure 3.7). This was a surprising result and didn't match the qPCR data. There may have been an issue with the nested qPCR for *eis*, or there may have been some bias in the sequencing for the *eis* amplicon. Investigation identified a base in the forward nested primer was misdesigned creating overly conservative efficiency judgements. Therefore, version 8 was selected for use in subsequent experimentation (Table 3.33).

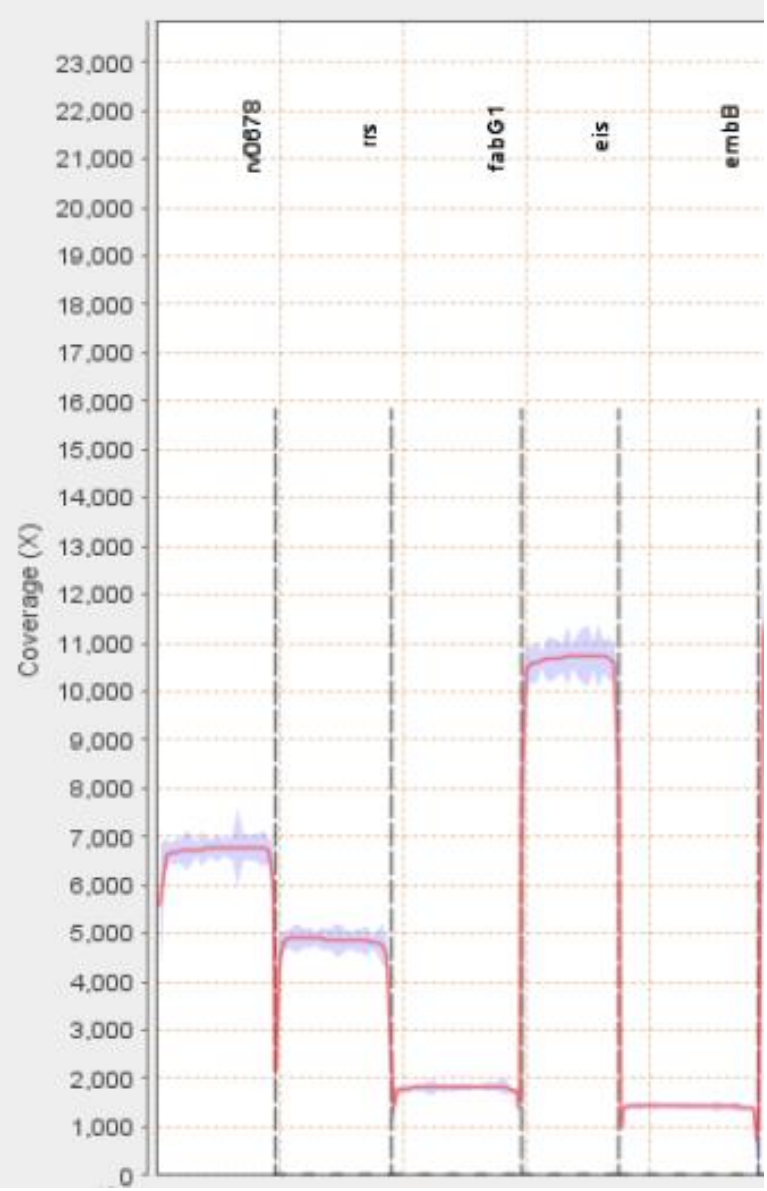

Figure 3.7: Qualimap coverage map of group 1 gene targets using eis redesign 8 primers from pooled triplicate samples for improved resolution

Table 3.33: Redesign history for eis primer pairs

***eis* Redesign Version**	**Forward Primer (5'-3')**	**Reverse Primer (5'-3)**	**Amplicon Length (bp)**
Original	GCGCTGTACATGGATCTGC	AAAAGCCCGTCAGCCTAGC	1063
Redesign 1	ACCGCGACGAAACTGAGAC	GGTAGTGGCGGTGCACATT	1009
Redesign 2	CCAGTAGGAACATCCCCGG	ATGACATCCACAAGCGCCA	904
Redesign 3	AGATCGCCTCAAACTCGCC	GATTCACGCGTTCGTCGC	1074
Redesign 4	CCGGAATCGGCTATGCGG	GTCGGGTACCTTTCGAGCC	1054
Redesign 5	GGACCGTGGAAAACTCGCC	TCGCTGATTCTCGCAGTGG	1083
Redesign 6	GGTGAGCAGGTGGGGTAAC	ACCGGTACTTGCTCTGCAC	1055
Redesign 7	TCATGCAAGGTGGTAGCGG	GTTCGCACTGTGAGCAACG	810
Redesign 8*	TCCATGTACAGCGCCATCC	TGTCGGGTACCTTTCGAGC	917
Redesign 9	CAGTAGGAACATCCCCGGC	GGTAGTGGCGGTGCACATT	806
Redesign 10	GGGATGCAGTAACGCGAAC	ATGGGACCGGTACTTGCTC	880
Redesign 11	TACCCGTCGGGATGCAGTA	TGTAGCGCGGTTGGACAAT	1189
Redesign 12	CTTCACCAGGCACCGTCAA	TGGGACCGGTACTTGCTCT	442
Redesign 13	GCCAGTAGGAACATCCCCG	GTTCGCACTGTGAGCAACG	453
Redesign 14	TCCCGACCACCTCAGAACC	CTTGTTCTGGTCCAACGGGT	593
Redesign 15	TCAGCTCATGCAAGGTGGT	TCGTCGCTGATTCTCGCAG	546

* Redesign version selected for use

3.2.2.17: Final Targets and Primers

Following primer redesign the assay consisted of 16 gene targets in 3 multiplex groups (Table 3.34). The removal of *hsp65* to improve *katG* performance, and the separation of *fabG1* and *inhA* into individual targets altered the total number of targets during optimization. Specificity, sensitivity, and LoD experiments were then performed using the optimized primer designs.

Table 3.34: Final optimized gene target primer sets for tNGS multiplex assay

Assay Drug Resistance Gene Target	Multiplex Group	Forward Primer (5'-3')	Reverse Primer (5'-3')
eis	1	TCCATGTACAGCGCCATCC	TGTCGGGTACCTTTCGAGC
embB		CGCCGTGGTGATATTCGGC	GCACACCGTAGCTGGAGAC
rrs		CTCTGGGCAGTAACTGACGC	GAGTGTTGCCTCAGGACCC
rv0678		GCTCGTCCTTCACTTCGCC	ATCAGTCGTCCTCTCCGGT
fabG1		CTTTTGCACGCAATTGCGC	AGCAGTCCTGTCATGTGCG
gyrA	2	TGACAGACACGACGTTGCC	CGATCGCTAGCATGTTGGC
rpoB		TCATCATCAACGGGACCGAG	ACACGATCTCGTCGCTAACC
ethA		GTCCAGGAGGCATTGGTGT	TGGATCCATGACCGAGCAC
rplC		AGTACAAGGACTCGCGGGA	TCGAGTGGGTACCCTGGC
katG		TGCCCGGATCTGGCTCTTA	CTGTGGCCGGTCAAGAAGA
gidB	3	TGACACAGACCTCAGGAGC	GCCCTTCTGATTCGCGATG
inhA		GGGCGCTGCAATTTATCCC	GGCGTAGATGATGTCACCC
rrl		GGTCCGTGCGAAGTCGC	TGAACCCGTGTTCTGCGG
pncA		TCACCGGACGGATTTGTCG	TCCAGATCGCGATGGAACG
rpsL		GCGGCGGGTATTGTGGTT	TAACCGGCGCTTCTCACC
tlyA		CGTTGATGCGCAGCGATC	GGTCTCGGTGGCTTCGTC

3.2.3: Optimization of Multiplex Groups for tNGS based DR-TB detection

3.2.3.1: *in silico* Multiplex Grouping

Multiplex PCRs are powerful diagnostic and research tools, however, this power comes with increased complexity and design difficulties. A 1997 paper by Henegariu, *et al.,* summarized the four primary issues associated with multiplex PCR; namely if all products are weak, only long products are weak, only short products are weak, or if non-specific amplification occurs [157]. These issues can often be attributed to primer-dimer formation and/or formation of unwanted products, both of which lower target amplification efficiency [158,159]. Unfortunately, in the 25 years since this

publication few methods have been developed to effectively remove the trial-and-error nature of multiplex design and optimization. This is especially so for design of multiplex primers, as unknown variables in DNA interactions make definitive design of non-competitive or -interactive primers difficult.

Software developers and bioinformaticians have developed several software tools to minimize the risk of non-specific interactions and streamline multiplex design. Examples include Oli2Go, PRIMEval, MultiPLX 2.1, and Ultiplex; all of which claim to remove the risk of primer interactions and allow greater multiplexing [160–163]. Oli2Go performs simultaneous cross-dimer checking as well as specificity testing against multiple Kingdoms and Phyla for increased assay performance in shotgun sequencing and environmental sampling projects [160].

MultiPLX 2.1 uses nearest neighbour DNA binding thermodynamic analysis to identify the optimal multiplex groupings of pre-designed primers [163]. This analysis aims to reduce primer-dimer formation and inhibition of primers while designing optimal multiplex groupings according to user defined parameters. Despite this, when tested on primers designed for the tNGS assay it returned only triplex mixes, regardless of parameters set. This prompted use of an alternative primer analysis and grouping tool.

PRIMEval, developed by many of the same individuals behind Oli2Go, also performs specificity and cross-dimer checks. However, PRIMEval improves on Oli2Go with added assessment of non-specific hybridization events, primer depletion, and amplification efficiency prediction [161]. This added utility was why PRIMEval was selected for *in silico* evaluation of Primer-BLAST designed primers for the tNGS assay.

More recently, Ultiplex multiplex analysis software was released by developers in China in 2021 [162]. Ultiplex surpasses PRIMEval or Oli2Go with incorporation of primer design directly in the program, as opposed to being solely a post-hoc analysis tool [162]. Validation testing by the developers saw successful design of a 108-plex through use of the Ultiplex pipeline; a degree of multiplicity that if

replicable could greatly improve efficiency of molecular assay design. This tool, however, wasn't available until 2021 after we had finished designing the primers [162].

Primers designed as described in section 3.2.2 were organized into multiplex groups. Initially, groups were selected using MultiPLX 2.1 web-based software program described previously [163]. This program assessed all the simplex primers *in silico* for interactions and chose those least most likely to work well in a multiplex reaction. The program suggested 5 triplex reactions would be most efficient (Table 3.35).

Table 3.35: Triplex groups as designed by use of MultiPLX 2.1 software

Triplex Group	**Gene Target 1**	**Gene Target 2**	**Gene Target 3**
1	*eis*	*ethA*	*embB*
2	*pncA*	*gyrA*	*rpoB*
3	*fabG1/inhA*	*rrs*	*gidB*
4	*rv0678*	*rplC*	*katG*
5	*tlyA*	*rpsL*	*rrl*

However, *in silico* analysis is generally insufficient on its own to guarantee a viable multiplex. Optimisation and troubleshooting must be performed *in vitro* to detect issues which software programs overlook. As seen in sections 3.2.2 and 3.2.3, even with the use of *in silico* analysis, extensive optimization was still required to create viable 5- and 6-plexes. Non-specific priming was not typically the reason for multiplex group redesign. Rather, variable primer efficiencies and primer interactions (homo/hetero-dimer formation) were more commonly the cause of multiplex failure.

3.2.3.2: *in vitro* Multiplex Optimisation

The decision was made to consolidate the triplexes into larger multiplexes to reduce assay complexity, cost, and risk of contamination. A set of three 5-plex reactions were created using

triplex groups 1, 2, and 3 as the backbone (Table 3.36). An additional multiplex mix was included in testing combining all target primers in a single reaction for comparison to the new 5-plex groups.

Table 3.36: Configuration 1 of the 5-plex primer mixes for the tNGS assay

5-Plex Group	Gene Target 1	Gene Target 2	Gene Target 3	Gene Target 4	Gene Target 5
1	*eis*	*ethA*	*embB*	*tlyA*	*rv0678*
2	*pncA*	*gyrA*	*rpoB*	*rpsL*	*rplC*
3	*fabG1* & *inhA**	*rrs*	*gidB*	*rrl*	*katG*

*Due to genomic proximity, targets for fabG1 and inhA were covered in a single primer set

The 5-plex mixes were tested in duplicate using *M. bovis* BCG DNA. The performance of each 5-plex group, and the 15-plex, was measured by qPCR of *M. bovis* BCG specific RD1 and RD3 region primers and TapeStation. Analysis by RD1/RD3 qPCR acted as a nested measure of amplification and identified an increase in variance in 5-plex groups, as compared to the triplex groups (mean range increase = 1.06 C_Ts). However, there was no evidence of amplification failure as detected by qPCR (Table 3.37). Analysis of 5-plex and multiplex amplification products by TapeStation demonstrated evidence of non-specific amplification in all 5-plex groups. Furthermore, this analysis showed a total lack of target amplification in 5-plex group 2 and the 15-plex mix (Figure 3.8).

Table 3.37: SYBR Green qPCR results for evaluation of 5-plex configuration 1 amplification performance on M. bovis BCG DNA using dual sets of triplicate samples

Sample Replicate Set	5-Plex Group 1 $\overline{X}$ C_T	5-Plex Group 2 $\overline{X}$ C_T	5-Plex Group 3 $\overline{X}$ C_T
1	19.60	17.12	19.82
2	19.60	17.11	19.84

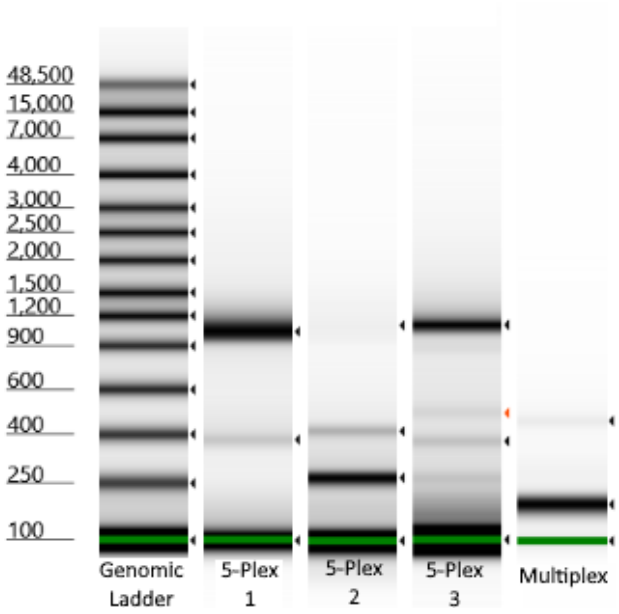

Figure 3.8: TapeStation gel image of 5-plex configuration 1 indicating non-specific amplification identified as of secondary and tertiary banding as well as, indicating no target amplicons in 5-plex 2 or the 15-plex reaction.

The 5-plex groups were then reconfigured to improve performance. This reconfiguration, and all subsequent reconfigurations, occurred in parallel to the primer redesigns covered in section 3.2.2. To improve assessment of multiplex performance a set of nested qPCR primers for each target were designed. This allowed us to measure the performance of each gene target in the multiplex without the need for sequencing (Table 3.38). Nested primers were designed to be approximately 100bp and have similar design characteristics to each other and the multiplex primers.

Table 3.38: Nested primer sequences for tNGS amplification analysis with design parameters

Nested Primer	Primer Length	Melting Temperature (°C)	GC%	Sequence (5'-3')	Amplicon Size (bp)
inhA & *fabG1* Forward	18	59.97	61.11	CAACAAGCTCGACGGGGT	101
inhA & *fabG1* Reverse	18	60.05	61.11	CCTTGGACACATCCGCGT	
pncA Forward	18	60.05	66.67	GGTGACCACTTCTCCGGC	110
pncA Reverse	18	60.13	61.11	TCGATTGCCGACGTGTCC	
katG Forward	18	59.89	61.11	TTATCCGGATGGCGTGGC	106
katG Reverse	18	60.05	61.11	TCGGGCCAGCTGTTAAGC	
eis Forward	19	60.08	57.89	CCGCTACCACCTTGCATGA	107
eis Reverse	18	59.65	66.67	GGGTCTGACCAACCGGAC	
rrs Forward	18	59.97	61.11	TTGTACACACCGCCCGTC	106
rrs Reverse	18	60.13	61.11	ACTTCGTCCCAATCGCCG	
tlyA Forward	18	60.13	66.67	GTCCTCGAGCGGACCAAC	108
tlyA Reverse	18	60.28	61.11	GGGCAACACGGTAGCCAA	
gyrA Forward	19	60.00	57.89	GGTCATGGGGCGGGTTAAA	92
gyrA Reverse	18	59.13	61.11	CGCGGCCAGTTTTGTAGG	
gidB Forward	18	60.13	61.11	CGTGGCCGTTGAGATCGT	109
gidB Reverse	18	60.05	61.11	CAACTTGTCCAACGCGGC	
rpsL Forward	18	59.81	61.11	ACGCTTGATGTAGGGGCG	100
rpsL Reverse	18	60.89	66.67	GCCGGGTGTGTCGCATAG	
ethA Forward	18	60.05	61.11	CCGCTGGACCGTTCACAT	110
ethA Reverse	18	59.80	61.11	ATCTCGGCGAGTAGCCCT	
rv0678 Forward	18	59.89	61.11	TGACCGTGTTGTCCAGCC	104
rv0678 Reverse	18	60.05	61.11	CAACGGCACCTGCGAAAC	
rrl Forward	18	59.57	61.11	CGCCCAAAGGTTCCCTCA	103
rrl Reverse	18	59.34	61.11	CCGACTTTCGTCCCTGCT	
rplC Forward	18	60.05	61.11	ATCAGCCCACGCAAGGTC	103
rplC Reverse	18	59.89	66.67	CATCCGAGTCGTCCAGCC	
embB Forward	18	60.05	61.11	CGACTTTACCGCCACCGT	108
embB Reverse	18	60.13	61.11	GCCTGCAAATTGGCGTCC	
rpoB Forward	18	60.13	66.67	GAGCGGTTCGGGTTCTCC	100
rpoB Reverse	18	60.13	61.11	GACGCAGCTTGCGGTAGA	

To determine the cause of the non-specific band in 5-plex group 1 a series of duplex reactions were examined. Each of the 5 primer pairs were included in a duplex PCR with each other, creating 10 total reactions, to identify which pairs were interacting. The interaction was identified between primers for *eis* and *rv0678*. Therefore, a second tNGS multiplex configuration was designed, swapping the primers for *pncA* in 5-plex group 2 with those for *rv0678* in 5-plex group 1 resulting in another three 5-plex mixes (Table 3.39).

Table 3.39: Configuration 2 of 5-plex primer mixes for tNGS amplification

5-Plex Group	**Gene Target 1**	**Gene Target 2**	**Gene Target 3**	**Gene Target 4**	**Gene Target 5**
1	*eis*	*ethA*	*embB*	*tlyA*	*pncA*
2	*gyrA*	*rpoB*	*rpsL*	*rplC*	*rv0678*
3	*fabG1* & *inhA*	*rrs*	*gidB*	*rrl*	*katG*

tNGS amplification results were assessed by both qPCR and TapeStation. Use of the nested primers to determine performance of each of the targets in the 3 multiplexes identified a single inhibited target in 5-plex group 1, *embB*. The remaining four group 1 targets had mean nested C_Ts between 8.68 to 11.38 while *embB* had a mean C_T of 19.77 (Table 3.40). TapeStation of this multiplex configuration did not show evidence of non-specific amplification indicating the swap of *pncA* for *rv0678* was successful (Figure 3.9). The swap also had a major impact on 5-plex group 2, which was now performing as desired.

Table 3.40: Mean results of nested SYBR Green qPCR analysis on amplification of assay targets using multiplex primer group configuration 2 using triplicate samples

Multiplex Group	**Gene Target**	**Nested qPCR Amplification ($\bar{X}$ C_T)**
1	*eis*	9.73
	ethA	8.82
	embB	19.77
	tlyA	8.99
	pncA	11.38

2	*gyrA*	10.56
	rpoB	9.31
	rpsL	10.04
	rplC	9.80
	rv0678	9.98
3	*fabG1* & *inhA*	8.68
	rrs	9.27
	gidB	10.34
	rrl	9.09
	katG	9.75

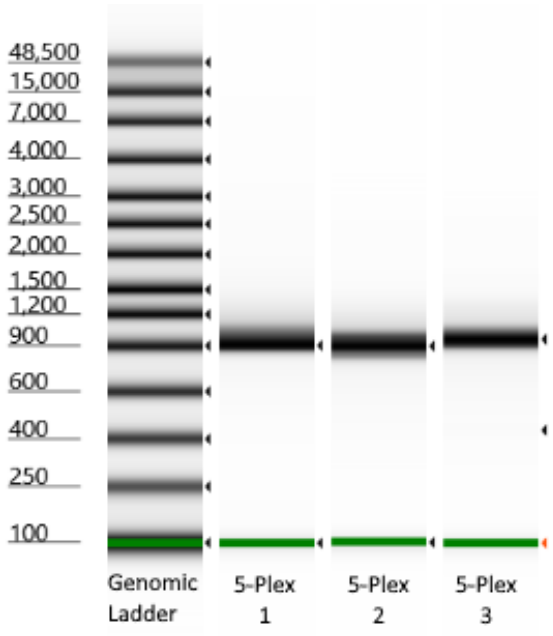

Figure 3.9: 5-plex configuration 2 TapeStation gel image showing no evidence of non-specific amplification identified as dual banding or loss of target amplicon in pooled triplicate samples for improved resolution.

Evidence of the *embB* target dropout ($\bar{X}$ C_T 8 later than the rest of the 5-plex group as shown in table 3.40) necessitated development of a third multiplex configuration. During reconfiguration, *fabG1* and *inhA* targets were separated due to lack of coverage of some important *fabG1* targets, and a *hsp65* primer pair was added for speciation of non-tuberculous mycobacteria (NTMs - see section 3.2.7). These changes resulted in two 6-plex primer mixes and one 5-plex primer mix (Table 3.41).

Table 3.41: Configuration 3 of multiplex primer mixes for tNGS amplification

Multiplex Group	Gene Target 1	Gene Target 2	Gene Target 3	Gene Target 4	Gene Target 5	Gene Target 6
1	*eis*	*embB*	*ethA*	*pncA*	*tlyA*	*hsp65*
2	*gyrA*	*rpoB*	*fabG1*	*rpsL*	*rplC*	*rv0678*
3	*inhA*	*rrs*	*gidB*	*rrl*	*katG*	N/A

M. bovis BCG spiked normal respiratory flora (NRF) sputum collected from the NNUH clinical microbiology laboratory was used for testing this multiplex configuration to simulate clinical samples. qPCR analysis identified that multiplex group 1 had two inhibited targets (*eis* and *embB*), while multiplex group 2 had one slightly inhibited target (*fabG1*). There was no inhibition evident in multiplex group 3 (Table 3.42). TapeStation analysis showed no evidence of non-specific amplification with this group configuration (Figure 3.10).

Table 3.42: Mean results of nested SYBR Green qPCR analysis on amplification of assay targets using multiplex primer group configuration 3 using triplicate samples

Multiplex Group	Gene Target	Nested qPCR Amplification ($\bar{X}$ C_T)
1	*eis*	15.12
	embB	22.75
	ethA	7.38
	pncA	8.60
	tlyA	7.22
	hsp65	9.13
2	*rv0678*	7.76
	gyrA	8.10
	rpoB	8.50
	fabG1	10.55
	rpsL	8.95
	rplC	7.26
3	*katG*	7.77
	gidB	7.99
	inhA	8.19
	rrs	8.91
	rrl	7.91

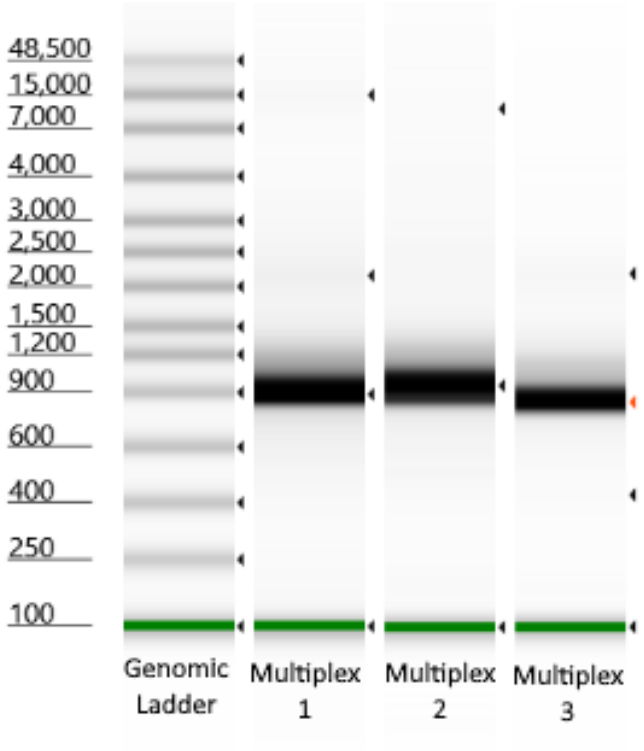

Figure 3.10: Multiplex configuration 3 TapeStation gel image showing no evidence of non-specific amplification identified as dual banding or loss of target amplicon in pooled triplicate samples for improved resolution.

A fourth multiplex configuration was then designed swapping *embB* from multiplex group 1 with *fabG1* from multiplex group 2 (Table 3.43). Redesigned primers for *rrs*, *rrl*, *tlyA*, *rpsL*, and *embB* were also introduced to normalize sequencing coverage between targets.

Table 3.43: Configuration 4 of multiplex primer mixes for tNGS amplification

Multiplex Group	**Gene Target 1**	**Gene Target 2**	**Gene Target 3**	**Gene Target 4**	**Gene Target 5**	**Gene Target 6**
1	*eis*	*fabG1*	*ethA*	*pncA*	*tlyA*	*hsp65*
2	*gyrA*	*rpoB*	*rpsL*	*embB*	*rplC*	*rv0678*
3	*katG*	*gidB*	*inhA*	*rrs*	*rrl*	N/A

Reconfigured multiplex groups were again tested using *M. bovis* BCG spiked NRF sputum. Nested qPCR identified inhibition of some multiplex group 1 (*eis, fabG1,* and *tlyA*), multiplex group 2

(*embB*), and multiplex group 3 (*rrs*) targets (Table 3.44). TapeStation analysis showed no evidence of non-specific amplification. (Figure 3.11).

Table 3.44: Mean results of nested SYBR Green qPCR experiment on amplification of multiplex configuration 4 multiplex primer group formulation using triplicate samples

Multiplex Group	**Gene Target**	**Nested qPCR Amplification ($\bar{X}$ C_T)**
1	*eis*	17.43
	fabG1	11.49
	ethA	8.01
	pncA	9.65
	tlyA	12.39
	hsp65	9.22
2	*rv0678*	9.63
	gyrA	11.06
	rpoB	10.52
	rpsL	10.71
	embB	14.86
	rplC	10.42
3	*katG*	8.60
	gidB	9.50
	inhA	8.75
	rrs	13.75
	rrl	8.40

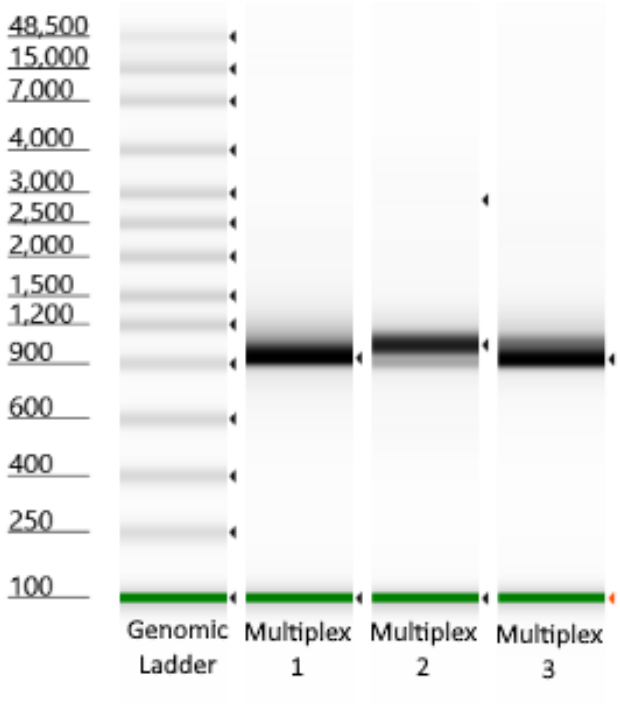

Figure 3.11: Multiplex configuration 4 TapeStation gel image showing no evidence of non-specific amplification identified as dual banding or loss of target amplicon in pooled triplicate samples for improved resolution.

Multiplex group 1 primer pairs worked well together. All but one primer pair in multiplex group 2 and multiplex group 3 amplified efficiently. Therefore, these groupings were chosen as the backbone for the next group configuration, and the poor performing primer pairs for *eis*, *fabG1*, *tlyA*, *embB*, and *rrs* were relocated (Table 3.45).

Table 3.45: Configuration 5 of multiplex primer mixes for tNGS amplification

Multiplex Group	**Gene Target 1**	**Gene Target 2**	**Gene Target 3**	**Gene Target 4**	**Gene Target 5**	**Gene Target 6**
1	*ethA*	*pncA*	*hsp65*	*rrs*	*embB*	N/A
2	*rv0678*	*gyrA*	*rpoB*	*rpsL*	*rplC*	*fabG1*
3	*katG*	*gidB*	*inhA*	*rrl*	*eis*	*tlyA*

This multiplex configuration was tested on M. bovis BCG spiked NRF sputum and assessed by TapeStation. Fragment size analysis identified no non-specific amplification or evidence of amplification failure. Multiplex performance was also tested using MinION sequencing. This

methodology allowed assessment of the breadth of coverage and relative amplification efficiency of each target. The samples were pooled and sequenced on the MinION as described in methods section 2.14. Reads were mapped against a reference FASTA file created by concatenating each target region (Methods section 2.16). Mapping with MiniMap2 and Qualimap (Methods section 2.16) identified a near total dropout of *embB*, low coverage of *fabG1*, *rrs* and *tlyA*, and notably high coverage of *hsp65* (Figure 3.12).

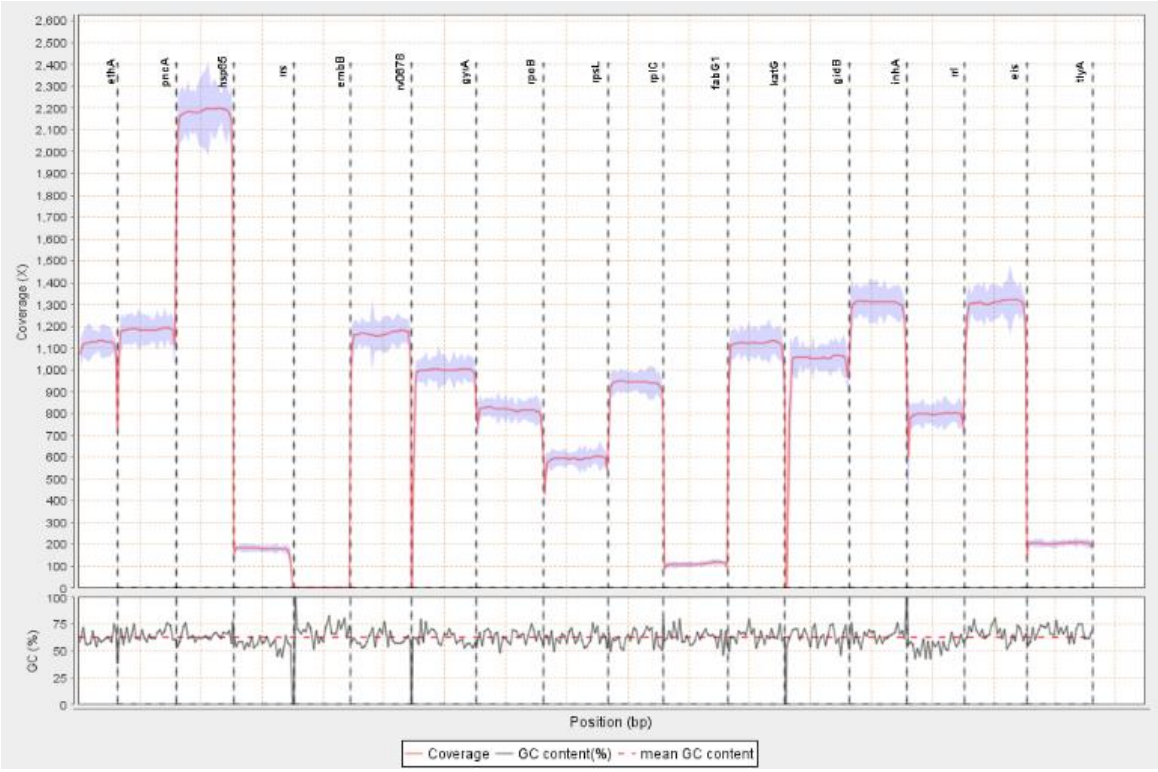

Figure 3.12: QualiMap visualization of multiplex configuration 5 sequenced reads mapped to a concatenated reference of assay gene targets using pooled triplicate samples for improved resolution.

Next, the problem *fabG1*, *rrs*, and *tlyA* target primers were grouped with *hsp65* (the best performing target) to promote even coverage in the other groups. The primer pair for *rpsL* was added to this group to create a five-plex. *embB* was moved out of multiplex group 1 into multiplex group 2 and the remainder of targets moved from multiplex group 1 were split between multiplex groups 2 and 3 (Table 3.46).

Table 3.46: Configuration 6 of multiplex primer mixes for tNGS amplification

Multiplex Group	Gene Target 1	Gene Target 2	Gene Target 3	Gene Target 4	Gene Target 5	Gene Target 6
1	*hsp65*	*rrs*	*rpsL*	*fabG1*	*tlyA*	N/A
2	*rv0678*	*gyrA*	*rpoB*	*ethA*	*rplC*	*embB*
3	*katG*	*gidB*	*inhA*	*rrl*	*eis*	*pncA*

The relative amplification efficiency of each target was assessed by nested qPCR. Results indicated inhibition of both *eis* and *embB*. The remainder of targets amplified with a mean range of 2.78 C_Ts, apart from *rpsL* which was a mean 2.29 C_Ts earlier than the next target (Table 3.47).

Table 3.47: Mean results of nested SYBR Green qPCR experiment on amplification of multiplex configuration 6 multiplex primer group formulation using triplicate samples

Multiplex Group	Gene Target	Nested qPCR Amplification ($\bar{X}$ C_T)
1	*hsp65*	10.86
	rrs	11.76
	rpsL	6.69
	fabG1	9.80
	tlyA	10.53
2	*rv0678*	10.98
	gyrA	9.23
	rpoB	10.22
	ethA	9.35
	rplC	9.58
	embB	19.77
3	*katG*	10.99
	gidB	8.98
	inhA	10.02
	rrl	9.93
	eis	18.94
	pncA	10.85

Multiple experiments indicated *eis* and *embB* were consistently inhibited. These target primer pairs were placed into a multiplex group together for focused redesign along with *rv0678*, *rrs*, and *fabG1*. *katG* and *hsp65* primers replaced *rv0678* and *embB* in multiplex group 2. Multiplex group 3 was completed with the addition of *tlyA* and *rpsL* (Table 3.48).

Table 3.48: Configuration 7 of multiplex primer mixes for tNGS amplification

Multiplex Group	**Gene Target 1**	**Gene Target 2**	**Gene Target 3**	**Gene Target 4**	**Gene Target 5**	**Gene Target 6**
1	*rv0678*	*eis*	*embB*	*rrs*	*fabG1*	N/A
2	*gyrA*	*rpoB*	*ethA*	*rplC*	*katG*	*hsp65*
3	*gidB*	*inhA*	*rrl*	*pncA*	*rpsL*	*tlyA*

Analysis of nested qPCR data showed inhibition in *eis* and *embB* primers (7.38 and 13.93 mean C_Ts later than the overall mean, respectively). The remainder of targets amplified more consistently exhibiting a total mean C_T range of 3.97 (Table 3.49).

Table 3.49: Mean results of nested SYBR Green qPCR experiment on amplification of multiplex configuration 7 multiplex primer group formulation using triplicate samples

Multiplex Group	**Gene Target**	**Nested qPCR Amplification ($\bar{X}$ C_T)**
1	*rv0678*	8.84
	eis	17.73
	embB	24.28
	rrs	10.95
	fabG1	10.63
2	*gyrA*	10.64
	rpoB	10.88
	ethA	7.7
	rplC	11.08
	katG	10.61
	hsp65	11.67
3	*gidB*	10.27
	inhA	9.62
	rrl	9.92
	pncA	11.26
	rpsL	9.57
	tlyA	11.66

Results indicated that while there was still some variation, e.g. multiplex group 2 with the early mean *ethA* C_T (7.7), multiplex groups 2 and 3 had relatively consistent amplification efficiency. There were continued issues with inhibition and/or competition with primers for *eis* and *embB*. Two experiments were performed in an attempt to solve these issues. First was the design of configuration 7 with doubled concentrations of primers for *eis* and *embB* (Table 3.50). Second, *embB* primers were swapped with multiplex group 2 member *ethA* and *eis was* swapped with *inhA* in multiplex group 3 to see if that would resolve primer interactions (Tables 3.51 & 3.52). Neither experiment improved the amplification efficiency of *eis* or *embB*. Doubling the primer concentrations caused significantly more inhibition in all groups.

Table 3.50: Mean results of nested SYBR Green qPCR experiment on amplification of multiplex configuration 7 multiplex primer group formulation with doubled eis and embB primer concentrations using triplicate samples

Multiplex Group	**Gene Target**	**Nested qPCR Amplification ($\bar{X}$ C_T)**
1	*rv0678*	18.46
	eis	25.22
	embB	27.29
	rrs	25.07
	fabG1	24.55
2	*gyrA*	19.84
	rpoB	20.89
	ethA	18.78
	rplC	22.62
	katG	20.51
	hsp65	21.23
3	*gidB*	18.77
	inhA	16.03
	rrl	16.26
	pncA	17.48
	rpsL	10.95
	tlyA	24.42

Table 3.51: Configuration 8 of multiplex primer mixes for tNGS amplification

Multiplex Group	**Gene Target 1**	**Gene Target 2**	**Gene Target 3**	**Gene Target 4**	**Gene Target 5**	**Gene Target 6**
1	*rv0678*	*rrs*	*fabG1*	*ethA*	*inhA*	N/A
2	*gyrA*	*rpoB*	*rplC*	*katG*	*hsp65*	*embB*
3	*gidB*	*rrl*	*pncA*	*rpsL*	*tlyA*	*eis*

Table 3.52: Mean results of nested SYBR Green qPCR experiment on amplification of multiplex configuration 8 multiplex primer group formulation using triplicate samples

Multiplex Group	**Gene Target**	**Nested qPCR Amplification ($\overline{X}$ C_T)**
1	*rv0678*	17.55
	rrs	16.16
	fabG1	10.94
	ethA	26.42
	inhA	15.88
2	*gyrA*	16.14
	rpoB	18.75
	rplC	22.43
	katG	20.30
	hsp65	20.69
	embB	24.97
3	*gidB*	15.78
	rrl	18.26
	pncA	17.04
	rpsL	21.12
	tlyA	24.83
	eis	25.31

Inefficient amplification of certain targets was a continuing issue, so alternative solutions were tested. Firstly, the multiplexes were reverted to configuration 7. Secondly, we tested increased $MgCl_2$ concentration to reduce stringency and improve efficiency. Three duplicate contrived clinical samples were prepared with additional 60mM $MgCl_2$; the first with 1µL, the second with 2µL, and the third with 3µL.

Increasing $MgCl_2$ concentrations reduced amplification efficiency in *eis* and *embB* compared to the control. No significant change in amplification efficiency for *rrs*, *rv0678*, or *fabG1* was detected with increased $MgCl_2$ concentrations (Table 3.53).

Table 3.53: Mean results of a nested SYBR Green qPCR experiment on the relative amplification of multiplex group 1 targets using multiplex primer configuration 7 with increased $MgCl_2$ concentrations using two sets of triplicate samples

Sample	**Replicate**	***eis* Nested qPCR Amplification ($\bar{X}$ C_T)**	***embB* Nested qPCR Amplification ($\bar{X}$ C_T)**	***rrs* Nested qPCR Amplification ($\bar{X}$ C_T)**	***rv0678* Nested qPCR Amplification ($\bar{X}$ C_T)**	***fabG1* Nested qPCR Amplification ($\bar{X}$ C_T)**
1µL 50mM $MgCl_2$	1	17.48	20.19	10.20	8.53	10.12
	2	18.12	20.80	10.83	8.29	10.64
2µL 50mM $MgCl_2$	1	18.84	21.57	10.00	8.74	9.87
	2	17.78	21.50	10.88	8.22	10.53
3µL 50mM $MgCl_2$	1	18.43	20.11	10.97	9.53	35.00
	2	19.13	20.83	10.51	9.04	10.62
Control	N/A	17.22	17.76	11.52	8.76	10.74

The most viable remaining option was to redesign the *eis* and *embB* primers. Multiple primer pairs were designed and tested for both targets – as detailed in section 3.2.2. The final primer design was selected by nested qPCR using configuration 7 groupings (Table 3.54). The inclusion of new primers resulted in efficient amplification of all multiplex group 1 targets, with the exception of *eis*. Primer design option 1 in multiplex configuration 7 was used for subsequent experimentation on specificity, sensitivity, LoD, and clinical validation. *eis* was redesigned to provide better amplification efficiency as described in section 3.2.2.

Table 3.54: Configuration 7 mean qPCR amplification results for relative amplification of multiplex group 1 targets using three redesigned eis primer pair options using triplicate samples

Sample	***eis* Nested qPCR Amplification ($\bar{X}$ C_T)**	***embB* Nested qPCR Amplification ($\bar{X}$ C_T)**	***rrs* Nested qPCR Amplification ($\bar{X}$ C_T)**	***rv0678* Nested qPCR Amplification ($\bar{X}$ C_T)**	***fabG1* Nested qPCR Amplification ($\bar{X}$ C_T)**
Option 1	13.61	8.03	6.00	6.00	7.39
Option 2	35.00	9.73	6.94	7.49	8.77
Option 3	16.70	9.82	6.51	6.00	8.19
H_2O Control	35.00	35.00	31.87	29.13	35.00

3.2.3.3: Multiplex Optimisation Summary

As presented throughout section 3.2.3, creating a sensitive multiplex configuration which yielded amplicons for all targets required multiple iterations. 5-plex PCRs were chosen as the minimum multiplex level to limit the number of total PCR reactions required per test. More PCR reactions would increase assay cost and increase risk of contamination. Early experiments to consolidate all primers into a single multiplex proved unsuccessful. These attempts consistently resulted in dropout of multiple target amplicons and would have required extensive redesign of target primers. Due to time limitations, one 5-plex and two 6-plex PCRs were designed and optimised for the assay. This use of multiple reactions is a disadvantage compared to e.g. the GenoScreen test that has a single multiplex PCR reaction. However, the sensitivity is better and the turnaround time significantly shorter than reported by GenoScreen [116]. It should be noted that the 3 multiplexes have since been consolidated into one by colleagues in the O'Grady Group.

3.2.4: Optimization of Sample Extraction for Amplification of Drug Resistance Gene Targets in Multiplex

3.2.4.1: Comparison of Nucleic Acid Extraction Methods for Sedimented Samples

Towards the end of the study the MagNA Pure was being discontinued by Roche so it was important to choose an alternative automated extraction device. The MagNA Pure was compared to the Promega Maxwell RSC from decontaminated sedimented samples (as our diagnostic methods needed to be capable of working on both sputum and sedimented decontaminated sputum for Seq&Treat). DNA yield for each system was compared by qPCR using two gene targets, *eis,* and *tlyA* (Table 3.55). Results showed a mean loss of 1.55 C_Ts (2.9 fold) for *eis* and a mean increase of 2.37 C_Ts (5.2 fold) for *tlyA* in Maxwell over MagNA Pure. As this did not indicate a significant difference in extraction efficiency (Paired T-Test: $p>0.05$) performance of the two machines was deemed similar, so MagNA Pure could safely be replaced with Maxwell.

Table 3.55: Comparison of two automated extraction methods for extraction of nucleic acids from sedimented spiked NRF sputum samples using triplicate samples

Nucleic Acid Extraction Method	**qPCR Amplification Nested Primer Set**	**SYBR Green qPCR Assay ($\overline{X}$ C_T)**
MagNA Pure Compact	eis	21.18
	tlyA	25.65
Promega Maxwell RSC	eis	22.73
	tlyA	23.28

The Promega Maxwell Extraction was superior to the MagNA Pure for extraction of sedimented samples. The cause for this difference is currently unknown. This superiority is potentially due to differences in bead concentration between machine reagent cartridges. Alternatively, the use of Roche BLB during mechanical lysis during both extraction methods may prove more compatible with the Roche MagNA Pure than the Promega Maxwell improving the DNA yield in the former. Use

of an alternative buffer during mechanical lysis may potentially improve DNA yields in the Promega Maxwell to the level seen in the MagNA Pure Compact. However, results were consistently better for MagNA Pure in raw sputum samples, and Promega Maxwell in sedimented sputum samples.

Optimisation of sedimented sample extraction (section 3.2.4.3) showed that altering the reagent in which bead-beating was performed, and adjusting the pre-extraction reagent volumes, increased Maxwell extraction efficiency.

3.2.4.2: Comparison of Maxwell Extraction Kits for Mycobacterial Extraction

To identify the most efficient Maxwell DNA extraction kit, two kits were tested; PureFood Pathogen and Cultured Cells. Two NaOH/NALC-NA decontaminated samples were bead beaten in PBS and another two were bead beaten in BLB (Bacterial Lysis Buffer, Roche) followed by extraction (one of each sample type) using the 2 Maxwell kits. Resulting elutes were quantified by SYBR Green qPCR using hsp65 primers to detect mycobacterial DNA (Table 3.56).

qPCR results showed PBS prepared samples were a mean 3.12 C_Ts earlier than BLB bead-beaten samples. This difference represented a significant difference in efficiency between the two kits (Paired T-Test: $p<0.0001$). Also, if PBS is used during mechanical lysis then the PureFood Pathogen and Cultured Cells kits perform similarly well but cultured cells kit had poorer extraction efficiency bead beating in BLB. The Maxwell PureFood Pathogen method using PBS was chosen for subsequent use.

Table 3.56: Mean qPCR quantification C_T results for comparison of two automated nucleic acid extraction kits using two different buffers during bead-beating using triplicate samples

Sample	**Sample Treatment**	**SYBR Green qPCR Assay ($\overline{X}$ C_T)**	**Difference between BLB and PBS ($\overline{X}$ ΔC_T)**
PureFood Pathogen	BLB	23.93	0.72 (1.6 fold)
	PBS	23.21	
Cultured Cells	BLB	27.05	4.15

	PBS	22.90	(17.7 fold)

3.2.4.3: Optimization of NaOH/NALC-Na Decontamination Protocol for Use with Low Sample Volumes

FIND initially provided us with sputum (~1.5ml/sample) for tNGS assay validation but later requested that validation be performed on decontaminated sputum. The WHO recommended sputum decontamination method is designed for 2-5mL sputum. We used half of the sample volume for sputum testing so had only 750ul sputum remaining for decontamination and testing. Therefore, working volumes were reduced and adjustments were made to avoid loss of biomass when working with invisible pellets.

The first method was approached mathematically to determine the maximum sample size effective within a 1.5mL Eppendorf to accommodate the maximum tube size for the benchtop centrifuge available and biosafety requirements of screw-cap tubes in the QIB CL3 facility. The decontamination method calls for equal volumes of liquid sample and NaOH/NALC-Na solution to be added, followed by a 2x volume of phosphate buffer solution, so 250µL of sample and 250µL decontaminant solution plus 1mL of phosphate buffer solution fit in the 1.5mL tube.

Following the change to 1.5mL Eppendorfs a direct comparison was conducted between decanting and pipetting supernatant (duplicate samples tested). Results indicated no significant difference (Paired T-Test: $p>0.05$) in nucleic acid yields between pipetting or decanting and so the more easily replicable pipetting method was selected (Table 3.57).

Table 3.57: Mean qubit quantification of paired samples for comparison of decanting and pipetting supernatant in a head-to-head trial using two sets of triplicate samples

Sample Set	**Sample Treatment**	**Mean Qubit Concentration (ng/µL)**	**Mean Difference Between Decanting and Pipetting (Δng/µL)_**
Replicate 1	Decanted	0.14	0.03
	Pipetted	0.11	

Replicate 2	Decanted	0.11	0.01
	Pipetted	0.10	

Further experimentation was performed to determine the amount of target DNA (*M. bovis* BCG) lost during NaOH/NALC-Na decontamination. Nucleic acid yield from non-decontaminated controls and decontaminated samples were quantified by SYBR Green qPCR using two target genes, *inhA* and *eis* (Table 3.58). Results showed that decontamination, even under optimized conditions, resulted in a significant loss of nucleic acid of approximately 380-fold (Paired T-Test: $p<0.0001$). However, prior to optimization the DNA loss was approximately 10,733-fold. Therefore, despite the continued loss of nucleic acid the optimized method was significantly improved over the original (Paired T-Test: $p<0.0001$).

Table 3.58: Mean qPCR C_T results of two M. bovis BCG gene targets for comparison of nucleic acid yields in decontaminated versus non-decontaminated samples using triplicate samples.

Sample	**qPCR Target**	**Sample Treatment**	**NaOH/NALC-Na qPCR Assay ($\overline{X}$ C_T)**	**DNA Loss ($\overline{X}$ ΔC_T)**
A338	eis	Decontaminated	24.77	9.21 (592.2 fold)
		Not Decontaminated	15.56	
	inhA	Decontaminated	25.19	7.40 (168.9 fold)
		Not Decontaminated	17.79	

Further optimization of decontaminated sample extraction adjusted incubation time and reagent volumes used prior to Maxwell extraction. 400µL of mechanically lysed sample was combined with 40µL Proteinase K and 200µL Lysis Buffer A from the Promega PureFood Pathogen kit. The mixture was incubated for 10 minutes at 65°C before adding 400µL of PBS and 300µLof Promega lysis buffer. The 1,300µL sample was loaded into the Maxwell cartridge instead of the smaller 400µL sample, 300µL lysis buffer, and 20µL Proteinase K (720µL total) sample used previously. This optimisation facilitated an LoD of 50-100 CFU/mL, equivalent to the Cepheid GeneXpert (Xpert) MTB/RIF test [164], and better than GenoScreen Deeplex Myc-TB (100-1,000 CFU/mL) [165] and was used for subsequent

extraction of decontaminated samples. However, for improved LoDs and extraction a selective depletion protocol would need to be developed to reduce competition and inhibition from commensal organisms.

3.2.4.3.1: Why Optimise Sputum Decontamination?

The tNGS assay was initially validated using primary sputum samples. This would be practical in settings where the tNGS assay is used for primary diagnosis and DST, as it decreases complexity and the risk of contamination. However, most labs will continue to perform culture and smear microscopy, alongside molecular techniques, requiring initial decontamination to inactivate commensal bacteria and concentrate samples. To accommodate these workflows, the tNGS assay also required validation with existing sample preparation protocols.

Preliminary testing of the decontamination and sedimentation protocol resulted in significant loss (99%) of available sample DNA, making optimisation a priority. While the protocol as written is practical for clinical sample volumes (>5mL), small volumes (<1mL) such as those used for experimentation and optimization are more sensitive to sample loss (section 3.2.4.3). One area of concern was loss of pellet mass during transfer of the supernatant. Experimentation with supernatant removal methods (decanting and pipetting) indicated that this was unlikely to be a cause of DNA loss, as both protocols yielded similar extracted DNA concentrations under experimental conditions.

Improved sample concentrations following optimisation of working volumes and tube size indicated that this may have been the source of sample loss. Use of smaller working volumes in a 15mL falcon tube was identified as the probable issue. Scaling down the reaction and performing decontamination in a 1.5mL Eppendorf tube reduced DNA loss by approximately 10,400 fold, resulting in similar performance of the test in decontaminated sputum compared to sputum.

3.2.5: Optimisation of PCR Conditions

Experimentation was performed to optimise reagents and cycling conditions for sensitive and specific amplification of *M. tuberculosis* DNA. Simplex reactions were initially amplified using Takara PrimeSTAR Max master mix with SYBR Green dye for quantification. 18µL of working master mix and 2µL *M. bovis* BCG DNA template were amplified in a LightCycler 480 following Takara protocols (Table 3.59)

Table 3.59: Cycling conditions for Takara simplex amplification of tNGS assay primers

Step	**Temperature (°C)**	**Time (mm:ss)**	**Cycles (#)**
Initial Denaturation	95	05:00	1
Denaturation	98	00:10	35
Annealing	60	00:05	
Extension	72	00:10	
Final Extension	72	05:00	1

Analysis identified no amplification failures, though amplification efficiency varied by target (Figure 3.13). However, TapeStation analysis showed non-specific amplification in 8/15 simplexes (Figure 3.14).

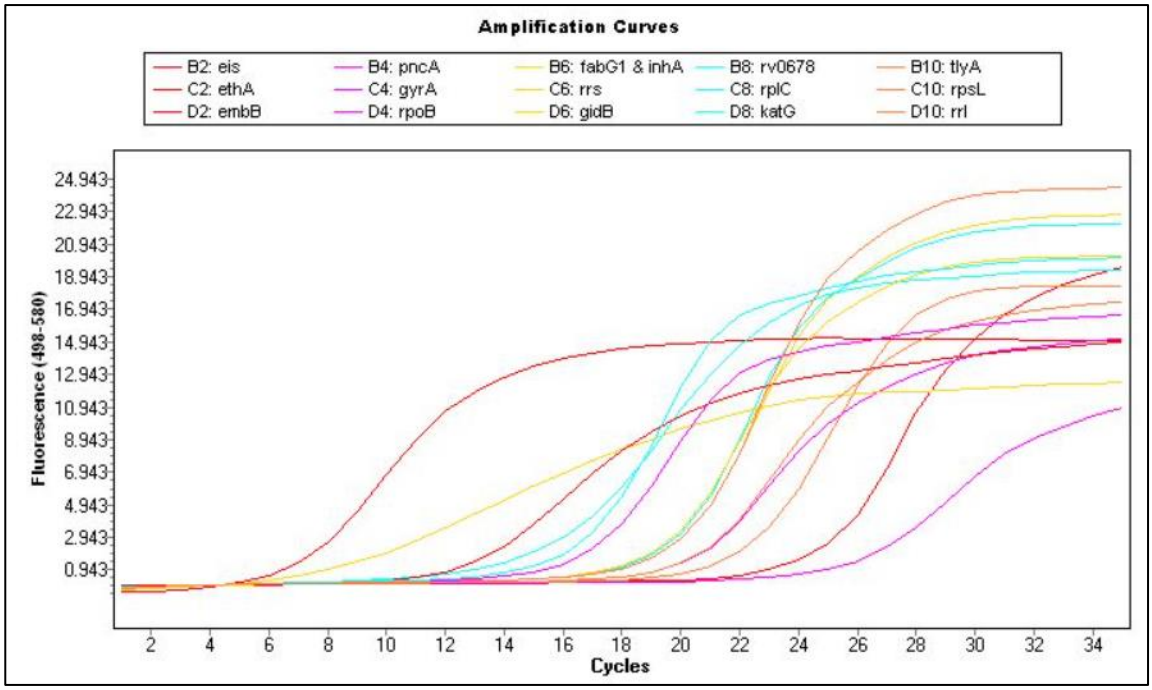

Figure 3.13: One of a triplicate set of qPCR amplification curves for simplex assay primers using Takara amplification

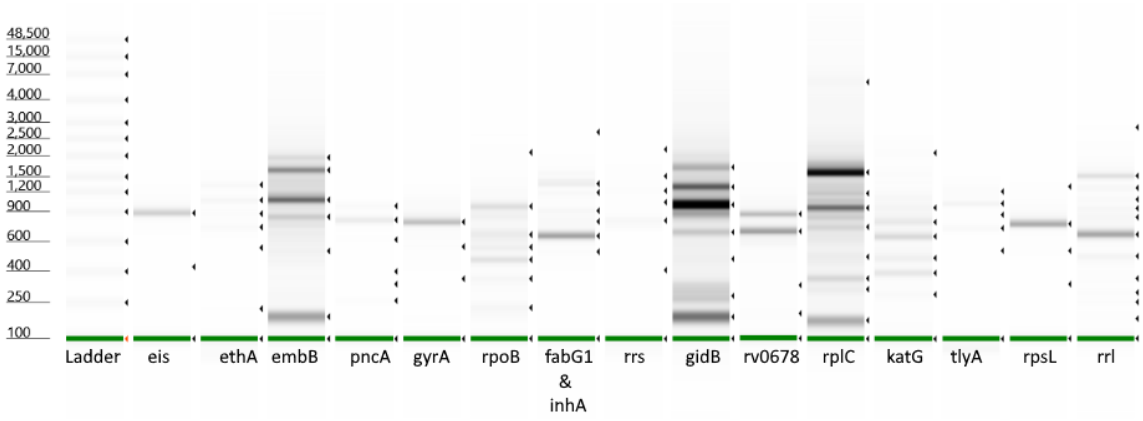

Figure 3.14: TapeStation analysis of PCR products indicating non-specific amplification in embB, rpoB, fabG1/inhA, gidB, rv0678, rplC, and katG reactions using pooled triplicate samples for improved resolution

To determine if the non-specific amplification was related to the mastermix; *ethA*, *embB*, *gidB*, and *rplC* were amplified using Roche SYBR Green master mix and recommended cycling conditions (Table 3.60). Analysis by TapeStation showed use of the SYBR Green master mix reduced non-specific amplification in all targets (Figure 3.15). These results demonstrated that the Takara mix was the cause of the non-specific amplification and an alternative was required.

Table 3.60: Cycling conditions for SYBR Green simplex amplification with Takara temperatures

Step	Temperature (°C)	Time (mm:ss)	Cycles (#)
Initial Denaturation	95	05:00	1
Denaturation	98	00:15	35
Annealing	60	00:15	
Extension	72	01:00	
Final Extension	72	05:00	1

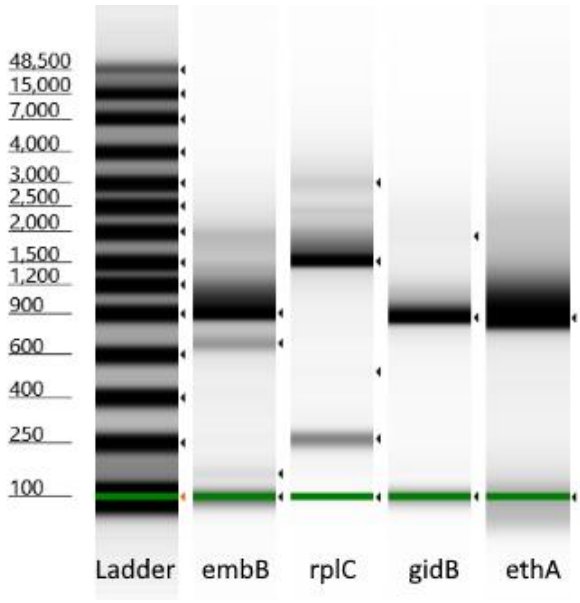

Figure 3.15: TapeStation analysis of PCR for embB, rplC, gidB, and ethA using Sybr Green mastermix using pooled triplicate samples for improved resolution

The SYBR Green master mix was used to amplify targets in triplex reactions. Cycling conditions were amended to be more suitable for the multiplex reaction (Table 3.61). Melt-curve analysis was included to identify non-specific amplification and primer dimers.

Table 3.61: NEB cycling conditions used for triplex amplification with SYBR Green master mix

Step	Time (mm:ss)	Temperature (°C)	Cycles
Initial Denaturation	05:00	98	1
Denaturation	00:30	98	35
Annealing	01:00	62	
Elongation	01:00	72	
Final Elongation	10:00	72	1
Melt	N/A	98	1

Cooling	01:00	37	1

Analysis showed triplex groups amplified at similar C_Ts with no evident inhibition or competition within groups (Figure 3.16). Melt-curve analysis showed some evidence of non-specific amplification and primer-dimer formation within triplex reactions (Figure 3.17). TapeStation analysis was also performed (Figure 3.18), showing no non-specific amplification in triplexes 1, 3, and 5. Triplex 2 had extra bands at 250bp and 400bp while triplex 4 had smearing from 1,000bp to approximately 1,400bp.

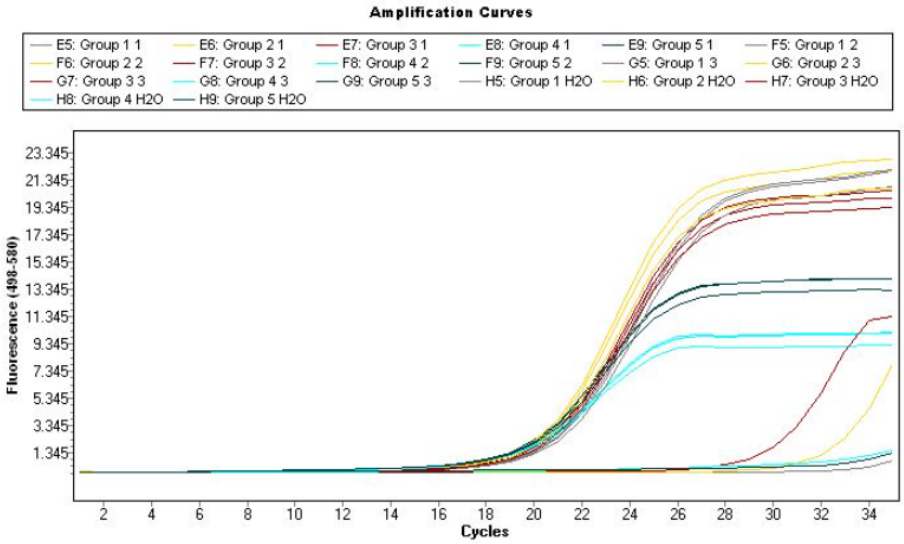

Figure 3.16: qPCR amplification curves for one of a triplicate set of triplex reactions amplified using NEB cycling conditions with SYBR Green master mix

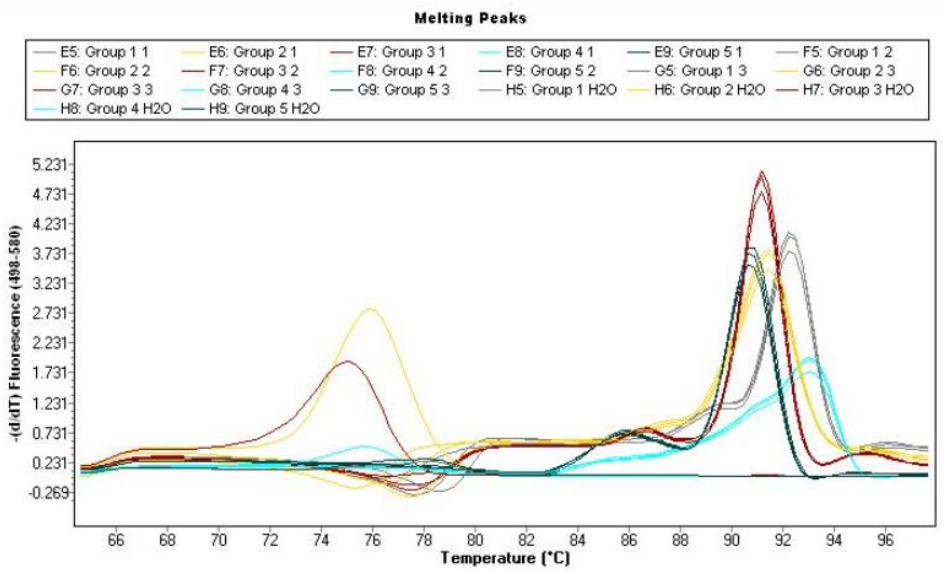

Figure 3.17: qPCR melt curves for one of a triplicate set of triplex reactions amplified using NEB cycling conditions with SYBR Green master mix

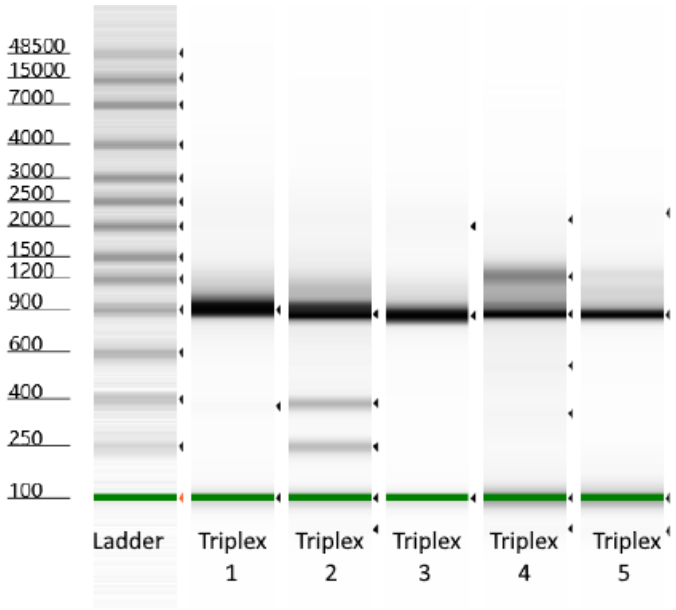

Figure 3.18: TapeStation analysis of five triplex PCRs amplified using the SYBR Green kit using pooled triplicate samples for improved resolution

Five triplex reactions would be too costly and laborious to perform, so it was decided to develop 3 multiplex assays each containing 5 targets instead. Roche probe master PCR mix was substituted for SYBR Green master mix (as the PCR products could not be sequenced with Sybr Green dye intercalated into the DNA backbone) using the same PCR conditions. qPCR incorporating *M. bovis* BCG probes showed similar amplification between each 5-plex groups (Figure 3.19). TapeStation

showed 5-plex group 1 exhibited no non-specific amplification. However, 5-plex group 2 failed to generate amplicons of the desired size and 5-plex group 3 exhibited smearing from 100bp to 1,000bp (Figure 3.20).

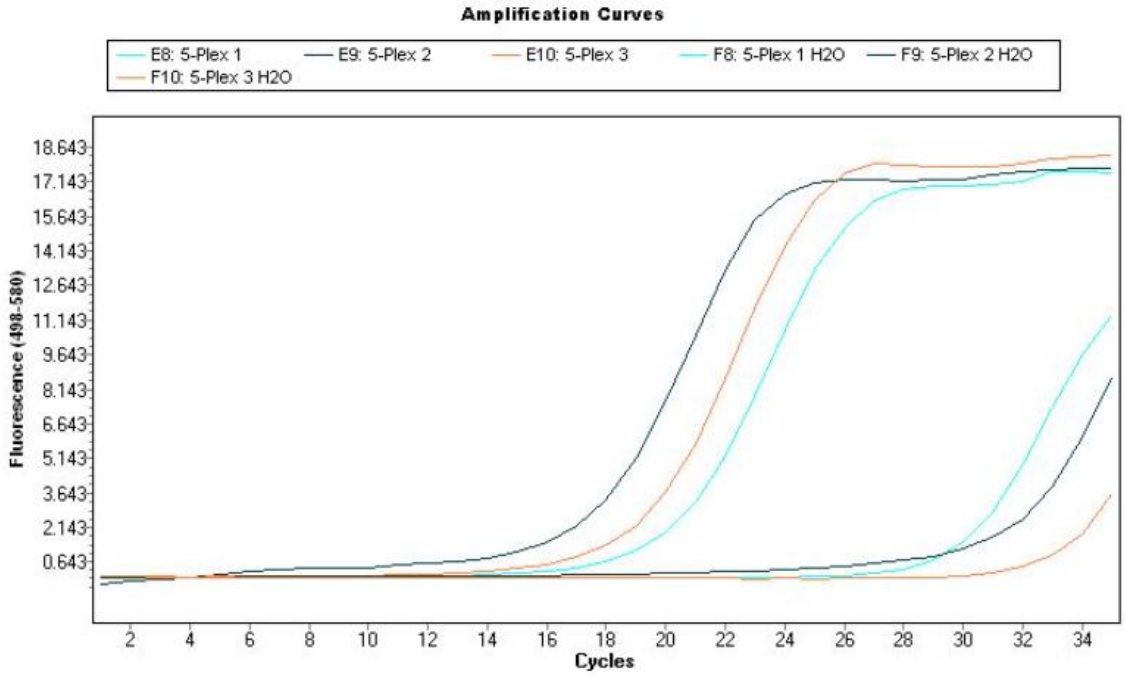

Figure 3.19: qPCR amplification curves for one of a triplicate set of 5-plex reactions

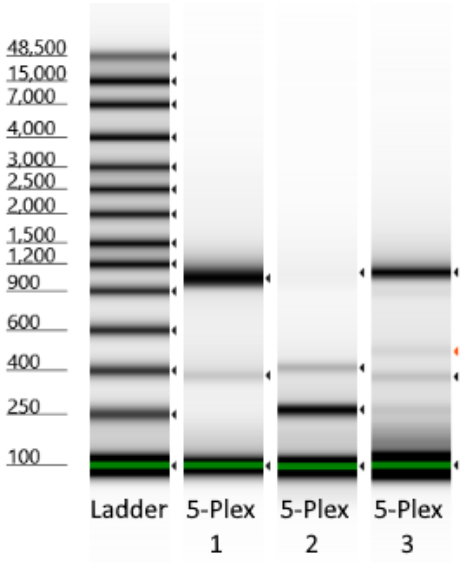

Figure 3.20: TapeStation fragment size analysis of three 5-plex reactions using pooled triplicate samples for improved resolution

Further amplification optimization was performed using master mixes designed specifically for multiplex amplification. The first was a multiplex kit from Qiagen and the second from NEB. Both methods were tested to determine the one with the best amplification yield and specificity. DNA from spiked NRF sputum was amplified according to manufacturers' protocols (Tables 3.62 & 3.63). Comparison by TapeStation indicated NEB exhibited greater variability in amplification specificity than Qiagen (Figure 3.21). Analysis also showed variability in amplicon yield for NEB (Range = 34.24 ng/µL) while Qiagen yields were generally higher and more consistent (Range = 6.3 ng/µL) with slightly less non-specific amplification and a cleaner negative control (Table 3.64).

Table 3.62: Qiagen Multiplex kit PCR cycling conditions

Step	**Time (mm:ss)**	**Temperature (°C)**	**Cycles**
Heat Activation	15:00	95	1
Denaturation	00:30	94	35
Annealing	01:30	60	
Extension	01:30	72	
Final Extension	10:00	72	1

Table 3.63: NEB Multiplex master mix PCR cycling conditions

Step	**Time (mm:ss)**	**Temperature (°C)**	**Cycles**
Initial Denaturation	01:00	95	1
Denaturation	00:20	95	35
Annealing	01:00	60	
Extension	01:30	68	
Final Extension	05:00	68	1

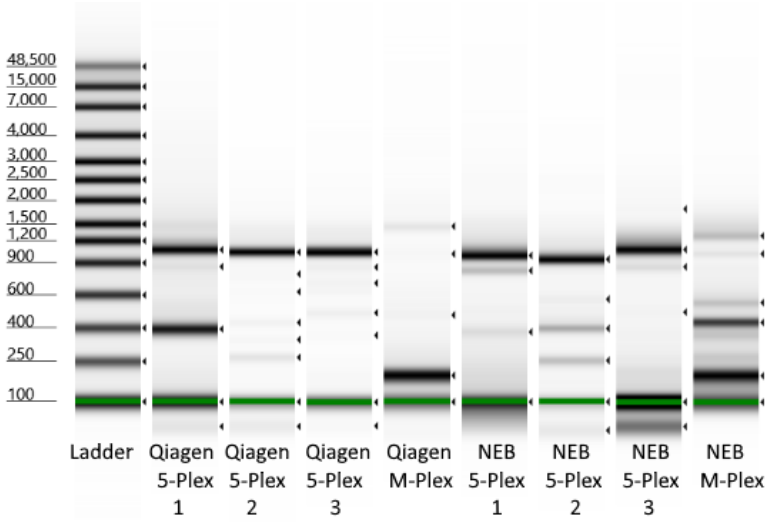

Figure 3.21: TapeStation analysis of the 3 5-plex reactions using Qiagen and NEB mastermixes (including a negative control for each mastermix) using pooled triplicate samples for improved resolution

Table 3.64: Mean post-amplification DNA concentrations for Qiagen and NEB mastermixes

Amplification Kit	**Sample**	**DNA Concentration ($\bar{X}$ ng/µL)**
Qiagen	5-Plex Group 1	17.9
	5-Plex Group 2	20.0
	5-Plex Group 3	13.7
NEB	5-Plex Group 1	7.46
	5-Plex Group 2	41.7
	5-Plex Group 3	8.08

The Qiagen kit was determined to be superior in sensitivity and specificity and was selected for multiplex PCR for the remainder of the study.

3.2.5.1: Why PCR Reagent Optimisation Matters

While metagenomic tests don't specifically amplify target DNA prior to sequencing, tNGS assays are defined by it. Identification of the optimal amplification protocol for target amplicons was needed, especially for multiplex reactions. Simplex and duplex qPCR assays can use most PCR master mixes

without a loss of sensitivity or specificity. However, as reaction complexity increases, tolerances decrease, requiring more careful selection of PCR reagents to avoid target loss, amplification failure, and/or non-specific amplification.

As addressed in section 3.2.5, use of PCR master mixes designed for simplex reactions [SYBR Green and Roche Probe] resulted in loss of target amplicons and significant non-specific amplification in multiplex. Two pre-optimised multiplex master mixes were tested. The Qiagen multiplex kit tested has been used in screening for genetic disorders and forensic investigation [166,167]. In addition, a study from 2014 showed this multiplex kit performed optimally for detection of bacterial infections [168].

The NEB multiplex master-mix tested has been used in conjunction with NGS for diagnosis and monitoring of cancers as well as assessing bacteria in environmental samples [169–171]. It has also been used in experiments directly diagnosing TB from clinical samples [172]. The combination of manufacturer optimised reagent concentrations and use of proprietary Q-solution for improved amplification of GC-rich template resulted in the Qiagen kit outperforming the NEB master mix for multiplex amplifications in our hands.

3.2.6: Development of External Assay Controls

To ensure all steps following DNA extraction performed as expected in every tNGS run, a set of external controls were designed in collaboration with the Garvan Institute for Medical Research in Australia. Three controls were created, one for each multiplex group. Controls were designed using concatenated gene target sequences approximately 6,000bp each (Appendix III). Controls were synthesized by Invitrogen and received at a stock concentration of 5ng/µL, approximately 758 x 10^6 DNA copies per µL. Stocks were diluted to approximately 10,000 copies per µL using three dilution steps. The controls were used as template in 3 multiplex groups, then combined and sequenced as with test samples. Amplification and sequencing indicated control sequences performed as expected for a fully susceptible target profile when assessed using the Epi2Me TB

Resistance Profile Pipeline (Figure 3.22). External controls were subsequently incorporated into every sequencing run. All 16 gene targets in the control must be detected, without any mutations, for the sequencing run to be considered valid.

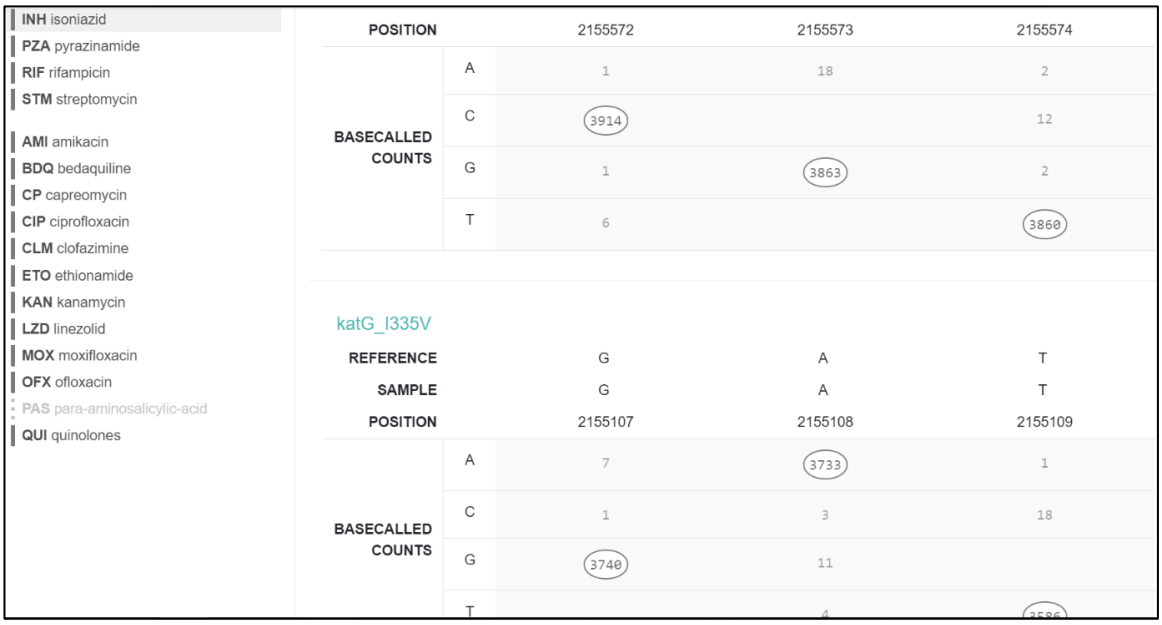

Figure 3.22: Screenshot of Epi2Me TB Resistance Profile Pipeline output for external controls showing expected fully susceptible profile as designed

As the multiplex amplifies multiple targets of a similar size it can be difficult to identify when a problem occurs, especially as there are a number of steps in library preparation that can lead to loss of amplicon; e.g. the bead washes. Contamination is also a concern as PCR is highly sensitive and TB amplicons will be present in the laboratory after the test starts to be used. These issues can lead to false positive and negative results, which are dangerous in a clinical setting - it is important the assay can monitor for problems that would lead to incorrect results. To monitor the test process post DNA extraction, three external controls (one per multiplex reaction) were developed for inclusion in the assay as discussed previously. These consisted of synthetic fragments of DNA containing all the targets for the relevant multiplex (approx. 6Kb long). The relevant control was added to a separate external control reaction for each multiplex and these were then processed in the exact same way as the test samples. The expected result for the external positive control after

sequencing and analysis was fully susceptible *M. tuberculosis* reads with minimum 20x coverage of all 17 targets. If there were target dropouts or any resistance SNPs detected, the run was considered invalid due to the risk of false positive or false negative results. The external controls are highly concentrated and the preparation and use of them needed to be performed with caution. Throughout testing and validation no contamination events were detected, however, for future implementation the use of internal controls (a human target or a spiked difficult to lyse bacterium that is not clinically relevant) is preferable.

Replacement of external controls with internal controls will further improve the viability of the tNGS assay in clinical settings. Removing the need for extra reagents, especially highly concentrated ones such as the controls, decreases risk of cross-contamination and false positives. The cost of controls will need to be addressed however as incorporation will likely increase the tNGS assay's cost per sample.

A no-template control, which swapped molecular grade water for template DNA, was also included for every test run to monitor for contamination. If more than three targets had >20 reads in the no-template control, the run was deemed invalid.

3.2.7: Inclusivity and Specificity Testing

After finalizing tNGS assay primer pairs and multiplex groups, specificity testing was performed. Inclusivity testing was included to assure the coverage of non *M. tuberculosis* members of the MTBC were detectable by the assay. Initial inclusivity/specificity testing used NRF sputum spiked with *M. tuberculosis* and *M. bovis* BCG DNA. Samples were extracted, multiplexed, and sequenced before uploading basecalled fastQ files to the Epi2Me WIMP pipeline for identification of reads. Any species identified above 1% of the microbial reads were recorded (Table 3.65).

Table 3.65: Total identified reads across all samples for mixed samples analyzed using the Epi2Me WIMP pipeline

Organism Identified at 1% Cutoff	**Reads Reported by Epi2Me WIMP Pipeline (#)**	**% of Total Reads**
Mycobacterium tuberculosis	148,365	45.9
Mycobacterium bovis	17,139	5.3
Pseudomonas aeruginosa	3,865	1.2
Homo sapiens	3,714	1.15

The tNGS assay amplified both *M. tuberculosis* and *M. bovis* as expected, however, there were also a small percentage of*Pseudomonas aeruginosa* reads identified. BLAST analysis of the reads revealed these to be 16S and 23S regions of the *P. aeruginosa* genome. Mapping the reads to our tNGS TB amplicon reference sequence using Qualimap (Figure 3.23) confirmed this to be the case, mapping to the *rrs* (16S) and *rrl* (23S) gene targets. Some low-level non-specific amplification of 16S and 23S regions of other bacteria wasn't unexpected and didn't affect the detection of the target species.

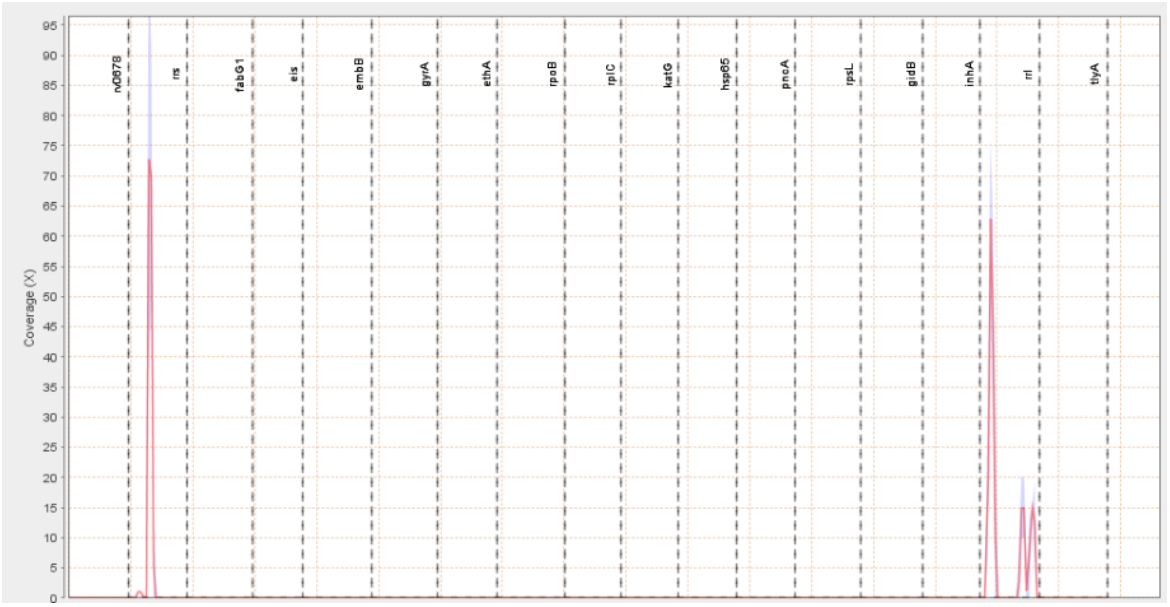

Figure 3.23: QualiMap visualization of Pseudomonas aeruginosa reads mapped onto the TB tNGS assay gene target reference to identify areas of cross-reactivity

A second inclusivity experiment used *Mycobacterium africanum* DNA to assess assay performance in other MTBC members. Approximately 100 cell equivalents (CE) of *M. africanum* DNA was spiked into an NRF sputum sample. Following amplification and sequencing reads were analysed by both Epi2Me WIMP and Epi2Me TB Resistance Profile pipelines.

WIMP analysis identified no non-target reads above the 1% cutoff. Due to the high homology between MTBC organisms, all *M. africanum* reads were identified as *M. tuberculosis* by the automated pipeline (Table 3.66). The TB Resistance Profile analysis did not identify any target dropouts and reads were also mapped for visualization of target coverage. This indicated that all resistance gene targets had at least 500x coverage with a relatively low input of 100 CE. Inclusion of non-*M. tuberculosis* MTBc species did not negatively impact the target coverage of the assay (Figure 3.24).

Table 3.66: Identified reads in three multiplex group samples spiked with M. africanum analyzed using the Epi2Me WIMP Pipeline

Sample	**Organism Identified at 1% Cutoff**	**Reads Reported by Epi2Me WIMP Pipeline (#)**	**% of Sample Reads**

Multiplex Group 1	*Mycobacterium tuberculosis*	4,390	44.6
Multiplex Group 2	*Mycobacterium tuberculosis*	5,506	37.9
Multiplex Group 3	*Mycobacterium tuberculosis*	5,754	41.8

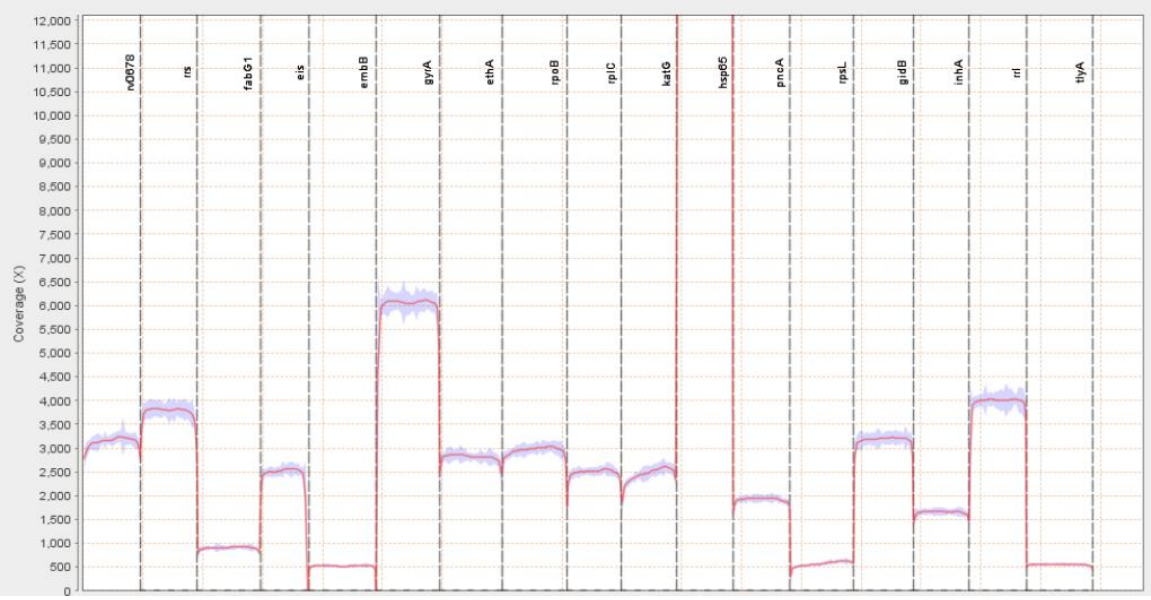

Figure 3.24: QualiMap visualization of M. africanum reads mapped onto the TB tNGS assay gene target reference

Further specificity experimentation was performed *in silico* using fastQ files of clinically important NTMs obtained from the NCBI database. Five NTM species as well as *M. leprae* were selected, the reads for each covered the full breadth of each genome (Table 3.67). Organism fastQ files were mapped to the tNGS assay reference using MiniMap2 and Qualimap (Figure 3.25). Locations exhibiting most non-specific reactivity were *rrs* and *rrl* gene targets which correspond with the 16S and 23S genes.

Table 3.67: A list of NTM and M. leprae genomes used for in-silico specificity testing

Organisms with Reference fastQ Files Obtained
Mycobacterium avium
Mycobacterium kansasii
Mycobacterium leprae

Mycobacterium marinum
Mycobacterium ulcerans
Mycobacterium abscessus

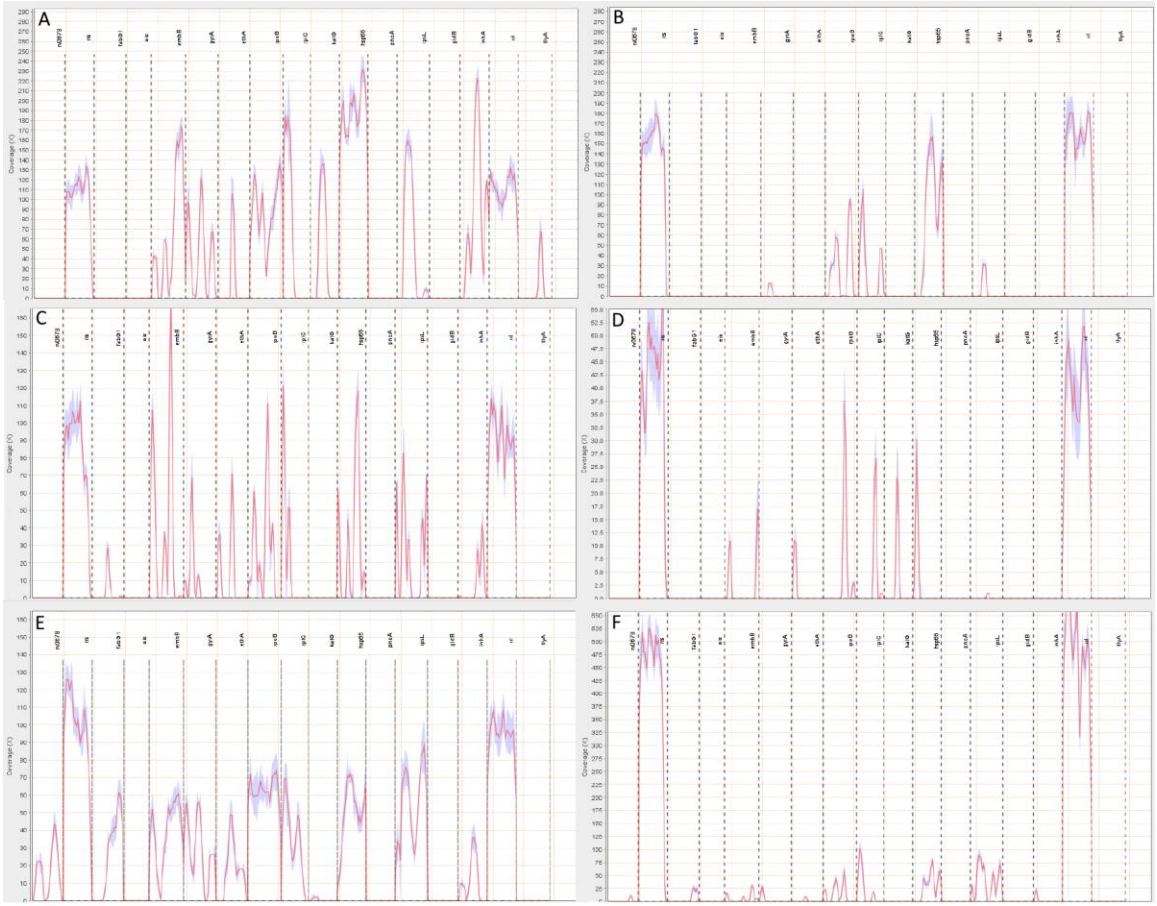

Figure 3.25: QualiMap visualization of 5 NTM genomes and M. leprae mapped onto the TB tNGS assay gene target reference to identify areas of potential nonspecific assay reactivity. A: M. avium, B: M. abscessus, C: M. kansasii, D: M. leprae, E: M. ulcerans, and F: M. marinum

Inclusivity of the assay in members of the MTBc was also tested *in silico*. As before, fastQ files were obtained from the NIH database for 6 MTBC members; *M. tuberculosis, M. africanum, M. bovis, M. bovis* BCG*, M. caprae,* and *M. pinnipedii*. fastQ files were mapped against the tNGS reference for identification of target dropouts (Figure 3.26). Analysis identified no target dropouts in the tested MTBc members. Lower coverage of *rrl* and *rrs* was detected in both *M. bovis* and *M. pinnipedii*, approximately 2- and 4- fold lower, respectively. The underlying cause for this discrepancy was unclear as all MTBC have identical 16S and 23S sequences.

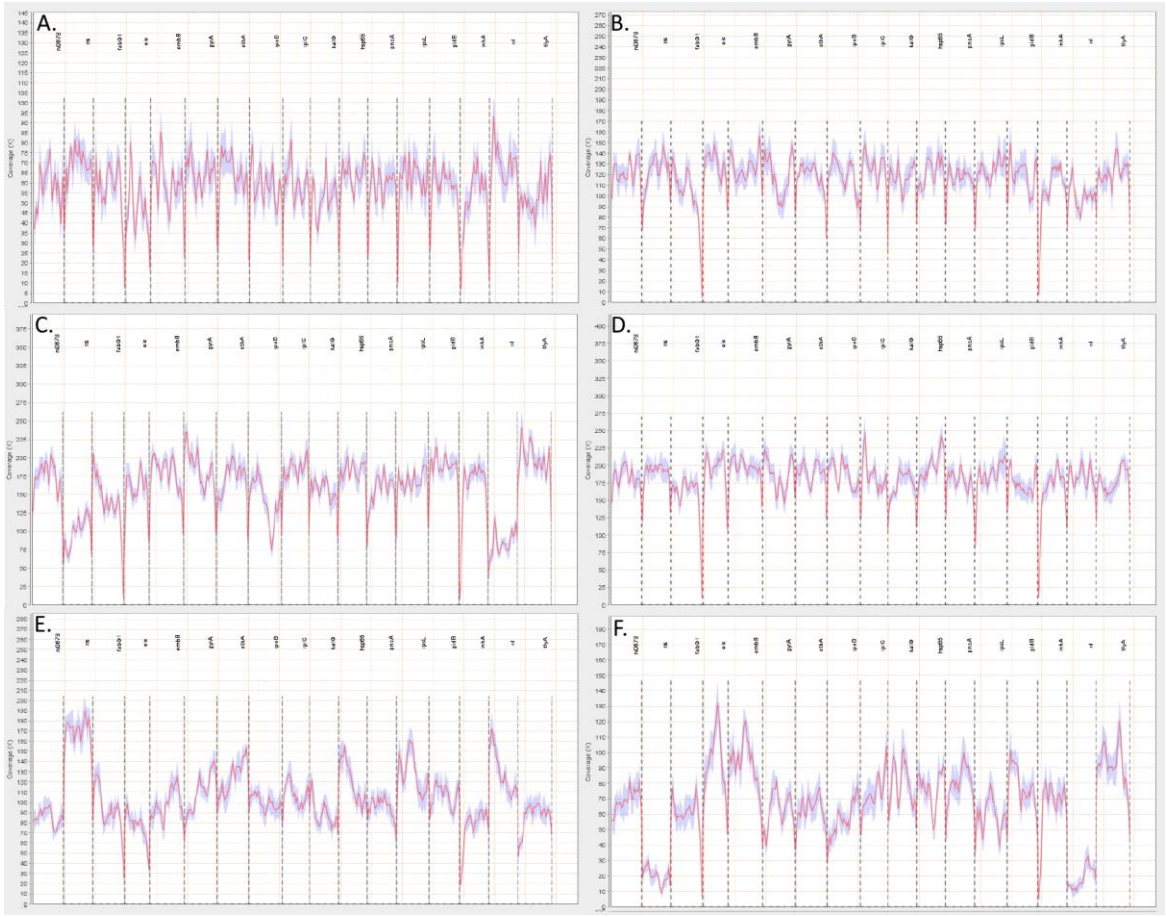

Figure 3.26: QualiMap visualization of 6 MTBC species genomes mapped onto the TB tNGS assay gene target reference. A: M. tuberculosis, B: M. africanum, C: M. bovis, D: M. bovis BCG, E: M. caprae, and F: M. pinnipedii

M. abscessus and *M. kansasii* were further used *in vitro* to test specificity of the tNGS assay in the presence of NTMs in sputum. Known concentrations of DNA (3.33 x 10^4 CFU) from the two NTMs were spiked into NRF sputum along with *M. tuberculosis* DNA (Table 3.68).

Table 3.68: Testing assay specificity in a sample containing equal concentrations of three mycobacteria

Sample Name	**Volume of 10^4 CE/µL *M. tuberculosis* DNA Added (µL)**	**Volume of 10^4 CE/µL *M. abscessus* DNA Added (µL)**	**Volume of 10^4 CE/µL *M. kansasii* DNA Added (µL)**
Mixed NTM Test	3.33	3.33	3.33

The triplicate samples were DNA extracted, amplified, sequenced, and reads were uploaded to the Epi2Me WIMP pipeline to assess detection of target DNA in the presence NTMs. Analysis identified

MTBC as the only microbes in the samples. No reads were identified as either *M. abscessus* or *M. kansasii,* indicating the tNGS assay is specific for *M. tuberculosis* even in samples with a high proportion of NTM DNA.

3.2.7.1: Specificity and Inclusivity Summary

As covered in throughout section 3.2.7, inclusivity and specificity of the tNGS assay were assessed repeatedly during development. Preliminary *in silico* analysis indicated the tNGS assay was highly specific in 15/17 targets with only *rrs* and *rrl*, (16S and 23S genes respectively), demonstrating nonspecific amplification. This was expected, as these genes are highly conserved in the genus and designing specific primers that are also compatible in the multiplex is extremely difficult. The non-specific amplification did not, however, cover the entirety of either target region and was non-disruptive to the assay. *In silico* analysis using 5 NTM and *M. leprae* fastQ files also demonstrated non-MTBC mycobacteria were not consistently or evenly covered by assay targets, indicating pulmonary infections by non-target mycobacteria do not negatively impact assay specificity. This also indicates the assay is incapable of accurately identifying NTM infections as currently designed.

In silico analysis of MTBC sequencing reads from the NCBI database mapped against a concatenated sequence of assay targets demonstrated full target region coverage. This coverage thereby indicates that the assay would work in true clinical samples regardless of MTBC causative agent assuming effective DNA extraction.

Following *in silico* testing, analytical specificity was assessed *in vitro* using cultures of *M. bovis* BCG, *M. kansasii,* and *M. abscessus* as well as pre-extracted *M. africanum* DNA. Extraction and sequencing of *M. kansasii* and *M. abscessus* culture did show full *rrs* and *rrl* target coverage. This is due to the highly conserved 16S and 23S genes in all mycobacteria mentioned previously. However, the specific MTBC targets did not see uniform target coverage demonstrating a high level of analytical specificity.

3.2.8: Limit of Detection

Experimentation after clinical validation was conducted to determine the minimum limit of detection (LoD) of the tNGS assay. NRF sputum was spiked with *M. bovis* BCG culture to simulate clinical samples. *M. bovis* BCG was cultured under conditions detailed in section 2.1 with the addition of Tween-80 to minimize clumping of mycobacterial cells. A dilution series from 1,000 CFU/mL – 10 CFU/mL was prepared (Table 3.69).

Table 3.69: Metagenomic sequencing LoD culture dilution series and spiking with Tween grown M. bovis BCG culture

Sample	**Mycobacterial Culture Concentration (CFU/mL)**	**Post-Spike Sample Mycobacterial Culture Concentration (CFU/mL)**
Dilution 1	1,000	100
Dilution 2	500	50
Dilution 3	100	10
Dilution 4	50	5
Dilution 5	10	1

Paired samples were prepared by spiking 100µL of each dilution into 900µL of NRF sputum. Spiked samples were extracted, amplified, and quantified by Promega GloMax for a preliminary assessment of LoD for each dilution step (Table 3.70). No sample quantified lower than the negative control. There was no significant (Paired T-Test: $p>0.05$) difference in amplification within each multiplex regardless of starting concentration, indicating uniformity at all concentrations.

Table 3.70: Mean DNA concentration quantifications for LoD determination of tNGS assay multiplex amplifications from two triplicate sets of 5 contrived clinical sample dilutions

Sample	**Replicate**	**Multiplex Group 1 Post-Amplification Concentration ($\overline{X}$ ng/µL)**	**Multiplex Group 2 Post-Amplification Concentration ($\overline{X}$ ng/µL)**	**Multiplex Group 3 Post-Amplification Concentration ($\overline{X}$ ng/µL)**
Dilution 1	1	48.88	7.42	15.00
	2	48.30	6.61	12.18
Dilution 2	1	50.37	5.40	10.08
	2	40.49	3.89	6.66
Dilution 3	1	50.07	4.89	8.64
	2	45.40	3.25	7.17
Dilution 4	1	48.54	4.72	8.23
	2	34.36	3.35	6.20
Dilution 5	1	46.74	3.30	6.91
	2	28.73	3.70	6.29
Negative Control	N/A	4.93	1.47	4.37

Multiplex groups were pooled and sequenced using the ONT Native Barcoding 96 Expansion kit as described in methods section 2.14.3. Analysis of reads indicated that at two hours of sequencing, only the 100 CFU/mL sample surpassed 50x coverage (mean gene target coverage = 1,733x). The 50, 10, 5, and 1 CFU/mL concentration samples failed to achieve 50x coverage for any gene target after 2 hours.

The gold standard for TB diagnostic analytical sensitivity (limit of detection/LoD) remains culture which is capable of detecting of 1-10 CFU/mL under optimal conditions [173]. For comparison, the LoD of the Xpert MTB/RIF assay is ~131 CFU/mL from primary sputum samples and between 10-100 CFU/mL from concentrated culture while the Xpert MTB/RIF Ultra assay has an LoD of 11.8 CFU/mL [174,175]. Likewise, the GenoType MTBDRplus assay exhibits an analytical LoD of 160 CFU/mL [176].

For further comparison, the tNGS multiplex assay demonstrates an LoD of 50-100 CFU/mL from primary sputum samples. With improved nucleic acid extraction efficiency it should be possible to reduce the tNGS assay LoD from primary samples yet further.

3.2.9: Clinical Validation of the tNGS Drug Resistance Assay

A set of 392 well characterized (phenotypic and genotypic {Illumina sequenced} susceptibility data), blinded, spiked sputum samples were provided by FIND for validation of the tNGS assay. Samples were unblinded after initial assessment of the assay by FIND. Samples were divided into three categories for assessing different properties of the tNGS assay. The largest set (A samples; n=312) consisted of triplicate samples of *M. tuberculosis* variants with resistance mutations for testing the assay's ability to detect a diverse range of drug-resistance associated SNPs. The second set (B samples) consisted of five triplicate mixtures of an XDR strain with a Pan-Susceptible strain including 50:50, 80:20, 90:10, 99:1, and 99.9:0.1 ratios at two concentrations (~10^5 and ~10^7 CFU/mL; n=30). This set was designed to determine the tNGS assay's ability to detect heteroresistance and measure what proportion of minor variants the assay can detect. Finally, five replicates of serial dilutions (10^7 – 10^3 CFU/mL) of a susceptible and a resistant strain spiked into sputum (C samples; n=50) were provided to test the dynamic range of the test.

The target product profile (TPP) set by FIND during validation for genotypic sensitivity and specificity was 98% for detection of drug-resistance associated SNPs against the genetic reference standard obtained by Illumina sequencing. In comparison, the TPPs for phenotypic sensitivity varied by drug, though all drugs were set at 95% phenotypic specificity. The phenotypic TPP for rifampicin sensitivity was highest at 95% while the desired sensitivity for isoniazid and fluoroquinolones was 90%. Finally, the desired amikacin, kanamycin, capreomycin, and pyrazinamide sensitivities were lowest (85%). The disparity between genotypic and phenotypic TPPs is due to variable resistance predictability by SNPs and that SNP based resistance prediction is better for some drugs than others.

For analytical sensitivity (limit of detection/LoD) the target was 1.8x10^3 CFU/mL in pan-susceptible samples and 4.7 x10^3 CFU/mL in XDR samples. In mixed samples the TPP for accurate genotypic resistance calls was at the 10% XDR to 90% pan-susceptible ratio. This is due to this being the

approximate cut-off at which phenotypic resistance can become dominant in mixed samples as determined by FIND.

3.2.9.1: Sequencing and Analysis of FIND Samples

All samples were processed for sequencing as described in methods section 2.14.2. Eighty samples were sequenced per MinION run for a minimum of 6 hours (resulting in 5 total sequencing runs). Analysis was performed using the Epi2ME TB Resistance Profile pipeline for resistance calling. During detailed analysis of the data in Epi2Me, we discovered that not all resistance SNPs were being reported by the software automatically. Therefore, the Epi2Me data had to be visualized and analysed manually to ensure SNPs were not omitted.

Appendix IV consists of the complete results compiled for FIND including genotypic and phenotypic results for 392 blinded samples. To make resistance calls the proportion of wildtype bases was compared to the proportion of resistance bases at each locus. Initially, resistance calls were divided into three categories; loci with resistance bases contributing ≥80% of reads were called resistant and were entered into a spreadsheet in red, loci with resistance bases contributing ≥50-<80% of reads were called as mixed infections but primarily resistant and were noted in orange, loci with resistance bases contributing ≥20 - <50% were called as mixed infections but non-resistant and were noted in green, any loci with <20% bases resistant were called pan-susceptible. This was summarily simplified to loci with resistance bases ≥15% being called phenotypically resistant regardless of mixed infection status (Table 3.71).

Table 3.71: Example results for phenotypic resistance prediction based on a 15% read threshold

Sample	Ethambutol	Isoniazid	Pyrazinamide	Rifampicin	Streptomycin	Amikacin	Bedaquiline	Capreomycin
A405	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A798	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible

Secondarily, Epi2Me WIMP analysis was performed to detect different MTBC members and check for contamination. This analysis identified only *M. tuberculosis* in each sample with no other MTBC causative agents or coinfectious agents.

Following initial analysis, all 392 samples were sequenced a second time using extractions from sedimented sputum to test assay reproducibility. Analysis identified no differences in resistance SNPs calls between replicates and the results were submitted to FIND for validation.

3.2.9.2: Genotypic Sensitivity and Specificity

One hundred and four "A" samples (pooled sputum) were spiked with *M. tuberculosis* strains with a wide selection of drug resistance mutations grown to an OD_{600} of 1 by FIND and aliquoted into 3 replicates. The majority call from each replicate set was used to determine sensitivity and specificity compared to the WGS reference. As mentioned previously the TPP criteria set by FIND required a minimum 98% sensitivity and specificity for detection of targeted SNPs. FIND assessed mutations for isoniazid, pyrazinamide, rifampicin, kanamycin, amikacin, and fluoroquinolones. Sensitivity and specificity results for each SNP were recorded, of which 95% exhibited sensitivity above the 98% threshold, while 99% exhibited specificity above the 98% threshold (Table 3.72).

Table 3.72: Overall genotypic sensitivity and specificity results for XDR+PZA resistance SNPs

Testing Criteria	**Sensitivity**	**Specificity**
Percent of Mutations Above 98%	95%	99%
Mutations at or Above 98%	70	73
Mutations Below 98%	4	1
Total	74	74

Four mutations fell below the 98% sensitivity threshold, of which one showed 0% sensitivity. This mutation (*rpob* D435A) was one of a double mutation at this codon, the other of which was D435Y. On review of Epi2Me results we found that both mutations occurred above the 15% reporting threshold in sample A361 and the loss in sensitivity was attributable to reporting error during manual analysis. Excluding this outlier, the sensitivity of the remaining three mutations (*rpoB* D435Y, *inhA* I194T, and *gyrA* D94A) ranged from 50%-80%. Related to the previous loss of sensitivity

in sample A361, a 66.7% sensitivity was calculated. This was again related to an error in reporting due to a double mutation which was remedied. The loss of sensitivity (50%) in *inhA* I194T was called wildtype in our test results for sample A229 but was a mixed infection (20%) in WGS indicating a false negative. Epi2Me results for sample A229 were reevaluated and were still identified as wildtype (10.1% reads resistant) indicating a potentially lowered LoD at this locus for mixed infections. Finally, sample A229 also exhibited 80% sensitivity for *gyrA* D94A. As before, Epi2Me results were reevaluated and found to be consistent, indicating a potential issue with intermittent dropouts for mixed infections at this locus.

One mutation fell below the 98% specificity threshold (*katG* S315T; 94.1%). In assay sequencing and analysis of sample A229 this locus was found to exhibit a proportion of resistance SNPS between 20-50%. In the WGS for comparison sample A229 this locus was categorized as wildtype. This disparity lead to reevaluation of Epi2Me results for this resistance call and resistance SNPs were found to account for 13.2% of reads. As this is below the 15% threshold the specificity loss was determined to be due to reporting error and summarily amended. The overall range in sensitivity was 67%-100% and in specificity was 75%-100% (Table 3.73).

Table 3.73: Overall genotypic sensitivity and specificity results for each tNGS assay gene target calculated from reported SNP findings

	Sensitivity		**Specificity**	
Gene Target	**Meet Criteria/Total**	**% Meet Criteria**	**Meet Criteria/Total**	**% Meet Criteria**
rpoB	22/24	92%	24/24	100%
fabG1	4/4	100%	4/4	100%
inhA	2/3	67%	3/3	100%
katG	4/4	100%	3/4	75%
gyrA	9/10	90%	10/10	100%
eis	1/1	100%	1/1	100%
rrs	5/5	100%	5/5	100%
pncA	25/25	100%	25/25	100%
embB	11/11	100%	11/11	100%
ethA	2/2	100%	2/2	100%
rpsL	2/2	100%	2/2	100%

Genotypic specificity and sensitivity were determined by FIND using comparison to Illumina sequences. Clinical validation quantified a genotypic specificity of 94-100% across all targets with an overall assay specificity of 99%. This demonstrated parity with existing Xpert MTB/RIF (99%), GenoType MTBDRplus (100%), and GenoType MTBDRsl (98.6%) assays [177–182]. Analysis further indicates improved accuracy of resistance calls in second-line anti-tuberculous drugs compared to technologies currently on the market.

Clinical validation of the tNGS assay quantified a total genotypic sensitivity of 95%. However, detailed analysis of validation results identified disparities in sensitivity among assay targets. Discussion with FIND researchers and manual analysis of results identified loss of genotypic sensitivity in individual targets was primarily attributable to issues in the Epi2Me analysis pipeline which omitted several pyrazinamide and kanamycin SNPs. Omitted SNPs have since been added to a newly developed analysis pipeline by collaborators at ONT. This new pipeline utilises the official curated list of resistance conferring SNPs from the WHO, standardising the SNPs detected [156].

3.2.9.3: Phenotypic Sensitivity and Specificity

Phenotypic sensitivity and specificity calling were performed using the same 104 triplicate samples as for the genotypic testing. Phenotypic DST was performed on all 104 *M. tuberculosis* strains by FIND using MGIT DST culture. Each drug had different optimal and minimum TPPs for phenotypic sensitivity and specificity assigned by FIND (Table 3.74). Kanamycin and pyrazinamide phenotypic resistance detection fell below the minimum sensitivity threshold. However, no phenotypic call fell beneath the required specificity threshold (Table 3.75). Thus, 4/6 tested drugs surpassed requirements for phenotypic resistance calling while 2/6 fell below the required sensitivity threshold.

Table 3.74: Optimum and minimum acceptable sensitivity and specificity TPPs for phenotypic resistance calling as determined by FIND for the analysis of the tNGS assay

	Sensitivity		Specificity	
Anti-TB Drug	**Optimal Sensitivity (%)**	**Minimum Sensitivity (%)**	**Optimal Specificity (%)**	**Minimum Specificity (%)**
Rifampicin	99	95	98	95
Isoniazid	90	90	98	95
Fluoroquinolones	90	90	98	95
Amikacin	90	85	98	95
Kanamycin	90	85	98	95
Capreomycin	90	85	98	95
Pyrazinamide	90	85	98	95

Table 3.75: Overall calculated phenotypic sensitivity and specificity of tNGS assay resistance calls as compared to a phenotypic DST reference

	Sensitivity		Specificity	
Drug	**Calculated Sensitivity (%)**	**95% Confidence Interval (%)**	**Calculated Specificity (%)**	**95% Confidence Interval (%)**
Rifampicin	97	91.5 – 99.0	100	20.7 - 100
Isoniazid	96	90.3 – 98.5	100	34.2 - 100
Fluoroquinolones	91	80.7 – 96.1	96	86.0 – 98.8
Amikacin	97	84.7 – 99.5	97	89.8 – 99.2
Kanamycin	66	52.7 – 76.4	100	92.3 - 100
Pyrazinamide	63	51.4 – 73.7	97	83.8 – 99.4

In kanamycin, poor sensitivity was related to failure of the Epi2Me TB Resistance Profile pipeline to detect a resistance SNP 10bp before the start of the *eis* gene in the promoter region. The loss of sensitivity in pyrazinamide was attributed to the Epi2Me pipeline not analysing 7 high-confidence mutations in *pncA*. A new resistance calling pipeline has now been developed which raises the phenotypic sensitivity above the required 90% threshold for both drugs.

Comparison to line-probe assay (LPA) DST performed by FIND demonstrated the tNGS assay is superior for making phenotypic resistance calls. According to this FIND analysis LPAs fell below the FIND specificity TPP for fluoroquinolones, amikacin, and kanamycin, and omitted pyrazinamide. In contrast, the tNGS assay met the FIND specificity TPP for all drugs; however, it fell below the sensitivity TPP for kanamycin and pyrazinamide (Table 3.76). As mentioned previously the development of a new analytical pipeline has since raised the sensitivity in phenotypic resistance calls for both of these drugs above the required threshold (90%).

Table 3.76: Comparison of tNGS DST assay to LPA DST as performed by FIND

	tNGS DST Assay			**LPA DST**		
Drug	**# of Samples**	**Sensitivity**	**Specificity**	**# of Samples**	**Sensitivity**	**Specificity**
Rifampicin	101	0.97	1.00	101	0.97	1.00
Isoniazid	104	0.96	1.00	103	0.97	1.00
Fluoroquinolones	104	0.91	0.96	104	0.96	0.91
Amikacin	100	0.97	0.97	100	0.97	0.90
Kanamycin	104	0.66	1.00	104	0.93	0.90
Pyrazinamide	99	0.63	0.97	---	---	---

Phenotypic specificity was assessed by comparison to culture. In comparison to the genotypic specificity, the tNGS assay showed a range of 96%-100% phenotypic specificity across all targets. This corroborates well with the genotypic specificity determined previously. Further, comparison of the tNGS assay to existing molecular diagnostic methods also demonstrated specificity superior to existing line probe assays for fluoroquinolones, amikacin, and kanamycin [103,183,184].

In comparison, the tNGS assay exhibited discrepancies between genotypic and phenotypic sensitivity, primarily when analysing mixed samples (section 3.2.9.5). One source of this discrepancy was the phenotypic resistance calling threshold initially selected, where only mutation SNPs greater

than 50% of total site reads were categorized as phenotypically resistant. Reduction of the threshold from 50% to 15% improved assay phenotypic sensitivity in mixed samples for all tested samples. This alteration improved the assay's phenotypic sensitivity to meet the FIND Seq&Treat TPP for each resistance.

Phenotypic sensitivity for rifampicin was of particular interest for comparison to the WHO endorsed Xpert MTB/RIF and Xpert MTB/RIF Ultra assays. A 2020 systematic review of the MTB/RIF Ultra test found a phenotypic sensitivity of 91.15% for rifampicin drug resistance [185]. For comparison, phenotypic sensitivity for rifampicin resistance was 79.2% for the GenoType MTBDRplus LPA [180,183]. However, the tNGS assay demonstrated a superior phenotypic sensitivity of 97% for rifampicin resistance compared to culture.

An issue with accurate phenotypic resistance calling in mixed samples was shown due to a non-resistance conferring mutation in the *katG* forward primer binding site. This mutation, which occurred only in one of the strains in the mixed sample, lowered the amplification efficiency of that strain effectively rendering the assay only able to detect one of the two strains present. Redesign of the forward primer to avoid this mutation locus (section 3.2.11) resolved this issue allowing accurate detection of mixed infections with *katG* involvement.

3.2.9.4: Indeterminate Rates and Reproducibility

The reproducibility of the tNGS assay was assessed using panels A and B (mixture panel; n=30). Individual mutations within targets demonstrated a disagreement rate of 0.16% (2 disagreement calls / 1,248 replicate calls). The first of these was in A139 with a mutation detected at *embB* M206I while the other two replicates had a mutation at *embB* M306I. This disagreement was due to reporting error during manual analysis and was corrected. The second disagreement occurred in A379 with the report of two double mutations at codon 445 while the other two replicates detected a single mutation at this codon. This double mutation was only 15% of reads which suggests that it

may be a false positive due to sequencing noise. Despite these two disagreement calls results indicated a very high level of assay reproducibility.

Analysis of samples and replicates demonstrated a 0% indeterminate rate across panels A and B, well below the FIND TPP of <5%. The overall target indeterminate rate was also assessed finding a 0% target indeterminate rate in panel B but a 1% (36/3,744) target indeterminate rate in panel A. By distinguishing between target failure and gene deletions in the targets the target indeterminate rate was reduced to 0.3% (12/3,744). Of the remaining 12 indeterminate targets, 3 came from sample A262 which was identified as a multiplex group 1 failure. The final 9 indeterminates occurred in targets *gidB* and *pncA*.

3.2.9.5: Mixed Clinical Samples to Measure Heteroresistance Detection

Mixed infection samples were created using a pan-susceptible and an XDR strain of *M. tuberculosis*, both grown to OD_{600}=1 and mixed in several different proportions; 50%, 20%, 10%, 1%, and 0.1% XDR:Pan-Susceptible. A 1/100 dilution of each stock was also prepared. Pooled sputum samples were then spiked in triplicate with neat and 1/100 dilutions of the 5 mixed samples resulting in 30 samples total. The minimum criteria according to TPP was accurate resistance calls in mixed samples with ≤10% XDR.

Initial resistance calls for mixed samples were accurate for *rpoB* (rifampicin), *fabG1* (isoniazid), *gyrA* (fluoroquinolones), and *rrs* (amikacin). However, initial resistance calls were unable to accurately call *katG* (isoniazid) and *pncA* (pyrazinamide) resistance (Figure 3.27). Multiple optimization methods were tested to improve sensitivity in *katG* and *pncA.* Firstly, adjustment of the call threshold from 20% to 15% of reads helped improve sensitivity in mixed samples. Additionally, optimization of the *katG* forward primer to mitigate the impact of a non-resistance conferring SNP improved sensitivity to *katG* mutations in mixed samples (Section 3.2.11). The loss of sensitivity in *pncA* was determined to be due to reporting error where *pncA* codon 171 results were mistakenly reported for *pncA* codon 71. Repeating analysis and reporting using reads from the correct codon

demonstrated accurate detection of mixed infection to the 10% threshold. Repeat analysis of results with the amended threshold showed the assay met requirements for calling phenotypic resistance in mixed (Figure 3.28).

ONT	rpoB 450L	katG 315T	fabG1 -15T	gyrA 94G	rrs 1484	pncA 71R
B-0.1%	0/3	0/3	0/3	0/3	0/3	0/3
B-0.1DIL	0/3	0/3	0/3	0/3	0/3	0/3
B-1%	0/3	0/3	0/3	0/3	0/3	0/3
B-1DIL	0/3	0/3	0/3	0/3	0/3	0/3
B-10%	1/3	0/3	3/3	3/3	3/3	0/3
B-10DIL	3/3	0/3	3/3	3/3	3/3	0/3
B-20%	3/3	0/3	3/3	3/3	3/3	0/3
B-20DIL	3/3	0/3	3/3	3/3	3/3	0/3
B-50%	3/3	0/3	3/3	3/3	3/3	3/3
B-50DIL	3/3	0/3	3/3	3/3	3/3	3/3
pure XDR	3/3	3/3	3/3	3/3	3/3	3/3
pure SUS	3/3	3/3	3/3	3/3	3/3	3/3

Figure 3.27: Initial FIND analysis of mixed infection detection. Green indicates both genotypic and phenotypic resistance calls were correct. Blue indicates genotypic calls were correct but phenotypic calls were incorrect. Red indicates neither genotypic nor phenotypic calls were correct.

ONT	rpoB 450L	katG 315T	fabG1 -15T	gyrA 94G	rrs 1484	pncA 71R
B-0.1%	0/3	0/3	0/3	0/3	0/3	0/3
B-0.1DIL	0/3	0/3	0/3	0/3	0/3	0/3
B-1%	0/3	0/3	0/3	0/3	0/3	0/3
B-1DIL	0/3	0/3	0/3	0/3	0/3	0/3
B-10%	3/3	3/3	3/3	3/3	3/3	3/3
B-10DIL	3/3	3/3	3/3	3/3	3/3	3/3
B-20%	3/3	3/3	3/3	3/3	3/3	3/3
B-20DIL	3/3	3/3	3/3	3/3	3/3	3/3
B-50%	3/3	3/3	3/3	3/3	3/3	3/3
B-50DIL	3/3	3/3	3/3	3/3	3/3	3/3
pure XDR	3/3	3/3	3/3	3/3	3/3	3/3
pure SUS	3/3	3/3	3/3	3/3	3/3	3/3

Figure 3.28: Analysis of mixed infection detection following adjustment of the phenotypic resistance threshold and correction of errors in katG and pncA calling. Green indicates both genotypic and phenotypic resistance calls were correct. Red indicates neither genotypic nor phenotypic calls were correct.

3.2.9.6: Dynamic Range

Dynamic range of the tNGS assay was determined using five serial dilutions of two *M. tuberculosis* strains; one XDR and one pan-susceptible. Dilutions ranged from $1.8x10^7$ CFU/mL to $1.8x10^3$ CFU/mL for the pan-susceptible strain and from $4.7x10^7$ – $4.7x10^3$ CFU/mL for the XDR strain. Diluted strains were spiked into sputum by FIND and five replicates were prepared for each dilution (n=50 samples total).

Analysis through the Epi2Me TB Resistance Profile pipeline showed no target dropouts at any dilution level. Assessment was repeated using decontaminated/sedimented samples for comparison with raw sputum results. Comparison identified no loss of detection at any dilution level when using sedimented sputum samples. Results indicated the clinical LoD of the tNGS assay is below $1.8x10^3$ CFU/mL. It should be noted that only 700µL sputum was used for extraction and only 1/10 of the extracted DNA was used for the PCR. Therefore, the analytical LoD of the PCR is <120 CE.

Dynamic range was also compared to three current molecular technologies by FIND; Genotype MTBDRPlus, GenoType MTBDRsl, and GeneXpert MTB/RIF. Analysis indicated that the dynamic range for the tNGS assay is equivalent to all three existing tests in the range tested (Table 3.77). Likewise, the dynamic range for DR-TB strain dilutions was equivalent to all three existing tests in the range tested (Table 3.78). However, despite 100% dynamic range sensitivity results the 1.8 x 10^4 CFU/mL concentration produced a small number of incorrect resistance calls. The reason for this is currently under investigation by collaborators at ONT.

Table 3.77: Results for comparison of dynamic detection range performed by FIND in pan-susceptible samples. Green indicates 100% detection by the test at the selected concentration.

	Concentration of Pan-Susceptible Strain (CFU/mL)				
	1.8×10^3	1.8×10^4	1.8×10^5	1.8×10^6	1.8×10^7
tNGS Assay	5/5	5/5	5/5	5/5	5/5
MTBDR Plus	1/1	1/1	1/1	1/1	1/1
MTBDR sl	1/1	1/1	1/1	1/1	1/1
GeneXpert MTB/RIF	1/1	1/1	1/1	1/1	1/1

Table 3.78: Results for comparison of dynamic detection range performed by FIND in XDR samples. Green indicates 100% detection by the test at the selected concentration.

	Concentration of XDR Strain (CFU/mL)				
	4.7×10^3	4.7×10^4	4.7×10^5	4.7×10^6	4.7×10^7
tNGS Assay	5/5	5/5	5/5	5/5	5/5
MTBDR Plus	1/1	1/1	1/1	1/1	1/1
MTBDR sl	1/1	1/1	1/1	1/1	1/1
GeneXpert MTB/RIF	1/1	1/1	1/1	1/1	1/1

The assay was shown to be capable of detecting all sample concentrations provided (minimum $1.8x10^3$ CFU/mL for pan-susceptible strains and $4.7x10^3$ CFU/mL for XDR strains) – the LoD of the assay is therefore $<10^3$ CFU/ml.

3.2.10: Assay Performance Summary

In assay phenotypic sensitivity for rifampicin (97%) the tNGS multiplex assay matched or outperformed all existing diagnostic assays to which it was compared, with the nearest being the Xpert MTB/RIF (97.6 %), Xpert MTB/RIF Ultra (92.7%) and GenoType MTBDRplus tests (96.2%)

[184,186,187]. Likewise, overall phenotypic specificity (99%) was equivalent to existing assays, with the nearest being smear microscopy (99.8%) and the Xpert MTB/RIF assay (99%) [177,188]. Phenotypic specificity for rifampicin resistance (100%) was superior to Xpert MTB/RIF and Xpert MTB/RIF Ultra (99% and 98% respectively) [186,187]. Culture based diagnosis and DST were excluded from comparison of sensitivity and specificity as they are the baseline by which all other assays are validated.

In direct comparison, the tNGS assay LoD was superior to Xpert MTB/RIF for primary sputum samples and second only to culture [173,174]. These findings indicate the tNGS multiplex assay is highly competitive for paucibacillary samples.

3.2.11: Post-Validation Optimisation

During clinical validation, *katG* primers required redesign to include a high confidence SNP at codon 315. To improve integration of redesigned *katG* primers with group 2 removal of *hsp65* was necessary. This was deemed an acceptable change during validation as the hsp65 target was only present to help speciate NTM if present and other targets could theoretically be used for the same purpose if necessary. The final multiplex configuration (config 9) is presented in Table 3.79 along with nested qPCR results (Figure 3.29) and QualiMap visualization (Figure 3.30).

Table 3.79: Configuration 9 of multiplex primer mixes for tNGS amplification following

Multiplex Group	**Gene Target 1**	**Gene Target 2**	**Gene Target 3**	**Gene Target 4**	**Gene Target 5**	**Gene Target 6**
1	*rv0678*	*eis*	*embB*	*rrs*	*fabG1*	N/A
2	*gyrA*	*rpoB*	*ethA*	*rplC*	*katG*	N/A
3	*gidB*	*inhA*	*rrl*	*pncA*	*rpsL*	*tlyA*

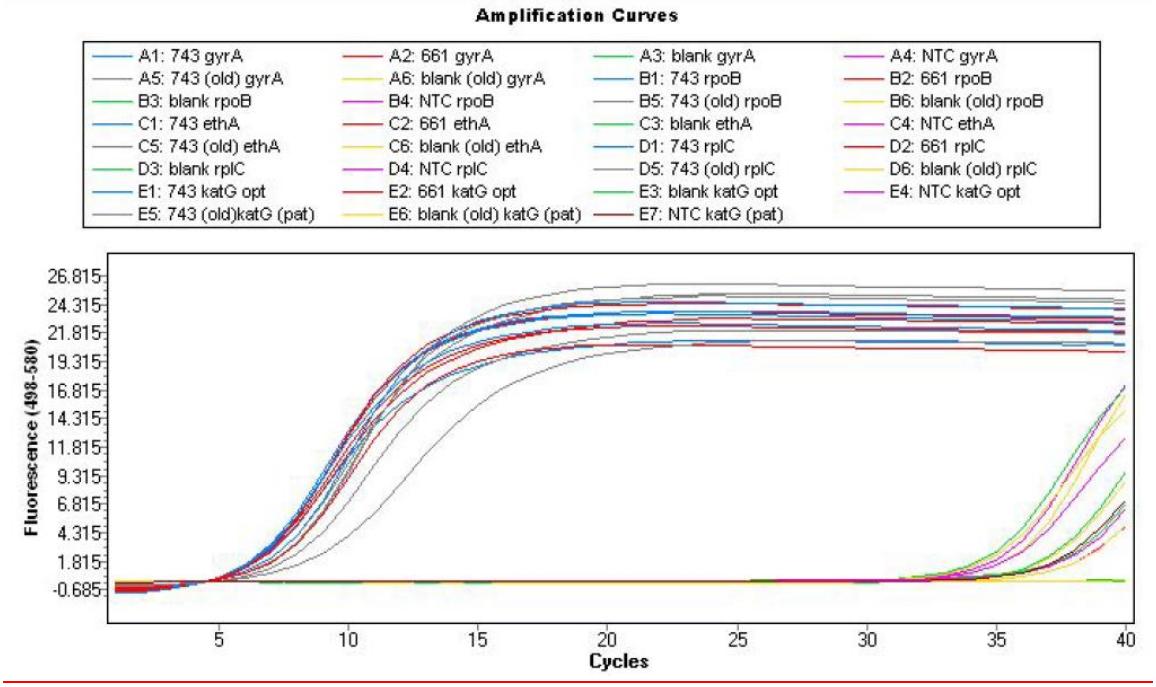

Figure 3.29: One of a triplicate set of nested qPCR C_Ts for configuration 9 multiplex group 2 gene targets with original and reformulated katG primer pairs. Used for visualization of amplification efficiency with the removal of hsp65

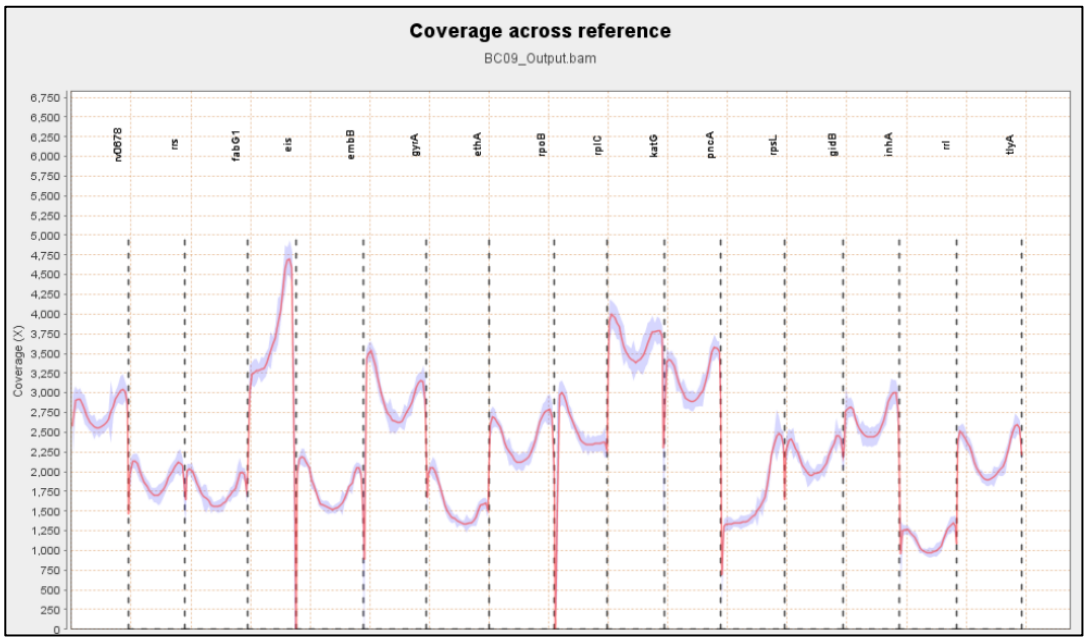

Figure 3.30: QualiMap visualization sequencing of a mixed infection sample showing equivalent coverage of all targets when using multiplex configuration 9 using one of a set of triplicate samples

Clinical validation experimentation identified a common, but non-resistance conferring, *katG* mutation in the forward primer site. This SNP promoted preferential amplification resulting in a loss of sensitivity in mixed infections. The mutation was located 5bp from the 5' end of the primer

requiring alternative forward primers to avoid its inclusion. A set of 5 primers were designed which shifted the primer location by 1bp each. All primer lengths were adjusted as needed to maintain melting temperature (Table 3.80). Alternative primers were tested on 50/50 mixed samples and analysed by sequencing. Analysis identified that primers which placed the SNP in the final 5' position, or excluded it entirely, were most sensitive to mixed infection (Table 3.81). As both primers performed similarly, the one completely avoiding the mutation site was selected for subsequent use (Table 3.82).

Table 3.80: Redesigned primers to mitigate and avoid the non-resistance conferring mutation site. The site of the SNP is bolded in red.

Base Pair Positions Shifted Toward 3' End	**Primer**
Original Primer	TGCC**C**GGATCTGGCTCTTA
1	GCC**C**GGATCTGGCTCTTAA
2	CC**C**GGATCTGGCTCTTAAGG
3	C**C**GGATCTGGCTCTTAAGGC
4	**C**GGATCTGGCTCTTAAGGCTG
5	GGATCTGGCTCTTAAGGCTGG

Table 3.81: Detection of heteroresistant reads using a forward primer shifted to mitigate the non-resistance conferring SNP site in 50/50 mixed samples.

Base Pair Positions Shifted Toward 3' End	**Mixed Resistance Reads Detected (Mutant/Wild-Type)**
Original Primer	102/1029
1	197/1029
2	451/381
3	906/414
4	1210/581
5	1365/608

Table 3.82: Redesign history for katG primers

katG Redesign Version	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Length (bp)
Original	TCCTCGAGATCCTGTACGGC	TGATACCCATGTCGAGCAGG	1005
Redesign 1	ATCGCGTCCTTACCGGTTC	GCAACACCCACCCATTACAG	930
Redesign 2	TAAGGCTGGCAATCTCGGC	CTTAACAGCTGGCCCGACA	982
Redesign 3	GGCCCAAGGTATCTCGCAA	TACGGGCCGCTGTTTATCC	995
Redesign 4	CCGCCTTTGCTGCTTTCTC	GTTACAGCGGTAAGCGGGA	1050
Redesign 5	TTGTCGCTACCACGGAACG	TAACAGCTGGCCCGACAAC	1068
Redesign 6	TGCCCGGATCTGGCTCTTA	CTGTGGCCGGTCAAGAAGA	951
Redesign 7	GCCCGGATCTGGCTCTTAA	CTGTGGCCGGTCAAGAAGA	950
Redesign 8	CCCGGATCTGGCTCTTAAGG	CTGTGGCCGGTCAAGAAGA	949
Redesign 9	CCGGATCTGGCTCTTAAGGC	CTGTGGCCGGTCAAGAAGA	948
Redesign 10	CGGATCTGGCTCTTAAGGCTG	CTGTGGCCGGTCAAGAAGA	947
Redesign 11*	GGATCTGGCTCTTAAGGCTGG	CTGTGGCCGGTCAAGAAGA	946

* Redesign version selected for use

3.2.12: Continuing Research

The tNGS project has the potential to generate sizable impact on the field of TB diagnostics through the opportunities afforded by the FIND/WHO Seq&Treat project. From multiple NGS technologies developed by varied research and development entities, our tNGS assay was chosen for further evaluation along with 2 others. The remaining three have moved into phase II trials where they will be assessed in reference laboratories in India, South Africa, and Georgia. Phase II trials will test the performance and viability of the assays in real-world high incidence conditions, after which the results will be analysed by FIND [189]. Assays which achieve FIND TPPs during this evaluation will undergo phase III trials globally where, if they perform as required, will receive WHO endorsement. Additionally, a patent has been filed for the tNGS assay method. This is in the process of being licensed by ONT for use in continuing development of the multiplex assay.

Consolidating reactions into a single multiplex must be a priority moving forward. While the assay as designed is comparable to existing technologies in sensitivity, specificity, and cost; the complexity and risk of contamination within the assay from three separate amplifications limits

practicality in clinical settings. By combining reagents into a single reaction; time, complexity, and cost may all be reduced. This in turn would increase the prospective implementation and reach of the assay for laboratories of all levels. Combination of the 3 multiplex reactions into a single reaction would reduce costs by an estimated £3.50 per sample. Colleagues in the O'Grady group have recently achieved this goal and continue to improve the assay moving forward.

3.3: Metagenomic Sequencing

Metagenomic sequencing has proven useful in the diagnosis of lower respiratory diseases and generation of epidemiological data in a single test [111,113]. This speed and breadth of information would be a boon both for clinical TB treatment and TB control efforts within populations. To that end, a metagenomic assay was designed using host and experimental commensal bacterial depletion methods to improve detection of *M. tb* and MTBC DNA.

3.3.1: Assessment of a Host DNA Depletion Method for Diagnosis of TB and Drug Resistance by Metagenomic Sequencing

The first step for the metagenomic approach for detecting *M. tuberculosis* in sputum was to remove human DNA. A saponin-based host depletion method, described by Charalampous, et al.[111], was tested on a spiked NRF sputum sample. Triplicate samples were spiked with 10-fold serial dilution of *M. bovis* BCG culture (~150-150,000 CE/mL). Post-depletion, samples were extracted and removal of human DNA was analysed by qPCR targeting the human RNA polymerase A gene. Analysis identified host depletion up to ~99.99%, or 10^4 fold, with a mean 3,257.5-fold reduction (Range = 159.8 – 6,165.5 fold) (Table 3.83).

Loss of target and overall bacterial DNA during depletion was also quantified (Tables 3.84 and 3.85). The loss of BCG ranged from 1.5-50 fold. This inconsistency is likely due to processing rather than an issue with saponin lysing *M. bovis* BCG. Commensal bacteria reduction was also monitored as

commensal bacteria would compete with target bacteria for sequencing reads. The saponin method showed reduction in commensal bacteria was similar to loss of target bacteria as expected.

Table 3.83: Mean human DNA qPCR results and calculated host depletion levels using triplicate samples

Sample	**Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)**	**Sample Treatment**	**Human RNA polymerase A qPCR Assay ($\overline{X}$ C_T)**	**Human DNA Depletion ($\overline{X}$ ΔC_T)**
BCG 10^5	150,000	Depleted	32.57	12.59 (6,165.5 fold Reduction)
		Undepleted	19.98	
BCG 10^4	15,000	Depleted	32.52	11.91 (3,848.3 fold Reduction)
		Undepleted	20.61	
BCG 10^3	1,500	Depleted	31.19	11.48 (2,856.4 fold Reduction)
		Undepleted	19.71	
BCG 10^2	150	Depleted	28.18	7.32 (159.8 fold Reduction)
		Undepleted	20.86	

Table 3.84: Mean M. bovis BCG DNA qPCR results and calculated target loss using triplicate samples

Sample	**Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)**	**Sample Treatment**	***M. bovis* BCG RD1 gene qPCR Assay ($\overline{X}$ C_T)**	***M. bovis* BCG DNA Loss/Gain ($\overline{X}$ ΔC_T)**
BCG 10^5	150,000	Depleted	24.76	2.36 (5.1 fold Loss)
		Undepleted	22.4	
BCG 10^4	15,000	Depleted	29.15	2.19 (4.6 fold Loss)
		Undepleted	26.96	
BCG 10^3	1,500	Depleted	30.66	0.62 (1.5 fold Loss)
		Undepleted	30.04	
BCG 10^2	150	Depleted	40	5.49 (44.9 fold Loss)
		Undepleted	34.51	

Table 3.85: Mean 16S rRNA gene qPCR results and calculated bacterial loss using triplicate samples

Sample	Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)	Sample Treatment	Bacterial 16S gene qPCR Assay ($\overline{X}$ C_T)	Total Bacterial DNA Loss/Gain ($\overline{X}$ ΔC_T)
BCG 10^5	150,000	Depleted	26.32	2.15 (4.4 fold Loss)
		Undepleted	24.17	
BCG 10^4	15,000	Depleted	26.38	2.35 (5.1 fold Loss)
		Undepleted	24.03	
BCG 10^3	1,500	Depleted	25.77	1.63 (3.1 fold Loss)
		Undepleted	24.14	
BCG 10^2	150	Depleted	26.73	1.9 (3.7 fold Loss)
		Undepleted	24.83	

To test reproducibility of host depletion in spiked NRF sputum the experiment was repeated with a second set of triplicate samples (Table 3.86). Analysis by qPCR showed host DNA was depleted up to ~99.99%, or 10^4, with a mean fold reduction of 5,742.9 (Range = 3,821.7 – 7,750.1 fold).

Target and overall bacterial DNA loss from the saponin method were assessed as before (Tables 3.87 & 3.88). Loss of target bacterial DNA was less than observed previously. However, a significant loss of total bacterial DNA was detected (Paired T-Test: p=0.016).

Table 3.86: Mean human DNA qPCR results and calculated host depletion levels using triplicate samples

Sample	Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)	Sample Treatment	Human RNA polymerase A qPCR Assay ($\overline{X}$ C_T)	Human DNA Depletion ($\overline{X}$ ΔC_T)
BCG 10^5	150,000	Depleted	34.79	12.92 (7,750.1 fold Loss)
		Undepleted	21.87	
BCG 10^4	15,000	Depleted	33.65	12.24 (4,837.3 fold Loss)
		Undepleted	21.41	
BCG 10^3	1,500	Depleted	33.67	11.9 (3,821.7 fold Loss)
		Undepleted	21.77	
BCG 10^2	150	Depleted	34.31	12.68 (6,562.4 fold Loss)
		Undepleted	21.63	

Table 3.87: Mean M. bovis BCG DNA qPCR results and calculated bacterial loss using triplicate samples

Sample	**Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)**	**Sample Treatment**	***M. bovis* BCG RD1 gene qPCR Assay ($\bar{X}$ C_T)**	***M. bovis* BCG DNA Loss/Gain ($\bar{X}$ ΔC_T)**
BCG 10^5	150,000	Depleted	24.97	1.75
		Undepleted	23.22	(3.4 fold Loss)
BCG 10^4	15,000	Depleted	30.41	2.82
		Undepleted	27.59	(7.1 fold Loss)
BCG 10^3	1,500	Depleted	29.63	0.71
		Undepleted	30.34	(1.6 fold Gain)
BCG 10^2	150	Depleted	34.60	0.05
		Undepleted	34.55	(1.0 fold Loss)

Table 3.88: Mean 16S rRNA gene qPCR results and calculated bacterial loss using triplicate samples

Sample	**Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)**	**Sample Treatment**	**Bacterial 16S gene qPCR Assay ($\bar{X}$ C_T)**	**Total Bacterial DNA Loss/Gain ($\bar{X}$ ΔC_T)**
BCG 10^5	150,000	Depleted	26.90	3.87
		Undepleted	23.03	(14.6 fold Loss)
BCG 10^4	15,000	Depleted	28.85	4.93
		Undepleted	23.92	(30.5 fold Loss)
BCG 10^3	1,500	Depleted	28.09	4.90
		Undepleted	23.19	(29.9 fold Loss)
BCG 10^2	150	Depleted	28.90	4.60
		Undepleted	24.30	(24.2 fold Loss)

Significant host depletion (Paired T-Test: p<0.0001) indicated this method did not require further optimization. However, there was need to develop a depletion protocol for commensal bacterial DNA for efficient and sensitive mycobacterial detection.

3.3.2: Development of a Commensal Bacteria DNA Depletion Method for Mycobacterial Samples

As the results previously demonstrated, there was a need for an optimized method for the removal of unwanted commensal DNA. We aimed to develop an additional depletion step to remove commensal bacteria within sputum samples without any loss of target (*M. bovis* BCG).

3.3.2.1: Assessment of Lysis Buffers for the Depletion of Commensal Bacterial DNA in Sputum Samples

Initially, two lysis buffers (MagNA Pure and Qiagen) were tested with and without the addition of lysozyme (Table 3.89). Triplicate samples were suspended in the lysis buffer solution for 10 minutes before undergoing host nucleic acid depletion. Following depletion, samples were amplified and assessed by qPCR to quantify DNA reduction.

Table 3.89: Lysis buffer solutions designed for testing in the optimization of commensal bacterial DNA depletion

Sample	Lysis Buffer Solution
BCG 10^5 1	400µL MagNA Pure Bacterial Lysis Buffer
BCG 10^5 2	200µL MagNA Pure Bacterial Lysis Buffer + 200µL 5M Lysozyme
BCG 10^5 3	400µL Qiagen Lysis Buffer
BCG 10^5 4	200µL Qiagen Lysis Buffer + 200µL 5M Lysozyme

Analysis indicated no significant depletion of commensal bacterial DNA (Paired T-Test: p>0.05), while host depletion remained significant (Tables 3.90 & 3.91) (Paired T-Test: p=0.0099). Target DNA (*M. bovis* BCG) was detected with no significant loss (mean loss = 3.57 fold) (Table 3.92).

MagNA Pure bacterial lysis buffer improved host DNA depletion (mean effect size = 162,491-fold) compared to Qiagen lysis buffer (mean effect size = 781.6-fold).

Table 3.90: Mean 16S rRNA gene qPCR results and calculated bacterial reduction using two sets of triplicate samples

Sample	**Replicate Set**	**Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)**	**Sample Treatment**	**Bacterial 16S gene qPCR Assay ($\overline{X}$ C_T)**	**Total Bacterial DNA Depletion ($\overline{X}$ ΔC_T)**
BCG 10^5 1	1	150,000	Depleted	25.66	1.26 (2.4 fold Reduction)
			Undepleted	24.40	
	2	150,000	Depleted	24.35	1.14 (2.2 fold Reduction)
			Undepleted	23.21	
BCG 10^5 2	1	150,000	Depleted	27.37	2.97 (7.8 fold Reduction)
			Undepleted	24.40	
	2	150,000	Depleted	24.89	1.68 (3.2 fold Reduction)
			Undepleted	23.21	
BCG 10^5 3	1	150,000	Depleted	23.88	0.52 (1.4 fold Gain)
			Undepleted	24.40	
	2	150,000	Depleted	24.56	1.35 (2.5 fold Reduction)
			Undepleted	23.21	
BCG 10^5 4	1	150,000	Depleted	25.03	0.63 (1.5 fold Reduction)
			Undepleted	24.40	
	2	150,000	Depleted	24.74	1.53 (2.9 fold Reduction)
			Undepleted	23.21	

Table 3.91: Mean human RNA Polymerase A gene qPCR results and calculated host depletion levels using four sets of triplicate samples

Sample	Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)	Sample Treatment	Human RNA polymerase A DNA Probe qPCR Assay ($\overline{X}$ C_T)	Human DNA Depletion ($\overline{X}$ ΔC_T)
BCG 10^5 1	150,000	Depleted	40.00	17.31 (162,491.0 fold Reduction)
		Undepleted	22.69	
BCG 10^5 2	150,000	Depleted	40.00	17.31 (162,491.0 fold Reduction)
		Undepleted	22.69	
BCG 10^5 3	150,000	Depleted	31.48	8.79 (442.6 fold Reduction)
		Undepleted	22.69	
BCG 10^5 4	150,000	Depleted	32.82	10.13 (1,120.6 fold Reduction)
		Undepleted	22.69	

Table 3.92: Mean M. bovis BCG qPCR results and calculated bacterial loss using two sets of triplicate samples

Sample	Replicate Set	*M. bovis* BCG Cells per Sample (CFU/mL)	Sample Treatment	*M. bovis* BCG RD1 Region Probe qPCR Assay ($\overline{X}$ C_T)	*M. bovis* BCG DNA Loss/Gain ($\overline{X}$ ΔC_T)
BCG 10^5 1	1	150,000	Depleted	27.49	5.18 (36.2 fold Loss)
			Undepleted	22.31	
	2	150,000	Depleted	24.13	1.01 (2.0 fold Loss)
			Undepleted	23.12	
BCG 10^5 2	1	150,000	Depleted	26.34	4.03 (16.3 fold Loss)
			Undepleted	22.31	
	2	150,000	Depleted	24.54	1.42 (2.7 fold Loss)
			Undepleted	23.12	
BCG 10^5 3	1	150,000	Depleted	22.69	0.38 (1.3 fold Loss)
			Undepleted	22.31	
	2	150,000	Depleted	24.27	1.15 (2.2 fold Loss)
			Undepleted	23.12	
BCG 10^5 4	1	150,000	Depleted	22.83	0.52 (1.4 fold Loss)
			Undepleted	22.31	
	2	150,000	Depleted	24.11	0.99 (2.0 fold Loss)
			Undepleted	23.12	

Analysis by ANOVA with a Tukey post-hoc test showed use of bacterial lysis buffers resulted in significantly less commensal depletion than using saponin treatment alone (ANOVA: p=0.001). This was shown for both MagNA Pure lysis buffer and Qiagen lysis buffer (Table 3.93) and was surprising, as these lysis buffers were predicted to lyse commensal bacteria and result in bacterial DNA depletion. Analysis further showed no significant difference in commensal DNA depletion between MagNA Pure buffer or Qiagen buffer (ANOVA: p=0.899).

Table 3.93: Tukey HSD Post-Hoc test results for commensal bacterial DNA depletion using two bacterial lysis buffer incubations

Commensal Depletion Comparison	**ANOVA P-Value**	**Tukey HSD Q-Value**	**α=0.05 Critical Q Value**
Saponin Only vs. MagnaPure Lysis Buffer Incubation	0.0010053	7.0872	3.4202
Saponin Only vs. Qiagen Lysis Buffer Incubation	0.0010053	6.8234	3.4202
MagnaPure Lysis Buffer Inclusion vs. Qiagen Lysis Buffer Incubation	0.8999947	0.2242	3.4202

3.3.2.2: Assessment of Reagents for the Depletion of Commensal Bacterial DNA in Sputum Samples

The effect of two detergents (triton x-100 and tween) and one reduction agent (DTT) was investigated for targeted depletion of commensal bacteria. Triplicate samples were incubated in each reagent for 10min followed by host nucleic acid depletion and DNA extraction. Extracted samples were amplified by qPCR to assess quantity of host DNA, commensal DNA, and target DNA in each sample with and without depletion.

Analysis showed a significant increase in commensal bacterial DNA following pre-incubation with 0.1% Tween, a 6.7 fold increase (Table 3.94). Exposure to 0.025% Triton, 1% DTT, and 0.1% DTT

showed no significant difference (Paired T-Test: p>0.05) in the amplification of commensal bacterial DNA during qPCR. Depletion of commensal bacterial DNA was only observed after exposure to triton x-100 (~99.99% reduction). However, this was observed only in a single replicate.

Table 3.94: Mean 16S rRNA gene qPCR results and calculated bacterial reduction using three sets of triplicate samples

Sample	**Replicate Set**	**Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)**	**Sample Treatment**	**Bacterial 16S DNA Probe qPCR Assay ($\overline{X}$ C_T)**	**Total Bacterial DNA Depletion ($\overline{X}$ ΔC_T)**
Triton 0.025%	1	150,000	Depleted	32.19	13.33 (10,297.4 fold Reduction)
			Undepleted	18.86	
	2	150,000	Depleted	16.32	-2.54 (5.8 fold Gain)
			Undepleted	18.86	
	3	150,000	Depleted	16.39	-2.47 (5.5 fold Gain)
			Undepleted	18.86	
Tween 0.1%	1	150,000	Depleted	16.40	-2.46 (5.5 fold Gain)
			Undepleted	18.86	
	2	150,000	Depleted	16.13	-2.73 (6.6 fold Gain)
			Undepleted	18.86	
	3	150,000	Depleted	15.88	-2.98 (7.9 fold Gain)
			Undepleted	18.86	
DTT 1%	1	150,000	Depleted	15.80	-0.18 (1.1 fold Gain)
			Undepleted	15.98	
	2	150,000	Depleted	15.40	-0.58 (1.5 fold Gain)
			Undepleted	15.98	
	3	150,000	Depleted	15.39	-0.59 (1.5 fold Gain)
			Undepleted	15.98	
DTT 0.1%	1	150,000	Depleted	15.34	-0.64 (1.6 fold Gain)
			Undepleted	15.98	
	2	150,000	Depleted	16.05	0.07 (1.1 fold Reduction)
			Undepleted	15.98	
	3	150,000	Depleted	15.72	-0.26 (1.2 fold Gain)
			Undepleted	15.98	

Assessment of host nucleic acid depletion found inclusion of detergents prior to depletion did not negatively impact performance (Table 3.95). There was no significant loss (Paired T-Test: $p>0.05$) of target DNA (*M. bovis* BCG) following exposure to 0.025% Triton (2.7 fold), 0.1% Tween (2.2 fold), and 0.1% DTT (1.7 fold) (Table 3.96). Findings indicate inclusion of these reagents does not effectively deplete commensal bacterial DNA. The increased concentration of bacterial DNA in most of the samples tested may indicate that commensals are being lysed more efficiently but that the commensal DNA is not being digested.

Table 3.95: Mean human RNA polymerase A qPCR for assessing DNA depletion using four pre-host depletion detergent incubations using three sets of triplicate samples

Sample	Replicate Set	*M. bovis* BCG Cells per Sample (CFU/mL)	Sample Treatment	Human DNA Probe qPCR Assay ($\overline{X}$ C_T)	Human DNA Depletion ($\overline{X}$ ΔC_T)
Triton 0.025%	1	150,000	Depleted	40	15.18 (37,122.3 fold Reduction)
			Undepleted	24.82	
	2	150,000	Depleted	40	15.18 (37,122.3 fold Reduction)
			Undepleted	24.82	
	3	150,000	Depleted	40	15.18 (37,122.3 fold Reduction)
			Undepleted	24.82	
Tween 0.1%	1	150,000	Depleted	40	15.18 (37,122.3 fold Reduction)
			Undepleted	24.82	
	2	150,000	Depleted	40	15.18 (37,122.3 fold Reduction)
			Undepleted	24.82	
	3	150,000	Depleted	40	15.18 (37,122.3 fold Reduction)
			Undepleted	24.82	
DTT 1%	1	150,000	Depleted	40	15.03 (33,456.5 fold Reduction)
			Undepleted	24.97	
	2	150,000	Depleted	40	15.03 (33,456.5 fold Reduction)
			Undepleted	24.97	
	3	150,000	Depleted	40	15.03 (33,456.5 fold Reduction)
			Undepleted	24.97	
DTT 0.1%	1	150,000	Depleted	40	15.03 (33,456.5 fold Reduction)
			Undepleted	24.97	
	2	150,000	Depleted	40	15.03 (33,456.5 fold Reduction)
			Undepleted	24.97	
	3	150,000	Depleted	40	15.03 (33,456.5 fold Reduction)
			Undepleted	24.97	

Table 3.96: Mean M. bovis BCG qPCR for assessing DNA loss using four pre-host depletion detergent incubations using three sets of triplicate samples

Sample	Replicate Set	Approximate Number of *M. bovis* BCG Cells per Sample (CFU/mL)	Sample Treatment	*M. bovis* BCG DNA Probe qPCR Assay ($\bar{X}$ C_T)	*M. bovis* BCG DNA Loss/Gain ($\bar{X}$ ΔC_T)
Triton 0.025%	1	150,000	Depleted	25.91	1.09 (2.1 fold Loss)
			Undepleted	24.82	
	2	150,000	Depleted	26.79	1.97 (3.9 fold Loss)
			Undepleted	24.82	
	3	150,000	Depleted	25.87	1.05 (2.1 fold Loss)
			Undepleted	24.82	
Tween 0.1%	1	150,000	Depleted	25.75	0.93 (1.9 fold Loss)
			Undepleted	24.82	
	2	150,000	Depleted	26.03	1.21 (2.3 fold Loss)
			Undepleted	24.82	
	3	150,000	Depleted	26.14	1.32 (2.5 fold Loss)
			Undepleted	24.82	
DTT 1%	1	150,000	Depleted	25.83	0.86 (1.8 fold Loss)
			Undepleted	24.97	
	2	150,000	Depleted	24.58	-0.39 (1.3 fold Gain)
			Undepleted	24.97	
	3	150,000	Depleted	25.87	0.90 (1.9 fold Loss)
			Undepleted	24.97	
DTT 0.1%	1	150,000	Depleted	25.52	0.55 (1.5 fold Loss)
			Undepleted	24.97	
	2	150,000	Depleted	25.71	0.74 (1.7 fold Loss)
			Undepleted	24.97	
	3	150,000	Depleted	25.93	0.96 (1.9 fold Loss)
			Undepleted	24.97	

3.3.2.3: Effectiveness of Depletion Methodologies

Assessment of host DNA depletion methods developed by Charalampous, *et al.*, proved effective in sputum samples containing mycobacteria [111]. However, this method is designed to remove human cells/DNA and to avoid disruption and depletion of any bacterial DNA. Optimisation and development of the method to remove commensal bacterial DNA and improve relative concentration of target DNA (*M. bovis* BCG*)* for sequencing proved ineffective.

Saponin depletion of host DNA works by creating pores in cell and nuclear membranes (phospholipid bilayers) [111]. These pores allow high salt HL-SAN buffer (which assists in DNA release from chromatin) and DNAse into the cell to digest the DNA. Saponin cannot penetrate bacterial cell walls, so while this method is effective for removal of host DNA (*H. sapiens*) it is unable to deplete bacterial DNA. This is useful for metagenomic diagnosis or microbiome studies but is a limitation when targeting a single pathogen, especially in infections where the pathogen can account for only a small proportion of the bacterial community present in the sample.

To improve the relative concentration of mycobacterial DNA in contrived sputum samples several lysis and depletion methods were tested. Initially, lysis buffers used in automated DNA extraction were tested (Section 3.3.2.1). Two buffers were tested, one for the MagNA Pure system and one from Qiagen. As these buffers are designed to lyse bacterial cells, but are inefficient for mycobacterial cells without mechanical disruption, it was hypothesized that these would preferentially lyse commensal bacterial. However, exposure to these buffers didn't result in reduction of commensal DNA.

Following these experiments the effects of three reagents (triton x-100, tween, and DTT) were assessed for depletion of commensal DNA in sputum samples. Rather than decrease the relative concentration of commensal bacteria DNA after extraction, tween instead increased the yield of commensal bacterial DNA. One hypothesis for why this occurred was that Tween improved bacterial cell lysis but inhibited HL-SAN DNase [190]. In comparison, exposure of sputum samples to DTT resulted in no significant change in the extraction of commensal bacterial DNA or the relative concentration of mycobacterial DNA. Finally, one replicate which was exposed to triton x-100 did show significant depletion of commensal bacteria, however this result was not replicable and the other two replicates showed slight increases in extracted commensal DNA. While this result indicates that further investigation into triton x-100 is warranted, the results were not consistent enough for continued research during this project.

While none of these reagents were effective for selective depletion of commensal bacteria, neither did they negatively impact depletion of host cells. This indicates that use of a detergent prior to saponin depletion does not inhibit saponin or DNAse.

3.3.2.4: Future Depletion Research

Future research to improve the relative concentration of mycobacterial DNA or remove commensal bacterial DNA for metagenomic sequencing can follow multiple paths. Two such options are development of a selective lysing and depletion process, or development of a hybridization capture system, as for enrichment in clinical samples for parasite and viral DNA [191–193]. Development of a targeted depletion methodology would allow efficient extraction of mycobacterial DNA from any primary sample type for sequencing. Hybridization capture allows highly specific enrichment of free DNA following mechanical lysis, removing the need for pre-processing for host or commensal depletion. Hybridization capture can streamline diagnostic workflows, however, use of specific bait molecules limits the breadth of coverage by sequencing in most samples [194].

Targeted enrichment using hybridization capture can be useful for detection and diagnosis of drug resistance mutations. However, in addition to the limited fragment sizes, hybridization capture methods also require longer sequencing runs to generate equivalent read-depth to targeted amplification. For example, Horn estimates that 20x coverage of a 1kb genetic fragment requires approximately 500,000 Illumina reads [194]. This inefficiency in sequencing hybridization captured DNA is therefore compounded in analysis of multiple targets in concurrent samples.

3.3.3: Preliminary Limit of Detection Experiment

LoD for the metagenomic assay was assessed by qPCR of triplicate samples and sequencing of two sets of triplicate *M. bovis* BCG spiked NRF sputum. Serial 10-fold dilutions of *M. bovis* BCG culture (10^5 to 10^1 CFU/mL) were spiked into sputum – host DNA depletion was performed, DNA extracted and then qPCR amplified using a BCG probe based qPCR assay. Results showed target DNA was only

detectable in samples containing 10^4 CFU/mL spikes and above (Table 3.97). This was surprising as previous experiments showed detection of BCG DNA down to 10^2 CFU/mL (e.g. Table 3.84)

Table 3.97: Mean Roche probe-based qPCR results for M. bovis BCG testing the concentration of DNA available for metagenomic sequencing following depletion protocols using triplicate samples

Sample	**BCG Probe qPCR Amplification ($\bar{X}$ C_T)**
10^5 BCG Spiked Sputum	26.49
10^4 BCG Spiked Sputum	28.90
10^3 BCG Spiked Sputum	37.31
10^2 BCG Spiked Sputum	40.00
10^1 BCG Spiked Sputum	40.00
Non-Depleted 10^5 Positive Control	25.85
Negative NRF Sputum Control	37.97

Following qPCR analysis 10^5 CFU/mL – 10^3 CFU/mL spiked extracted samples were sequenced to determine the working LoD of the method. Following 6 hours of sequencing, reads were uploaded to the Epi2Me WIMP pipeline and MTBC reads were recorded (Table 3.98).

Lack of amplification observed in the 10^3 CFU/mL spiked sample correlated with lack of coverage from sequencing. Analysis also showed amplification during qPCR did not directly correlate with sequencing coverage. MTBC reads accounted for only 7.62% of total reads in the $1x10^5$ CFU/mL spiked sample but 41.48% of total reads in the $1x10^4$ CFU/mL spiked sample, despite a qPCR product concentration 5.3 fold greater in the $1x10^5$ CFU/mL sample. This is likely related to varying levels of bacterial and commensal DNA present in the samples. These preliminary results demonstrate an LoD of approximately 10,000 CFU/mL.

Table 3.98: Epi2Me WIMP pipeline results of three 10-fold serially diluted M. bovis BCG samples in NRF sputum

Sample	MTBC Reads (#)	Total Mycobacterial Reads (% of Total Reads)
$1x10^5$ BCG Spiked Sputum	802	7.62
$1x10^4$ BCG Spiked Sputum	23,709	41.48
$1x10^3$ BCG Spiked Sputum	0	0

Based on these results focus was returned to the tNGS method to minimize the impact of commensal bacteria on diagnosis and DST.

3.3.4: Metagenomic Assay Performance Summary

The metagenomic protocol exhibits an LoD of approximately 10,000 CFU/mL; roughly equivalent to smear microscopy, though with increased complexity [188]. This poor analytical sensitivity largely precludes competition with existing molecular technologies. One method for improving the LoD is by increasing sequencing times, although this would quickly inflate turnaround time (TaT) for the assay.

Currently, the metagenomic method does exhibit a TaT of approximately 8 hours from receipt of sample for majority genome coverage. Use of this method following a positive smear test could prove clinically useful given the depth and breadth of information possible. This use is limited, however, by the poor analytical specificity seen during preliminary testing. As with the analytical sensitivity, the metagenomic method displayed poor analytical specificity (41%).

The poor analytical performance of the metagenomic assay is further hampered by its cost. Current metagenomic techniques have an average cost of £25.57/sample [111]. This price point is higher than existing molecular techniques on the market and would need to be significantly reduced to promote uptake of the method.

Chapter 4 – Implementation and Conclusion

4.1: Implementation of the tNGS Assay

4.1.1: Current tNGS Assay Landscape

Although primarily new diagnostics for TB have utilized amplification without sequencing, tNGS assays assays have been developed for DST. The GenoScreen® Deeplex Myc-TB and the TGEN® Next-Gen RDST assay are both currently on the market although neither is, as yet, endorsed by the WHO [116,195,196]. Each test is used following a positive TB diagnosis for comprehensive DST. The Deeplex Myc-TB has a cited genotypic sensitivity of 97.1% [165]. This test also exhibits a high phenotypic sensitivity and specificity (95.3% and 97.4% respectively) for first and second-line medications [165].

A 2016 study of the Next-Gen RDST method showed a genotypic sensitivity of 97.8% as compared to pyrosequencing [196]. This study also cited phenotypic sensitivities and specificities for three first-line medications; isoniazid (95%, 100%), rifampicin (97.6%, 98.9%) , and kanamycin (96.2%, 93.9%) [196]. For second-line medications phenotypic sensitivity ranged from 42.9% (amikacin) to 86.7% (moxifloxacin). The phenotypic specificity had a narrower range in second-line medications from 85.7% (oxifloxacin) to 98% (capreomycin) [196].

However, neither of these methods is currently endorsed by the WHO. This means that the primary molecular methods for TB diagnosis and DST remain amplification or line-probe based.

4.1.2: TB Diagnostic Time Requirements

One of the primary limitations with gold standard culture techniques for TB is the time required. This is frequently compounded by patient delays in seeking treatment which average a median 23 days from the onset of symptoms [197]. In LMICs where frequently the only DST tool routinely available is culture the turnaround time (TaT) is often seen as unavoidable. Delays in diagnosis can result in negative patient outcomes, increased community transmission, and an increased risk of

drug resistance emerging. Conversely, while smear microscopy is rapid it suffers from low sensitivity and an inability to inform drug regimens. These limitations have been primary motivators behind the development of improved technologies.

The most widely used of these, the Xpert MTB/RIF assay, reduces diagnosis and rifampicin resistance calling TaT to 24 hours on average, although under optimal conditions it can take as little as 1 hour [185,198]. The primary source of delays for the performance of an Xpert MTB/RIF assay lie in infrastructure, both physical and human. The median delay attributable to such issues within the healthcare system is 7 days [197]. However, this ability to rapidly provide a TB diagnosis, and basic DST in the MTB/RIF assay, can result in improved patient outcomes. While this assay is only able to detect resistance to rifampicin, this is often a good surrogate for MDR increasing the utility possible within the rapid timeframe [199,200].

Another such technology, the Genotype MTBDRplus line probe assay, reduced time from sample to diagnosis of TB and drug resistance to 1 week on average [201]. While this marked a significant decrease in TaT from culture it is still longer then preferable for optimal patient outcomes. Often, by the one-week mark, patients have already begun a treatment regimen based on clinical diagnosis. However, as a treatment course for TB currently takes several months of concerted effort, one week of ineffective treatment will have minimal healthcare implications except in the most extreme of cases.

Delays in the use of the Genotype MTBDRplus line probe assay are exacerbated by its use as a reflexive test following a positive TB diagnosis by either culture or Xpert MTB/RIF which further extends the time between diagnosis and comprehensive DST result. This is a concern when using the Genotype assay as administration of drugs despite resistance, or later alteration of a treatment regimen, can increase the risk of drug-resistance propagation in patients and the population. However, use of the MTBDRplus and MTBDRsl (second-line) assays do provide comprehensive drug resistance information and should not be discounted due to this lag time.

The tNGS multiplex assay can reduce the TaT of diagnosis and provide comprehensive DST in ~13 hours from the receipt of samples (Figure 4.1) when testing 80 samples at a time. However, in real lab conditions this is likely to translate to approximately two days from sample to patient. This can provide clinically useful treatment information the day after a TB diagnosis. The TaT is dependent on the number of samples tested and can be cut to approximately 8 hours when testing 24 samples. Further development of the test by colleagues in the O'Grady group has further reduced the TaT and it is now possible to test 24 samples in approximately 4 hours.

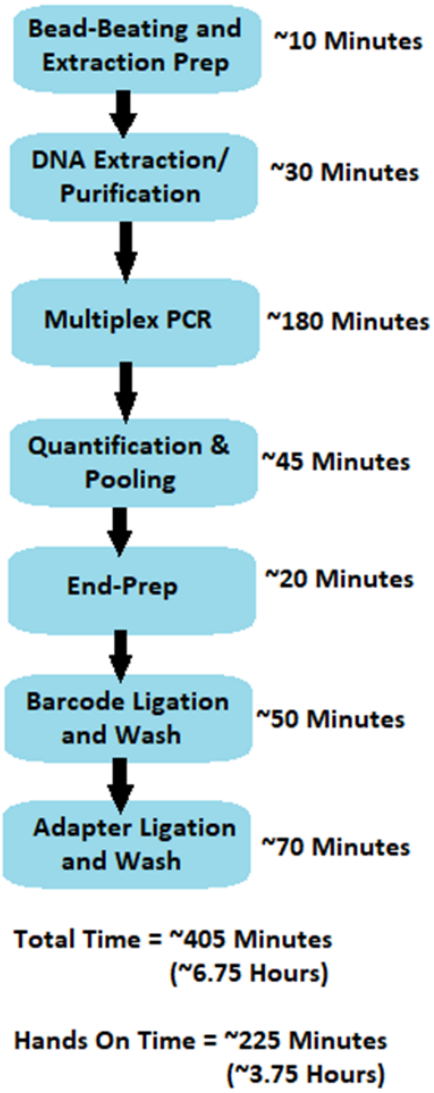

Figure 4.1: Flow and time requirements for each step of the tNGS multiplex assay following receipt of a sample excluding 6 hour recommended sequencing time

As mentioned previously, TaT is not solely impacted by the length of a diagnostic procedure. Rather, numerous factors from collection of the sample, distance to the appropriate laboratory, and levels of staffing play large roles in the TaT of any given assay.

4.1.3: Cost of TB Diagnosis

Beyond the clinical utility and performance of a diagnostic and DST assay, cost per sample is an essential factor in implementation. Any new assays should first be compared to the gold standards of culture and smear microscopy. Culture, due to necessary sample preparation and biosafety considerations, costs between £10.02 and £21.58, depending on if DST is conducted [201,202]. Smear microscopy is significantly cheaper at an average £1.77– £1.85 per sample and generally requires less infrastructure increasing accessibility [201]. When combined, fluorescent smear microscopy with culture DST costs approximately £13.91 per sample [201]. The majority of the costs for these methods lies in consumables which can fluctuate from country to country [203]. However, these costs are not absolute and can be subsidized in LMICs to improve access and remove barriers to diagnostic care and improve patient outcomes.

New diagnostic and DST tests are also likely to be compared to existing WHO endorsed assays such as the previously discussed Xpert MTB/RIF, MTB/RIF Ultra, GenoType MTBDRplus and MTBDRsl. Of these, the Xpert MTB/RIF has the lowest average cost per sample (£12.30 – £27.58) when including privatised healthcare markets in India and Brazil, two high-incidence countries [198,201,204,205]. The GenoType MTBDRplus assay is more expensive at £18.47 – £19.33 per sample [201,206]. Unlike culture and smear microscopy the bulk of the cost for these tests lies not with consumables but instead with the equipment and assay cartridges themselves [203]. Also, staff costs accounted for approximately 29% of the cost for an Xpert MTB/RIF assay compared to only 5.4% of the cost for smear microscopy in one study from 2021 [203]. However, as with culture and smear microscopy, endorsed molecular assays are often subsidized by the WHO for use in LMICs to improve global health outcomes. These costs make the currently endorsed assays equivalent to, or slightly better

than, culture with DST. However, no molecular assay has yet approached the cost-effectiveness of smear microscopy.

WHO has called for new NGS technologies with costs below £49.43/sample [207]. The tNGS assay has calculated costs slightly higher than culture but which fall below the desired threshold; £23.22/sample for 80-samples/run, less than half of WHO's upper cost limit. This calculated cost would decrease to £19.52/sample, equivalent to the MTBDRplus assay, should all three multiplex reactions be combined into a single reaction. Cost could potentially be further reduced by sequencing with alternative library preparation methods, sequencing more samples per flow cell and washing/reusing flowcells.

4.2: Benefits of Nanopore Sequencing for TB Diagnosis

Both tNGS and metagenomic methods benefit from the use of Nanopore sequencing instead of Illumina or pyrosequencing. Nanopore sequencing allows real-time results which improves TaT of assays and can decrease costs by ceasing runs after adequate results have been generated. This allows wash and reuse of flowcells reducing per sample costs [208]. Illumina, however, requires completion of a run before results can be analysed decreasing analytical flexibility.

One major advantage of nanopore sequencing is cost-effectiveness. Starter kits from ONT cost £823.80 and don't require service contracts [208]. In comparison the iSeq 100 from Illumina has a list price of £16393.62 and requires a service contract for operation [209]. This cost disparity limits the uptake of Illumina in LMICs outside of central reference laboratories but can allow for use of nanopore methods at the point-of-care.

Another benefit of nanopore sequencing which can assist uptake at point-of-care and near-patient facilities is its portability. Both the tNGS and metagenomic methods utilise the MinION sequencing platform which is highly portable (10.5cm x 2.3cm x 3.3cm, 85g) and has been used in remote locations around the world, and above it (the ISS) [210]. Use of the MinION only requires connection

to a laptop and a power source, one study performed 24 hours of sequencing off of portable solar panels, demonstrating utility in remote areas with limited infrastructure [211]. The new MinION Mk1C removes the requirement for an external laptop for use further improving portability.

All of these factors make nanopore sequencing in general, and the MinION platform in particular, powerful resources for disease control in LMICs.

4.3: Conservation and Ecology Applications

Use of the tNGS assay for monitoring TB status in livestock and wildlife is a potential avenue of investigation for future consideration. In conversation with zoo keepers at the National Zoonotic Gardens of South Africa and Brandywine Zoo in Delaware, as well as trackers and game keepers at the Phinda Private Game Reserve I was told that herd animals have a high incidence of TB which is largely undetectable until the infection is terminal. This is especially problematic in zoos where death by TB can result in a cull of the entire herd [212]. By using high throughput sequencing such as used in validation of the tNGS assay, it should be feasible to include TB screening in annual wellness checks.

Using the assay in such a way would require some measure of redesign in the targets to optimize coverage of *M. bovis, M. orygis*, and *M. caprae*. This redesign would also need to reincorporate a speciation target to improve determination of an infection's causative agent. However, the drug resistance detection pipeline is not predicted to require modification simplifying adaptation of the tNGS assay for use in animals instead of humans.

4.4: Conclusions

While TB will continue to be a global health issue for the foreseeable future, constant research and development of new diagnostic technologies and treatment regimens should help stem the increase in DR-TB. The tNGS assay developed in this study will help in the fight against DR-TB. This was effectively shown compared to existing diagnostic assays where the tNGS assay exhibited

equivalent or superior sensitivity, specificity and LoD. However, the metagenomic methodology, despite showing promise for lower respiratory infections in general [113], proved ineffective for TB specific diagnoses due to commensal bacteria. Design of a targeted depletion or capture methodology for metagenomic sequencing could improve this technology's viability in coming years.

As molecular methods continue to improve the use of culture and smear microscopy should be phased out and replaced with methods that can guide effective anti-TB therapy on the day the patient is tested. With the aid of WHO and FIND in validating and subsidizing these new technologies, this is possible in the foreseeable future.

Appendix I: Catalogue of Known Drug-Resistance SNPs for TB

The following is a comprehensive list of known high confidence mutations associated with the development of drug resistance in MTBC species. Mutations are annotated as either nucleotide changes (lower case) or amino acid changes (upper case) along with the gene locus at which the mutation may occur.

Gene	Drug	Mutation (Wildtype:Site:Mutation)	Mutation Type (Nucleotide or Amino Acid)
rrs	Amikacin	a514c	Nucleotide
		a514t	Nucleotide
		c517t	Nucleotide
		a1338c	Nucleotide
		a1401*	Nucleotide
		a1401g	Nucleotide
		c1402*	Nucleotide
		c1402t	Nucleotide
		g1484*	Nucleotide
		g1484t	Nucleotide
	Capreomycin	a1401*	Nucleotide
		a1401g	Nucleotide
		c1402*	Nucleotide
		c1402t	Nucleotide
		g1484*	Nucleotide
		g1484t	Nucleotide
	Kanamycin	a514c	Nucleotide
		c517t	Nucleotide
		a1401*	Nucleotide
		a1401g	Nucleotide
		c1402*	Nucleotide
		c1402t	Nucleotide
		g1484*	Nucleotide
		g1484t	Nucleotide
	Streptomycin	c462t	Nucleotide
		c492t	Nucleotide
		c513t	Nucleotide
		a514c	Nucleotide
		a514t	Nucleotide
		c517t	Nucleotide
		c905a	Nucleotide
		c905g	Nucleotide
		a906g	Nucleotide

		a907c	Nucleotide
		a907t	Nucleotide
		a908g	Nucleotide
		t1239c	Nucleotide
		a1325c	Nucleotide
rv0678	Bedaquline	S63R	Amino Acid
	Clofazamine	S63R	Amino Acid
gyrA	Ciprofloxin	A74S	Amino Acid
		S91*	Amino Acid
		S91P	Amino Acid
		D94*	Amino Acid
		D94A	Amino Acid
		D94G	Amino Acid
		D94H	Amino Acid
		D94N	Amino Acid
	Moxifloxacin	A90*	Amino Acid
		A90V	Amino Acid
		S91*	Amino Acid
		S91P	Amino Acid
		D94*	Amino Acid
		D94A	Amino Acid
		D94G	Amino Acid
		D94H	Amino Acid
		D94N	Amino Acid
		D94Y	Amino Acid
		A90V	Amino Acid
		D89N	Amino Acid
		G88A	Amino Acid
		G88C	Amino Acid
	Ofloxacin	A90*	Amino Acid
		A90V	Amino Acid
		S91*	Amino Acid
		S91P	Amino Acid
		D94*	Amino Acid
		D94A	Amino Acid
		D94G	Amino Acid
		D94H	Amino Acid
		D94N	Amino Acid
		D94Y	Amino Acid
		G89N	Amino Acid
		G88A	Amino Acid
		G88C	Amino Acid
	Quinolones	H70R	Amino Acid

		A74S	Amino Acid
		H85*	Amino Acid
		P86*	Amino Acid
		H87*	Amino Acid
		G88*	Amino Acid
		D89*	Amino Acid
		A90*	Amino Acid
		A90V	Amino Acid
		S91*	Amino Acid
		S91P	Amino Acid
		I92*	Amino Acid
		Y93*	Amino Acid
		D94*	Amino Acid
		D94A	Amino Acid
		D94G	Amino Acid
		D94H	Amino Acid
		D94N	Amino Acid
		L96*	Amino Acid
		V97*	Amino Acid
gidB	Capreomycin	Y195H	Amino Acid
	Streptomycin	I11N	Amino Acid
		A19P	Amino Acid
		L26F	Amino Acid
		G30D	Amino Acid
		G34V	Amino Acid
		V41I	Amino Acid
		R47W	Amino Acid
		H48N	Amino Acid
		H48Q	Amino Acid
		C52F	Amino Acid
		R64W	Amino Acid
		V65G	Amino Acid
		G69D	Amino Acid
		S70N	Amino Acid
		G73A	Amino Acid
		P75L	Amino Acid
		P75R	Amino Acid
		L79S	Amino Acid
		L79W	Amino Acid
		A80P	Amino Acid
		R83P	Amino Acid
		D85A	Amino Acid
		V88A	Amino Acid

		L91P	Amino Acid
		E92D	Amino Acid
		P93L	Amino Acid
		G117V	Amino Acid
		R118L	Amino Acid
		R118S	Amino Acid
		Q125.	Amino Acid
		A134E	Amino Acid
		S136.	Amino Acid
		R137P	Amino Acid
		R137W	Amino Acid
		A138T	Amino Acid
		A138V	Amino Acid
		S149R	Amino Acid
		I162S	Amino Acid
		E173.	Amino Acid
		A200E	Amino Acid
		V203L	Amino Acid
		A205E	Amino Acid
tlyA	Capreomycin	c-83t	Nucleotide
		N236K	Amino Acid
embB	Ethambutol	N296H	Amino Acid
		S297A	Amino Acid
		M306*	Amino Acid
		A313V	Amino Acid
		Y319C	Amino Acid
		Y319S	Amino Acid
		D328G	Amino Acid
		D328V	Amino Acid
		D328Y	Amino Acid
		Y334H	Amino Acid
		S347I	Amino Acid
		D354A	Amino Acid
		A356V	Amino Acid
		V377G	Amino Acid
		E378A	Amino Acid
		P397T	Amino Acid
		E405D	Amino Acid
		G406A	Amino Acid
		G406C	Amino Acid
		G406D	Amino Acid
		G406S	Amino Acid
		Q497K	Amino Acid

		Q497P	Amino Acid
		Q497R	Amino Acid
		E504D	Amino Acid
		A659T	Amino Acid
		Q853P	Amino Acid
		H1002R	Amino Acid
		D1024N	Amino Acid
		N1033K	Amino Acid
		T1082A	Amino Acid
ethA	Ethionamide	M1R	Amino Acid
		G43C	Amino Acid
		T61M	Amino Acid
		T232A	Amino Acid
		I338S	Amino Acid
		T342K	Amino Acid
		A381P	Amino Acid
fabG1	Ethionamide	g-17t	Nucleotide
	Isoniazid	g-17t	Nucleotide
		a-16*	Nucleotide
		c-15*	Nucleotide
		t-8*	Nucleotide
		G609A	Amino Acid
inhA	Ethionamide	c-15t	Nucleotide
		I21T	Amino Acid
		S49A	Amino Acid
		S94A	Amino Acid
		I194T	Amino Acid
	Isoniazid	c-15t	Nucleotide
		I21T	Amino Acid
		I21V	Amino Acid
		S94A	Amino Acid
		I194T	Amino Acid
katG	Isoniazid	Y155C	Amino Acid
		Y155S	Amino Acid
		L159P	Amino Acid
		T180K	Amino Acid
		G182R	Amino Acid
		W191G	Amino Acid
		W191R	Amino Acid
		P232R	Amino Acid
		P241P	Amino Acid
		M257I	Amino Acid
		T275A	Amino Acid

		Q295P	Amino Acid
		G297V	Amino Acid
		G299C	Amino Acid
		W300C	Amino Acid
		W300S	Amino Acid
		S302R	Amino Acid
		D311G	Amino Acid
		S315*	Amino Acid
		S315I	Amino Acid
		S315N	Amino Acid
		S315T	Amino Acid
		W321.	Amino Acid
		W328L	Amino Acid
		I335V	Amino Acid
		L378P	Amino Acid
		A379V	Amino Acid
		D419H	Amino Acid
		A424G	Amino Acid
eis	Kanamycin	g-10a	Nucleotide
		c-12t	Nucleotide
		c-14t	Nucleotide
		g-37t	Nucleotide
rplC	Linezolid	C154R	Amino Acid
pncA	Pyrazinamide	t-12c	Nucleotide
		a-11g	Nucleotide
		t-7c	Nucleotide
		M1T	Amino Acid
		A3E	Amino Acid
		L4S	Amino Acid
		L4W	Amino Acid
		I5S	Amino Acid
		I6L	Amino Acid
		I6T	Amino Acid
		V7F	Amino Acid
		V7G	Amino Acid
		V7L	Amino Acid
		D8A	Amino Acid
		D8G	Amino Acid
		D8N	Amino Acid
		D8E	Amino Acid
		V9A	Amino Acid
		Q10.	Amino Acid
		Q10P	Amino Acid

		Q10R	Amino Acid
		D12A	Amino Acid
		D12N	Amino Acid
		C14.	Amino Acid
		C14G	Amino Acid
		C14R	Amino Acid
		C14Y	Amino Acid
		G17D	Amino Acid
		L19P	Amino Acid
		V21G	Amino Acid
		G24D	Amino Acid
		L27P	Amino Acid
		I31S	Amino Acid
		S32I	Amino Acid
		Y34.	Amino Acid
		Y34D	Amino Acid
		L35R	Amino Acid
		V44G	Amino Acid
		A46E	Amino Acid
		A46V	Amino Acid
		T47A	Amino Acid
		T47P	Amino Acid
		K48E	Amino Acid
		K48T	Amino Acid
		D49A	Amino Acid
		D49G	Amino Acid
		D49N	Amino Acid
		H51Q	Amino Acid
		H51R	Amino Acid
		H51Y	Amino Acid
		P54L	Amino Acid
		P54Q	Amino Acid
		P54S	Amino Acid
		H57D	Amino Acid
		H57R	Amino Acid
		H57P	Amino Acid
		H57Y	Amino Acid
		F58L	Amino Acid
		F58S	Amino Acid
		S59P	Amino Acid
		T61P	Amino Acid
		P62L	Amino Acid
		P62Q	Amino Acid

		P62T	Amino Acid
		P62R	Amino Acid
		D63A	Amino Acid
		D63G	Amino Acid
		Y64D	Amino Acid
		S66P	Amino Acid
		S67P	Amino Acid
		W68.	Amino Acid
		W68C	Amino Acid
		W68G	Amino Acid
		W68R	Amino Acid
		P69L	Amino Acid
		H71Q	Amino Acid
		H71R	Amino Acid
		H71Y	Amino Acid
		H71D	Amino Acid
		C72R	Amino Acid
		C72Y	Amino Acid
		T76I	Amino Acid
		T76P	Amino Acid
		G78C	Amino Acid
		C78D	Amino Acid
		F81V	Amino Acid
		H82D	Amino Acid
		H82R	Amino Acid
		L85P	Amino Acid
		L85R	Amino Acid
		T87M	Amino Acid
		I90S	Amino Acid
		F94L	Amino Acid
		F94S	Amino Acid
		K96E	Amino Acid
		K96N	Amino Acid
		K96Q	Amino Acid
		K96T	Amino Acid
		K96R	Amino Acid
		G97C	Amino Acid
		G97D	Amino Acid
		G97R	Amino Acid
		G97S	Amino Acid
		Y99.	Amino Acid
		A102V	Amino Acid
		Y103*	Amino Acid

		Y103.	Amino Acid
		Y103H	Amino Acid
		S104G	Amino Acid
		S104R	Amino Acid
		G108R	Amino Acid
		T114P	Amino Acid
		L116P	Amino Acid
		L116R	Amino Acid
		L120P	Amino Acid
		R123P	Amino Acid
		V125G	Amino Acid
		V125F	Amino Acid
		V128G	Amino Acid
		V130G	Amino Acid
		G132A	Amino Acid
		G132D	Amino Acid
		G132S	Amino Acid
		I133T	Amino Acid
		A134V	Amino Acid
		T135P	Amino Acid
		T135N	Amino Acid
		D136G	Amino Acid
		D136N	Amino Acid
		H137P	Amino Acid
		H137R	Amino Acid
		C138R	Amino Acid
		C138S	Amino Acid
		C138Y	Amino Acid
		V139A	Amino Acid
		V139G	Amino Acid
		V139L	Amino Acid
		C139M	Amino Acid
		Q141.	Amino Acid
		T142A	Amino Acid
		T142M	Amino Acid
		T142K	Amino Acid
		A146T	Amino Acid
		A146V	Amino Acid
		A148Insertion	Amino Acid
		L151S	Amino Acid
		R154G	Amino Acid
		V155A	Amino Acid
		V155G	Amino Acid

		V155L	Amino Acid
		L159V	Amino Acid
		L159P	Amino Acid
		T160P	Amino Acid
		A161P	Amino Acid
		G162D	Amino Acid
		T168P	Amino Acid
		A171V	Amino Acid
		A171E	Amino Acid
		L172P	Amino Acid
		L172R	Amino Acid
		M175R	Amino Acid
		M175T	Amino Acid
		M175V	Amino Acid
		M175I	Amino Acid
		V180F	Amino Acid
		V180G	Amino Acid
rpoB	Rifampicin	V170F	Amino Acid
		A286V	Amino Acid
		V359A	Amino Acid
		T400A	Amino Acid
		F424L	Amino Acid
		F424S	Amino Acid
		F424V	Amino Acid
		F425*	Amino Acid
		G426*	Amino Acid
		T427*	Amino Acid
		S428*	Amino Acid
		Q429*	Amino Acid
		L430*	Amino Acid
		S431*	Amino Acid
		Q432*	Amino Acid
		F433*	Amino Acid
		M434*	Amino Acid
		D435*	Amino Acid
		Q436*	Amino Acid
		N437*	Amino Acid
		N438*	Amino Acid
		P439*	Amino Acid
		L440*	Amino Acid
		S441*	Amino Acid
		G442*	Amino Acid
		L443*	Amino Acid

		T444*	Amino Acid
		H445*	Amino Acid
		K446*	Amino Acid
		R447*	Amino Acid
		R448*	Amino Acid
		L449*	Amino Acid
		S450*	Amino Acid
		A451*	Amino Acid
		L452*	Amino Acid
		P454H	Amino Acid
		P454L	Amino Acid
		E460G	Amino Acid
		I480T	Amino Acid
		I480V	Amino Acid
		I491F	Amino Acid
		S493L	Amino Acid
		T676P	Amino Acid
		E761D	Amino Acid
		G981D	Amino Acid
rpsL	Streptomycin	K43*	Amino Acid
		K43R	Amino Acid
		K43T	Amino Acid
		K88*	Amino Acid
		K88Q	Amino Acid
		K88R	Amino Acid
		T40I	Amino Acid

Appendix II – Gene Maps With Primer Sites and Known SNPs

The following are annotated FastA maps of each gene targeted by the assay. Maps are annotated with the locations of the final assay primers, nested primers, known resistance SNPs, and any extragenic buffer included for the design of amplicons.

WHOLE GENE COVERAGE INCLUDES 300bp BRACKETING KNOWN GENE

Red = Non-Gene Extension Region
Green = Primer
Pink = Nested Primer
Yellow = Known Resistance SNP

>NC_000962.3:c2289600-2288300 Mycobacterium tuberculosis H37Rv, pncA Pyrazinamide Resistance Gene **(TOTAL GENE COVERAGE)**

AATGCACTTCGCTTTCATCGCTTACGTCCTTGCCGGCGGTTTCCTTGCCCTGCGGTGGCGACGCACGATG
TGGCTGCATGTTCCGGCGGTGATATGGGGGATCGGCATCGCCGCTAAGCGGGTCGACTGCCCGCTGACCT
GGGTGGAGCGCTGGGCTCGCACCAAGGCCGCGATGACACCTCTGTCACCGGACGGATTTGTCGCTCACTA
CATCACCGGCGTGATCTATCCCGCCGGTTGGGTGGCCGCCGCTCAGCTGGTCATGTTCGCGATCGTCGCG
GCGTCATGGACCCTATATCTGTGGCTGCCGCGTCGGTAGGCAAACTGCCCGGGCAGTCGCCCGAACGTAT
GGTGGACGTATGCGGGCGTTGATCATCGTCGACGTGCAGAACGACTTCTGCGAGGGTGGCTCGCTGGCGG
TAACCGGTGGCGCCGCGCTGGCCCGCGCCATCAGCGACTACCTGGCCGAAGCGGCGGACCTACCATCACGT
CGTGGCAACCAAGGACTTCCACCATCGACCCGGGTGACCACTTCTCCGGGCACACCGGACCTATTCCTCGTCG
TGGCCACCGCATTGCGTCAGCGGTACTCCCGGCGCGGACTTCCATCCCAGTCTGGACACGTCGGCAATCG
AGGCGGTGTTCTACAAGGGTGCCTACACCGGAGCGTACAGCGGCTTCGAAGGAGTCGACGAGAACGGCAC
GCCACTGCTGAATTGGCTGCGGCAACGCGGCGTCGATGAGGTCGATGTGGTCGGTATTGCCACCGATCAT
TGTGTGCGCCAGACGGCCGAGGACGCGGTACGCAATGGCTTGGCCACCAGGGTGCTGGTGGACCTGACAG
CGGGTGTGTCGGCCGATACCACCGTCGCCGCGCTGGAGGAGATGCGCACCGCCAGCGTCGAGTTGGTTTG
CAGCTCCTGATGGCACCGCCGAACCGGGATGAACTGTTGGCGGCGGTGGAGCGCTCGCCGCAAGCGGCCG
CCGCGCACGACCGCGCCGGCTGGGTCGGGTTGTTCACCGGTGACGCGCGGGTCGAAGACCCGGTGGGTTC
GCAGCCGCAGGTGGGGCATGAGGCCATCGGCCGCTTCTACGACACCTTCATCGGGCCGCGGGATATCACG
TTCCATCGCGATCTGGATATCGTCTCCGGCACGGTGGTGCTGCGCGATCTCGAACTCGAGGTCGCGATGG
ACTCGGCTGTGACGGTGTTCATTCCCGCCTTCCTACGCTATGACCTACGACCGGTTACCGGCGAGTGGCA
GATTGCCGCACTGCGGGCGTACTGGGAGTTGCCGGCGATGA

>NC_000962.3:4246514-4249810 Mycobacterium tuberculosis H37Rv, embB Ethambutol Resistance Gene **(SNP at 306bp)**

ATGACACAGTGCGCGAGCAGACGCAAAAGCACCCCAAATCGGGCGATTTTGGGGGCTTTTGCGTCTGCTC
GCGGGACGCGCTGGGTGGCCACCATCGCCGGGCTGATTGGCTTTGTGTTGTCGGTGGCGACGCCGCTGCT
GCCCGTCGTGCAGACCACCGCGATGCTCGACTGGCCACAGCGGGGGCAACTGGGCAGCGTGACCGCCCCG
CTGATCTCGCTGACGCCGGTCGACTTTACCGCCACCGTGCCGTGCGACGTGGTGCGCGCCATGCCACCCG
CGGGCGGGGTGGTGCTGGGCACCGC**A**CCCAAGCAAGGC**A**AGGACGCC**A**ATTTGCAGGCGTTGTTCGTCGT
CGT**C**AGCGCCCAGCGCGTGGACGTCACCGACCGCAACGTGGTGAT**C**TTGT**C**CGTGC**C**GCGCGAGCAGGTG
ACGTCCCCGCAGTGTCAACGCATCGAGGTCACCTCTACCCACGCCGGCACCTTCGCCAACTTCGTCGGGC
TCAAGG**A**CCCGTCGGGCGCGCCGCTGCGCAGCGGCTTCCCCGACCCCAACCTGCGCCCGCAGATTGTCGG
GG**T**GTTCACCGACCTGACCGGGCCCGCGCCGCCCGGGCTGGCGGTCTCGGCGACCATCGACACCCGGTTC
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ACCTACCTGTCCCGCGACTGGGCCCGCGATTGGGGTTCCCTGCGCAAGTTCGACACCCTGGTCGATGCCC
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TCCATAG

>NC_000962.3:759807-763325 Mycobacterium tuberculosis H37Rv,
rpoB Rifampicin Resistance Gene **(SNPs at 508-534bp)**

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CAGTCGCTGTGCCTCAACGTCGAGGTGCTATCGAGTGACGGTGCGGCGATCGAACTGCGCGAAGGTGAGG
ACGAGGACCTGGAGCGGGCCGCGGCCAACCTGGGAATCAATCTGTCCCGCAACGAATCCGCAAGTGTCGA
GGATCTTGCGTAA

>NC_000962.3:1673100-1674500 Mycobacterium tuberculosis H37Rv, fabG1 Isionazid & Ethionamide Resistance Gene **(TOTAL GENE COVERAGE, SNP at 15)**

ACCTTCAAATCGGTGGCCTGTATCAGGGCGTCGACGGCCGCGACGGCGGGGTCCAATGGAAATCGACTGG
TCAGGTCGAGCGCCGTTCGCTCCGGTGTGGTCACGCGCATGCCCTCGATGACGCAGATCTCGTCGGGCTC
GATGCGCTCTTCCCAGACTTGCAGCCCCGGGGCACGGCGGCGGTTGGTGTCGATGATCGCGGCGGGAAGA
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GACGATAGGTTGTCGGGGTGACTGCCACAGCCACTGAAGGGGCCAAACCCCCATTCGTATCCCGTTCAGT
CCTGGTTACCGGAGGAAACCGGGGGATCGGGCTGGCGATCGCACAGCGGCTGGCTGCCGACGGCCACAAG
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GCGACGCCGTCGATCGCGCCTTCACGGCGGTAGAAGAGCACCAGGGTCCGGTCGAGGTGCTGGTGTCCAA
CGCCGGCCTATCCGCGGACGCATTCCTCATGCGGATGACCGAGGAAAAGTTCGAGAAGGTCATCAACGCC
AACCTCACCGGGGCGTTCCGGGTGGCTCAACGGGCATCGCGCAGCATGCAGCGCAACAAATTCGGTCGAA
TGATATTCATAGGTTCGGTCTCCGGCAGCTGGGGCATCGGCAACCAGGCCAACTACGCAGCCTCCAAGGC
CGGAGTGATT

>NC_000962.3:c2156111-2153889 Mycobacterium tuberculosis H37Rv, katG Isionazid Resistance Gene **(SNP at 315bp)**

GTGCCCGAGCAACACCCACCCATTACAGAAACCACCACCGGAGCCGCTAGCAACGGCTGTCCCGTCGTGG
GTCATATGAAATACCCCGTCGAGGGCGGCGGAAACCAGGACTGGTGGCCCAACCGGCTCAATCTGAAGGT
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CCAACGACTTCTTCGTGAACCTGCTCGACATGGGTATCACCTGGGAGCCCTCGCCAGCAGATGACGGGAC
CTACCAGGGCAAGGATGGCAGTGGCAAGGTGAAGTGGACCGGCAGCCGCGTGGACCTGGTCTTCGGGTCC
AACTCGGAGTTGCGGGCGCTTGTCGAGGTCTATGGCGCCGATGACGCGCAGCCGAAGTTCGTGCAGGACT
TCGTCGCTGCCTGGGACAAGGTGATGAACCTCGACAGGTTCGACGTGCGCTGA

>NC_000962.3:1673900-1675300 Mycobacterium tuberculosis H37Rv,
inhA Isionazid & Ethionamide Resistance Gene **(TOTAL GENE COVERAGE)**

CAGCCTCCAAGGCCGGAGTGATTGGCATGGCCCGCTCGATCGCCCGCGAGCTGTCGAAGGCAAACGTGAC
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GCCTACAACTGGATGACGGTCGCCAAGAGCGCGTTGGAGTCGGTCAACAGGTTCGTGGCGCGCGAGGCCG
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AGAACGTTACCCGGGGCCGCGGTGTGCCTGCCGAACGGTTGGACGCGGTGGCCGAGCACTACCTGCATTT
CGGTGGGGTATCACCGATCAATGGCATTAATCGCACACTGATCGCGGAGCTGGAGGCGCAGCAAGAACTG
CCGGTGTACTTCGGTAACCGCAACTGGGAGCCGTATGTAGAAGATGCCGTTACGGC**C**ATGCGCGACAACG
G

>NC_000962.3:c2713000-2714124 Mycobacterium tuberculosis H37Rv,
eis Kanamycin Resistance Gene **(SNP at 12-37bp, EXTENDED TO ALLOW)**

TCGTGATGGCATTCACTGGAATTTTGAAGCCCACCAGGCGGTCGCCGAACTGATGCTCAAGGCACTGGCC
GAAGCCGGGGTGCCGAACGAGAAATCGCGCGGCTGAGACATGCCCCGCGCCGGCGACGATGCAGAGCGAA
GCGATGAGGTGGGGGCACCTCCCGCTTGCGGGGGAGAGCGGCGCCGGTGACCGTTGTGGTGGTGACCGAT
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GACCGCGTTCGAGTTCTGAAGGCCGTGCTAGGCCGGCGCTAGGCTGACGGGCTTTTCGGCGTGGTCAGCG
ACCCGCGTGCTGCGCGCCGGCTTCGGTCGCCACACGCCATGGATGGGGATCGGCCGCGCGGCTCAGCACT
CGCGGATCGCGTTGCCGAGTACACTCTCGATCGCGGTGAGCCGAGTCCGGATGGACGTCGCAGCCCGGTG
CTGGTTGTTCGGGGATGTTGGCGCGGTTTGTCCCATCTTGATCCATCGCCAAAGCGGCTTGTCCACAGCC
TCGGATTGATCCACAGCAGGGCAGCGCGACGCCGTCGTG

>NC_000962.3:1471846-1474000 Mycobacterium tuberculosis H37Rv, rrs Amikacin Resistance Gene **(SNP at 1401bp, EXTENDED TO ALLOW, Partial overlap with RRL)**

TTTTGTTTGGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAAC
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CGGGGAGCTGTCAACCGAGCGTGGATCCGAGGATTTCCGAATGGGGAAACCCAGCACGAGTGATGTCGTG
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GGAGAAAACAATTGTGATTCCGCAAGTAGTGGCGAGCGAACGCGGAACAGGCTAAACCGCACGCATGGGT
AACCGGGTAGGGGTTGTGTGTGCGGGGTTGTGGGAGGATATGTCTCAGCGCTACC

>NC_000962.3:1917640-1919046 Mycobacterium tuberculosis H37Rv, tlyA Capreomycin Resistance Gene **(TOTAL GENE COVERAGE)**

GCCGATCTTCACCAGCGGCCACTGCGCATCGAGGCCGGCGACGAGCGGGCCCGTGCGGCCTTGCAACGCT
GGTCGTTGATGCGCAGCGATCATCCGGTGACTAGCGTAGGAACGCAATGACCATCGATCCTGACCAGATC
CGTGCCGAAATCGACGCCCTACTTGCTTCGCTGCCCGACCCCGCCGACGCCGAGAACGGACCGTCTCTGG
CCGAACTCGAAGGCATCGCACGTCGTCTTTCCGAGGCGCACGAGGTGTTGTTGGCCGCCCTGGAGTCGGC
GGAGAAGGGTTGAGTGCGGCGTGGCACGACGTGCCCGCGTTGACGCCGAGCTAGTCCGGCGGGGCCTGGC
GCGATCACGTCAACAGGCCGCGGAGTTGATCGGCGCCGGCAAGGTGCGCATCGACGGGCTGCCGGCGGTC
AAGCCGGCCACCGCCGTGTCCGACACCACCGCGCTGACCGTGGTGACCGACAGTGAACGCGCCTGGGTAT
CGCGCGGAGCGCACAAACTAGTCGGTGCGCTGGAGGCGTTCGCGATCGCGGTGGCGGGCCGGCGCTGTCT
GGACGCGGGCGCATCGACCGGTGGGTTCACCGAAGTACTGCTGGACCGTGGTGCCGCCCACGTGGTGGCC
GCCGATGTCGGATACGGCCAGCTTGGCGTGGTCGCTGCGCAACGATCCTCGGGTGGTGGTCCTCGAGCGGA
CCAACGCACGTGGCCTCACACCGGAGGCGATCGGCGGTCGCGTCGACCTGGTAGTGGCCGACCTGTCGTT
CATCTCGTTGGCTACCGTGTTGCCCGCGCTGGTTGGATGCGCTTCGCGCGACGCCGATATCGTTCCACTG
GTGAAGCCGCAGTTTGAGGTGGGGAAAGGTCAGGTCGGCCCCGGTGGGGTGGTCCATGACCCGCAGTTGC
GTGCGCGGTCGGTGCTCGCGGTCGCGCGGCGGGCACAGGAGCTGGGCTGGCACAGCGTCGGCGTCAAGGC
CAGCCCGCTGCCGGGCCCATCGGGCAATGTCGAGTACTTCCTGTGGTTGCGCACGCAGACCGACCGGGCA
TTGTCGGCCAAGGGATTGGAGGATGCGGTGCACCGTGCGATTAGCGAGGGCCCGTAGTGACCGCTCATCG
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TTGGGCGACAATAAAATTGCGCTTCGCGTGCTCTCGGCCGAAGCAGTCGACCGAGGGTCGTTGCATCTGC
CTCCCGACGACATGCGGGCCATGGGCGTCGAGATCGAGGTGGTTGACGCGGACCAGCACGCAGCCGACGG
CTGCGAACTGGTGCTGGTTTTGGGCGGCGATGGCACCTTTTTGCGGGCAGCCGAGCTGGCCCGCAACGCC
AGCATTC

>NC_000962.3:7302-9818 Mycobacterium tuberculosis H37Rv,
gyrA Fluoroquinolones Resistance Gene **(SNP at 74bp)**

ACGGTCTGCTGGAGGCGGGGCTGAAGGCCGGGAAGAAGATCAACAAGGAAGACGGCATTCAGCGGTACAA
GGGTCTAGGTGAAATGGACGCTAAGGAGTTGTGGGAGACCACCATGGATCCCTCGGTTCGTGTGTTGCGT
CAAGTGACGCTGGACGACGCCGCCGCCGCCGACGAGTTGTTCTCCATCCTGATGGGCGAGGACGTCGACG
CGCGGCGCAGCTTTATCACCCGCAACGCCAAGGATGTTCGGTTCCTGGATGTCTAACGCAACCCTGCGTT
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CGGCGACGCGTCGATCTACGACAGCCTGGTGCGCATGGCCCAGCCCTGGTCGCTGCGCTACCCGCTGGTG
GACGGCCAGGGCAACTTCGGCTCGCCAGGCAATGACCCACCGGCGGCGATGAGGTACACCGAAGCCCGGC
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CGGCCGGGTGCAAGAGCCGACGGTGCTACCCAGCCGGTTCCCCAACCTGCTGGCCAACGGGTCAGGCGGC
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GGCACGCCAGGTTCGCAAGGCGGGACGGCAGACCAAGGGTGTTCGGTTGATGAATCTGGGCGAGGGCGAC
ACACTGTTGGCCATCGCGCGCAACGCCGAAGAAAGTGGCGACGATAATGCCGTGGACGCCAACGGCGCAG
ACCAGACGGGCAATTAA

>NC_000962.3:c4408500-4407200 Mycobacterium tuberculosis H37Rv, gidB Streptomycin Resistance Gene **(TOTAL GENE COVERAGE)**

ACGAGGTGGCGCGGCGAGTGGCCGAAACCGGTGACCGCGAGGAACTCGTTCCAATGACGCCGTTCGAACG
GAAGATCGTCCACGATGCGGTTGCAGCGGTGCCAGGTGTGCACAGCGAAAGCGAAGGCGTGGAGCCAGAA
CGCCGAGTCGTTGTGCTCCGCGACTAGCTCGCGAGCCAGCGGCTCCGACCGACGCCGCAGTAAGCGATGC
GTGGCCGAGCGGCTGGGCCAGCGTCTCGAGAGCGGAGAATGTTTCACGTGAAACATGACACAGACCTCAC
GAGCCGGCGGAGTGCGTAATGTCTCCGATCGAGCCCGCGGCGTCTGCGATCTTCGGACCGCGGCTTGGCC
TTGCTCGGCGGTACGCCGAAGCGTTGGCGGGACCCGGTGTGGAGCGGGGGCTGGTGGGACCCCGCGAAGT
CGGTAGGCTATGGGACCGGCATCTACTGAACTGCGCCGTGATCGGTGAGCTCCTCGAACGCGGTGACCGG
GTCGTGGATATCGGTAGCGGAGCCGGGTTGCCGGGCGTGCCATTGGCGATAGCGCGGCCGGACCTCCAGG
TAGTTCTCCTAGAACCGCTACTGCGCCGCACCGAGTTTCTTCGAGAGATGGTGACAGATCTGGGCGTGGC
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GTGTCACGGGCGGTGGCCGCGTTGGACAAGTTGACGAAATGGAGCATGCCGTTGATACGGCCGAACGGGC
GAATGCTCGCCATCAAAGGCGAGCGGGCTCACGACGAAGTACGGGAGCACCGGCGTGTGATGATCGCATC
GGGCGCGGTTGATGTCAGGGTGGTGACATGTGGCGCGAACTATTTGCGTCCGCCCGCGACCGTGGTGTTC
GCACGACGTGGAAAGCAGATCGCCCGAGGGTCGGCACGGATGGCGAGTGGAGGGACGGCGTGAGTGCTCC
GTGGGGCCCGGTGGCCGCTGGACCGTCCGCGCTCGTAAGGTCGGGCCAGGCTTCAACTATCGAACCATTC
CAGCGGGAAATGACACCACCGACACCGACGCCTGAGGCCGCGCACAATCCGACGATGAATGTTTCACGTG
AAACATCGACAGAATTCGACACCCCCATCGGCGCTGCAGCAGAACGTGCGATGCGGGTCCTGCACACCAC
CCACGAGCCGCTGCAGCGGCCGGGTCGACGCCGGGTGCTCAGCATCGCGAATCAGAAGGGCGGGGTCGGT
AAGACGACCACCGCCGTCAATATCGCTGCCGCGCTTGCTGT

>NC_000962.3:781000-782300 Mycobacterium tuberculosis H37Rv,
rpsL Streptomycin Resistance Gene **(TOTAL GENE COVERAGE)**

GCCCACCACCACCCGGGTCGGTACTCGGCGTTCACCCGGATG**C**CGCTGGGCGGTGACGATCCCGAATACA
CCGCTGCGACTAGGGGC**G**CAGCCGCGCCCGTCATCGCCGTGCTGTCCTCGTACGGCCTCGACGGTGAGCA
GGCTTTCTACGCGGCGCTCGAGTTTTGGTCGGCACTGCATGGGTTTGTGTTGCTGGAAATGACCGGCGTC
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GACTGGCGGCGGGTATTGTGGTTGCTCGTGCCTGGCGGCTTACGCTTGATGTAGGGGCGTGGATGCCGGG
CCAATTCGCATGTCCGCGATGCCTCGGATGAGACGAATCGAGTTTGAGGCAAGCTATGCGACACACCCGG
CCGCGGGTAACCGTGGCGGGGCATGGCCGACAAACAGAACGTGAAAGCGCCCAAGATAGAAAGCCGGTAG
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TGAAGGGCAGCCCGCAG**C**GTCGTGGTGTATGCACCCGCGTGTACACCACCACTCCGAAGAAGCCGAACTC
GGCGCTTCGGAAGGTTGCCCGCGTGAAGTTGACGAGTCAGGTCGAGGTCACGGCGTACATTCCCGGCGAG
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CGGTCTACGGATCGCAGTTGGTCACCCAGTTGGTGAACAAGGTTCTGTTGAAGGGGAAAAAATCGCTGGC
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AGATTGGTGAGGTGCACGACGGCGCAGCCACCATGGACTGGATGGAACAGGAACAGGAGCGCGGCATCAC
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AAGAGGGTGTCGAACCGCAGTCCGAACAGGTGTGGCGGCAGGCCGACAAATACGATGTCCCCCGAATCTG
CTTCGTCAACAAGATGGACAAGATCGGTGCGGACTTCTACTTCTCGGTTCGCACGATGGGGGAGCGGCTT
GGGGCCAACGCCGTGCCCATTCAGCTTCCCGTCGGTGCGGAG

>NC_000962.3:c4327800-4326004 Mycobacterium tuberculosis H37Rv, ethA Ethionamide Resistance Gene **(SNP at 11bp and 110bp, EXTENDED TO ALLOW)**

GTCGGCTTGATTGACCACCCGGTCCAGCAGGGTCAGCAGCACCGCTTCCTTGGATGGGAAATAGAAGTAG
AACGTCGGCCTCGAGATACCGGCGCCCTTGGCCAGATCGTCGACCGAGATATCGGCCAGCGGACGGTCCT
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GCCCCTAGGCAGCGAAGCCTGACTGGCCGCGGAGGTGGTCACCCTGGCAGCTTACTACGTGTCGATAGTG
TCGACATCTCGTTGACGGCCTCGACATTACGTTGATAGCGTGGATCCATGACCGAGCACCTCGACGTTGT
CATCGTGGGCGCTGGAATCTCCGGTGTCAGCGCGGCCTGGCACCTGCAGGACCGTTGCCCGACCAAGAGC
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GCGGCCCTTCATGGAGTTCACCCCAGGTTACGTGCTGCGCTCGCTGGACGAGCTGCCCAAGCAGGGTTCG
CGTACACCGTGGCGCCTGAATCAGAACTACCTACGTGACATCCGGCTCATCCGGCGCGGCAAGATCGACG
ACGAGGGTCTGCGGTTCGCCAAAAGGCCTGCCCCGGTGGGGGTTTAG

>NC_000962.3:778690-779800 Mycobacterium tuberculosis H37Rv, rv0678 Bedaquiline & Clofazimine Resistance Gene **(TOTAL GENE COVERAGE)**

AGGTCAGGGCATCCACGCCGGTTGCGGTCCGCTCGTCCTTCACTTCGCCATCGACGGTGATTCGGCAGGT
GATGGAAGTGCCGTCGCCTTGCGCGAGGATGTTGGGGGCCGCGGACGGCGCCGTGGTCTTCAAGGTGAGC
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CTTCGGCGTCACCAAGATGCCTTCGGAACCAAAGAAAGTGCGGATCCGCTGCACCGTGAAGCCGGCGATG
GCGACCACAACCAGGATGAGCAGCGGTATCCAGGCACGCTTGAGAGTTCCAATCATCGCCCTCCGCCTCT
GCCGCATGAAGTTCACGCCGGTCTGGTGACGCATACCGAACGTCACAGATTTCAGAGTACAGTGAAACTT
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TCCCGAGCGGCAGTCCTCGGAGGAACTGGCGACGGCGCTGGCGGCCAGCAGCGGGGGGATCAGCACCAAT
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GGTTGCGGCCCAACGCTTTCGCGGCTGGCGAGCGTGAACGCATCCGGGCAATGGCCGAACTGCAGGACCT
GGCTGACGTGGGGCTGAGGGCGCTGGGCGACGCCCCGCCGCAGCGAAGCCGACGGCTGCGGGAGATGCGG
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GCGCAGTCAAGGGTGGCGGTAGTCCCGATGGATCCGTAGGTCAGCATGTCACCGATCTGGATCGAAGCGG
TTGTGGATTCTCCGGTCGTCACGGTCGGCGCCGCGGTCGGGCCGCTCGTCGCTGTCGTGGTGGTGGCGGT
C

>NC_000962.3:1473658-1476795 Mycobacterium tuberculosis H37Rv, rrl Linezolid Resistance Gene **(SNP at 2058bp)**

TTGTAAGTGTCTAAGGGCGCATGGTGGATGCCTTGGCATCGAGAGCCGATGAAGGACGTGGGAGGCTGCG
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GAGGCAGTCAGAAAGTGTCGTGGTTAGCGGAAGTGGCCTGGGATGGTCTGCCGTAGACGGTGAGAGCCCG
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CTGGAAGCTCAGTAATGGGTGTAGGGAACTGGTGCTAACCGGCCGAAAACTTACAACA

>NC_000962.3:800500-801800 Mycobacterium tuberculosis H37Rv, rplC Linezolid Resistance Gene **(TOTAL GENE COVERAGE)**

AGATCCGCATCAGGCTGAAGGCCTACGACCATGAGGCCATTGACGCTTCGGCGCGCAAGATCGTCGAAAC
CGTCGTCCGCACCGGTGCCAGCGTCGTAGGGCCGGTGCCGCTACCGACTGAGAAGAACGTGTATTGCGTC
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GCAGGTATTCGACGAAAGCAACAGAGTAGTACCGGTGACCGTGGTCAAGGCCGGGCCCAACGTGGTAACC
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GCTTCCGCGGTCAGGGCGCCAGTCACGGTGCCCAGGCGGTGCACCGCCGTCCGGGCTCCATCGGCGGATG
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CTTAACCTTTTGGTGCATAAGGTCGATGCCGAGAACGGCGTGCTGCTGATCAAGGGTGCGGTTCCTGGCC
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>NC_000962.3:528608-530230 Mycobacterium tuberculosis H37Rv, complete genome

Hsp65 Gene (Partial Gene Coverage)

ATGGCCAAGACAATTGCGTACGACGAAGAGGCCCGTCGCGGCCTCGAGCGGGGCTTGAACGCCCTCGCCG
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GTCCACCCTGGTCGTCAACAAGATCCGCGGCACCTTCAAGTCGGTGGCGGTCAAGGCTCCCGGCTTCGGC
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CATGGATTTCTGA

Appendix III – External Control Sequences

The following are FastA sequences of the external controls designed for inclusion in the assay. Each sequence is made of the concatenated gene targets within one of the multiplex groups to observe the success or failure of amplification and sequencing of every targeted locus throughout assessment.

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ACACCGCCTCGATTGCCGACGTGTCCAGACTGGGATGGAAGTCCGCGCCGGGAGTACCGCTGACGCAATG
CGGTGGCCACGACGAGGAATAGTCCGGTGTGCCGGAGAAGTGGTCACCCGGGTCGATGTGGAAGTCCTT
GGTTGCCACGACGTGATGGTAGTCCGCCGCTTCGGCCAGGTAGTCGCTGATGGCGCGGGCCAGCGCGGC
GCCACCGGTTACCGCCAGCGAGCCACCCTCGCAGAAGTCGTTCTGCACGTCGACGATGATCAACGCCCGCA
TACGTCCACCATACGTTCGGGCGACTGCCCGGGCAGTTTGCCTACCGACGCGGCAGCCACAGATATAGGG
TCCATGACGCCGCGACGATCGCGAACATGACCAGCTGAGCGGCGGCCACCCAACCGGCGGGATAGATCAC
GCCGGTGATGTAGTTCACAGCCTAGTGGCCTGGATGTTCGTAGTGAGCGACAAATCCGTCCGGTGAGCTTC
AAGTCCTGGCCTACGATGTCTTTGGTGTCTCAATCCCGAGGACCTAATACGCGCAGCGGCGGGTATTGTGG
TTGCTCGTGCGGGACCCGGGAGCTTAATTAGTAGTCGGCTCTGGCGGCTTACGCTTGATGTAGGGGCGTG
GATGCCGGGCCAATTCGCATGTCCGCGATGCCTCGGATGAGACGAATCGAGTTTGAGGCAAGCTATGCGA
CACACCCGGCCGCGGGTAACCGTGGCGGGGCATGGCCGACAAACAGAACGTGAAAGCGCCCAAGATAGA
AAGCCGGTAGATGCCAACCATCCAGCAGCTGGTCCGCAAGGGTCGTCGGGACAAGATCAGTAAGGTCAAG
ACCGCGGCTCTGAAGGGCAGCCCGCAGCGTCGTGGTGTATGCACCCGCGTGTACACCACCACTCCGAAGA
AGCCGAACTCGGCGCTTCGGAAGGTTGCCCGCGTGAAGTTGACGAGTCAGGTCGAGGTCACGGCGTACAT
TCCCGGCGAGGGCCACAACCTGCAGGAGCACTCGATGGTGCTGGTGCGCGGCGGCCGGGTGAAGGACCT
GCCTGGTGTGCGCTACAAGATCATCCGCGGTTCGCTGGATACGCAGGGTGTCAAGAACCGCAAACAGGCA
CGCAGCCGTTACGGCGCTAAGAAGGAGAAGGGCTGATGCCACGCAAGGGGCCCGCGCCCAAGCGTCCGT
TGGTCAACGACCCGGTCTACGGATCGCAGTTGGTCACCCAGTTGGTGAACAAGGTTCTGTTGAAGGGGAA
AAAATCGCTGGCCGAGCGCATTGTTTATGGTGCGCTTGAGCAAGCTCGCGACAAGACCGGCACCGATCCG
GTGATCACCCTCAAGCGGGCTCTCGACAATGTCAAACCCGCCCTGGAGGTGCGCAGCCGTCGCGTCGGCG
GCGCGACCTATCAGGTGCCTGTCGAGGTGCGCCCCGACCGGTCGACCACGCTGGCGCTGCGCTGGCTCGT
CGGCTACTCGCGGCAACGCCGTGAGAAGACGATGATCGAGCGCCTGGCAAATGGAGATCCTGGATGCCA
GCAATGGCCTTGGGGCCTCCGTCAAGCGGCGTGAGGACACCCACAAGATGGCCGAGGCGAACCGAGCCTT
TGCGCATACCTTGTTAGGGCAGTACGAGTTCCAGCCTTATCGCTGGTGAGAAGCGCCGGTTAACAGGCAAT
CTGCACTCTACTCGAATAAACTAGGCGCGTCCCTTGCGGTGCTCCTCATTTGGTCTCGGTGGCTTCGTCGCG
CCCGGTACATGTACACCCGTCTTCCCGCTGAGTGCTGTGGACGACCAGCAGAACACTGCGATGAGCGGTCA
CTACGGGCCCTCGCTAATCGCACGGTGCACCGCATCCTCCAATCCCTTGGCCGACAATGCCCGGTCGGTCT
GCGTGCGCAACCACAGGAAGTACTCGACATTGCCCGATGGGCCCGGCAGCGGGCTGGCCTTGACGCCGAC
GCTGTGCCAGCCCAGCTCCTGTGCCCGCCGCGCGACCGCGAGCACCGACCGCGCACGCAACTGCGGGTCA
TGGACCACCCCACCGGGGCCGACCTGACCTTTCCCCACCTCAAACTGCGGCTTCACCAGTGGAACGATATC
GGCGTCGCGCGAAGCGCATCCAACCAGCGCGGGCAACACGGTAGCCAACGAGATGAACGACAGGTCGGC
CACTACCAGGTCGACGCGACCGCCGATCGCCTCCGGTGTGAGGCCACGTGCGTTGGTCCGCTCGAGGACC
ACCACCCGAGGATCGTTGCGCAGCGACCACGCCAGCTGGCCGTATCCGACATCGGCGGCCACCACGTGGG
CGGCACCACGGTCCAGCAGTACTTCGGTGAACCCACCGGTCGATGCGCCCGCGTCCAGACAGCGCCGGCC
CGCCACCGCGATCGCGAACGCCTCCAGCGCACCGACTAGTTTGTGCGCTCCGCGCGATACCCAGGCGCGTT
CACTGTCGGTCACCACGGTCAGCGCGGTGGTGTCGGACACGGCGGTGGCCGGCTTGACCGCCGGCAGCCC
GTCGATGCGCACCTTGCCGGCGCCGATCAACTCCGCGGCCTGTTGACGTGATCGCGCCAGGCCCCGCCGG

ACTAGCTCGGCGTCAACGCGGGCACGTCGTGCCACGCCGCACTCAACCCTTCTCCGCCGACTCCAGGGCGG
CCAACAACACCTCGTGCGCCTCGGAAAGACGACGTGCGATGCCTTCGAGTTCGGCCAGAGACGGTCCGTTC
TCGGCGTCGGCGGGGTCGGGCAGCGAAGCAAGTAGGGCGTCGATTTCGGCACGGATCTGGTCAGGATCG
ATGGTCATTGCGTTCCTACGCTAGTGACAATACGAAGATCTTAATCCGGAATAGACACCGGATGATCGCTG
CGCATCAACGCATCTCTCGGACAATACATATCAATGTCGTATACGTCCAGTGCCGGCGAGTGGGAACGTCG
ATGGGTGCGCCGCGGATCCGTCACACGCGTCCGTGAATCCTATGTATAACGGCTCGTGATGTGATAGGCAC
ACCGGTTGGTCCACCAGA

Appendix IV – Total Phenotypic Resistance Calls for FIND Samples

The following is a table of complete resistance calls for 16 anti-tuberculous medications following performance of the DST assay on 392 blinded samples provided by FIND. This table, in conjunction with the table in Appendix V were provided to FIND for validation and assessment of the DST method.

Sample	Ethambutol	Isoniazid	Pyrazinamide	Rifampicin	Streptomycin	Amikacin	Bedaquiline	Capreomycin
A405	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A798	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A614	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A656	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A762	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A659	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A675	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A202	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A431	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A521	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A320	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A284	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A872	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A565	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A648	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A053	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A581	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A199	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A045	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A783	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A847	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A316	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A750	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A205	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A267	Resistant	Resistant	Not Detected	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A743	Resistant	Resistant	Not Detected	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A152	Resistant	Resistant	Not Detected	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A246	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A921	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A726	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A554	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A371	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A056	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A107	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible

A418	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A167	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A537	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A519	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A187	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A062	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A818	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A217	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A605	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A273	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A298	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A119	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A877	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A827	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A759	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A701	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A524	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A067	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A971	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A886	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A998	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A128	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A809	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A310	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A240	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A430	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A661	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A274	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A929	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A249	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A490	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A922	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A410	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A139	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A349	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A197	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A670	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A832	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A594	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A312	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A820	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A082	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A391	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A362	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible

A277	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A423	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A582	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A908	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A222	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A174	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A305	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A844	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A458	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A807	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A242	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A563	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A394	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A871	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A338	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A596	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A512	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A980	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A505	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible
A662	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible
A399	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible
A299	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A057	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A992	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A125	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A984	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A471	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A272	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A474	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A558	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A528	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A555	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A440	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A547	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A808	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A439	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A104	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A244	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A459	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A191	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A940	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A768	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A276	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A791	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible

A539	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A730	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A894	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A870	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A727	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A172	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A496	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A891	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A071	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A852	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A830	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A690	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A928	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A422	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A347	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A785	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A034	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A156	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A988	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A213	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A700	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A453	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A510	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A134	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A158	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A861	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A879	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A271	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A723	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A245	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A479	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A845	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A991	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A421	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A186	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A556	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A890	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A229	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A337	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A383	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A515	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A001	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A007	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A035	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant

A234	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A446	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A286	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A838	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A570	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A754	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A022	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A342	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A223	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A883	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A412	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A972	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A983	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A257	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A215	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A428	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A742	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A967	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A396	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A606	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A777	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A066	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A193	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A171	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A553	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A073	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
A164	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A335	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A261	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A046	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A196	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A962	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A332	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A860	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A684	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A642	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A198	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A502	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A911	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A769	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A375	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A901	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A252	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A918	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible

A824	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A379	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A309	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A117	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A678	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A348	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A664	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A842	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A432	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A953	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A741	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A293	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A513	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A433	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A179	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A253	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A774	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A030	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A270	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A781	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A087	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A905	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A720	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A880	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A414	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A275	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A718	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A764	Resistant	Resistant	Not Detected	Resistant	Susceptible	Resistant	Susceptible	Resistant
A674	Resistant	Resistant	Not Detected	Resistant	Susceptible	Resistant	Susceptible	Resistant
A532	Resistant	Resistant	Not Detected	Resistant	Susceptible	Resistant	Susceptible	Resistant
A993	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A385	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A708	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A483	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A463	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A511	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A258	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A749	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A623	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A520	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A916	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A878	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A254	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A122	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible

A665	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A562	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A924	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A452	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A368	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A334	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A518	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A608	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A473	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A814	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A869	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A401	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A794	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A343	Resistant	Resistant	Not Detected	Resistant	Resistant	Resistant	Susceptible	Resistant
A447	Resistant	Resistant	Not Detected	Resistant	Resistant	Resistant	Susceptible	Resistant
A544	Resistant	Resistant	Not Detected	Resistant	Resistant	Resistant	Susceptible	Resistant
A443	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A468	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A522	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A797	Resistant	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible
A340	Resistant	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible
A455	Resistant	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible
A397	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A333	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A420	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A686	Resistant	Resistant	Not Detected	Resistant	Resistant	Resistant	Susceptible	Resistant
A114	Resistant	Resistant	Not Detected	Resistant	Resistant	Resistant	Susceptible	Resistant
A029	Resistant	Resistant	Not Detected	Resistant	Resistant	Resistant	Susceptible	Resistant
A477	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Resistant
A597	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Resistant
A729	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Resistant
A966	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A243	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A265	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A945	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Resistant
A937	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Resistant
A297	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Resistant
A710	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A355	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A417	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Resistant
A444	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A486	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A226	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A841	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible

A835	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A816	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A036	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A218	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A663	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A123	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A822	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A456	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A111	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A975	Resistant	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
A262	Not Detected	Resistant	Susceptible	Resistant	Susceptible	Not Detected	Susceptible	Not Detected
A361	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A793	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
A247	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible
B829	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
B564	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
B739	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
B771	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B899	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B177	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B858	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B888	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B084	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B694	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B583	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B679	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B682	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B772	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B687	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B839	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
B740	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
B184	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
B311	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B416	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B351	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B944	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B572	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B698	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B671	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B567	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B370	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B590	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B579	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B573	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible

C345	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C589	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C685	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C817	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C150	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C137	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C149	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C773	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C497	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C560	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C958	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C834	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C504	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C364	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C859	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C357	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C210	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C568	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C705	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C737	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C404	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C955	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C141	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C765	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C366	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C192	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C534	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C982	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C088	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C864	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C268	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C231	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C542	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C514	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C744	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C220	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C913	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C752	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C147	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C040	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C232	Resistant	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant
C695	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant
C873	Resistant	Resistant	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant
C549	Resistant	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant

C548	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant
C026	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C203	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C543	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C097	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant
C796	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Susceptible	Resistant

Sample	Ciprofloxacin	Clofazimine	Ethionamide	Kanamycin	Linezolid	Moxifloxacin	Ofloxacin	Quinolones
A405	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A798	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A614	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A656	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A762	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A659	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A675	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A202	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A431	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A521	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A320	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A284	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A872	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A565	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A648	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A053	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A581	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A199	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A045	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A783	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A847	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A316	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A750	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A205	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A267	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A743	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A152	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A246	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A921	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A726	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A554	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A371	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A056	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A107	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A418	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A167	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible

A537	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A519	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A187	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A062	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A818	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A217	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A605	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A273	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A298	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A119	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A877	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A827	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A759	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A701	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A524	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A067	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A971	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A886	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A998	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A128	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A809	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A310	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A240	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A430	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A661	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A274	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A929	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A249	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A490	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A922	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A410	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A139	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A349	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A197	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A670	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A832	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A594	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A312	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A820	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A082	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A391	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A362	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A277	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant
A423	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant

A582	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant
A908	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A222	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A174	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A305	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A844	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A458	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A807	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A242	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A563	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A394	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A871	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A338	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A596	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A512	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A980	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A505	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A662	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A399	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A299	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A057	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A992	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A125	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A984	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A471	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A272	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A474	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A558	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A528	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A555	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A440	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A547	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A808	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A439	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A104	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A244	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A459	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A191	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A940	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A768	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A276	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A791	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A539	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A730	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant

A894	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A870	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A727	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A172	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A496	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A891	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A071	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A852	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A830	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A690	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A928	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A422	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
A347	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
A785	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
A034	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A156	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A988	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A213	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A700	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A453	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A510	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A134	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A158	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A861	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A879	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A271	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A723	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A245	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A479	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A845	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A991	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A421	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A186	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A556	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A890	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A229	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A337	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A383	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A515	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
A001	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
A007	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
A035	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A234	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A446	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant

A286	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A838	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A570	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A754	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A022	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A342	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A223	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A883	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A412	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A972	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A983	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A257	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A215	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A428	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A742	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A967	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A396	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A606	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A777	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
A066	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
A193	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant
A171	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A553	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A073	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A164	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A335	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A261	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
A046	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A196	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A962	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A332	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A860	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A684	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A642	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A198	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A502	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A911	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A769	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A375	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A901	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A252	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A918	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A824	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A379	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant

A309	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A117	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A678	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A348	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A664	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A842	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A432	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A953	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A741	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A293	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A513	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A433	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A179	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A253	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A774	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A030	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A270	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant
A781	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant
A087	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Resistant	Resistant	Resistant
A905	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A720	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A880	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A414	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A275	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A718	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A764	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A674	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A532	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A993	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A385	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A708	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A483	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A463	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A511	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A258	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A749	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A623	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A520	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A916	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A878	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A254	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A122	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A665	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A562	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible

A924	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A452	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A368	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A334	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A518	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A608	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A473	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A814	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A869	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A401	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A794	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A343	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A447	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A544	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A443	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A468	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A522	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A797	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A340	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A455	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A397	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A333	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A420	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant
A686	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A114	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A029	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A477	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A597	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A729	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
A966	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A243	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A265	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A945	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A937	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A297	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A710	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A355	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A417	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A444	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A486	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A226	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A841	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A835	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
A816	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible

A036	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A218	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A663	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A123	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A822	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A456	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A111	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A975	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
A262	Susceptible	Susceptible	Susceptible	Not Detected	Susceptible	Resistant	Resistant	Resistant
A361	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A793	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
A247	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Resistant
B829	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
B564	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
B739	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
B771	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B899	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B177	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B858	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B888	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B084	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B694	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B583	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B679	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B682	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B772	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B687	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B839	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
B740	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
B184	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
B311	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B416	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B351	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B944	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B572	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B698	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B671	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B567	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B370	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B590	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B579	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
B573	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C345	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C589	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible

C685	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C817	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C150	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C137	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C149	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C773	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C497	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C560	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C958	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C834	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C504	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C364	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C859	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C357	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C210	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C568	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C705	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C737	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C404	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C955	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C141	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C765	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C366	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C192	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C534	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C982	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C088	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C864	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C268	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C231	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C542	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C514	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C744	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C220	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C913	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C752	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C147	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C040	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C232	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
C695	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
C873	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C549	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
C548	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Resistant	Resistant	Resistant
C026	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant

C203	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C543	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C097	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant
C796	Resistant	Susceptible	Resistant	Resistant	Susceptible	Resistant	Resistant	Resistant

Appendix V – Example Genotypic Resistance Calling for SNPs

The following is an example section of table used for manual observation of SNP loci and resistance calling. This section focuses on 9 high confidence SNP loci in the *embB* gene associated with resistance to ethambutol. Recording of SNPs in this manner was performed for all covered SNP loci and all 392 blinded FIND samples.

Colour key	
	≥80% of bases resistant at SNP position
	51% to 79% bases resistant at SNP position
	20% to 50% bases resistant at SNP position
	Gene deletion or target dropout
	wild type

	Ethambutol								
Sample	**embB 378**	**embB 306**	**embB 354**	**embB 497**	**embB 406**	**embB 297**	**embB 296**	**embB 405**	**embB 397**
A405									
A798									
A614									
A656									
A762									
A659									
A675									
A202									
A431									
A521									
A320									
A284									
A872									
A565									
A648									
A053									
A581									
A199									
A045									
A783									
A847									
A316									
A750									
A205									
A267									
A743									

A152									
A246									
A921									
A726									
A554									
A371									
A056									
A107									
A418									
A167									
A537									
A519									
A187									
A062									
A818									
A217									
A605									
A273									
A298									
A119									
A877									
A827									
A759									
A701									
A524									
A067									
A971									
A886									
A998									
A128									
A809									
A310									
A240									
A430									
A661									
A274									
A929									
A249									
A490									
A922									
A410									
A139									
A349									
A197									

A670									
A832									
A594									
A312									
A820									
A082									
A391									
A362									
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A423									
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A908									
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A174									
A305									
A844									
A458									
A807									
A242									
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A984									
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A272									
A474									
A558									
A528									
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A440									
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A104									
A244									
A459									
A191									
A940									
A768									
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A539									
A730									
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A870									
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A891									
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A830									
A690									
A928									
A422									
A347									
A785									
A034									
A156									
A988									
A213									
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A271									
A723									
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A991									
A421									
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A556									

A890									
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A337									
A383									
A515									
A001									
A007									
A035									
A234									
A446									
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A570									
A754									
A022									
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A883									
A412									
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A742									
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A606									
A777									
A066									
A193									
A171									
A553									
A073									
A164									
A335									
A261									
A046									
A196									
A962									
A332									
A860									
A684									
A642									

A198									
A502									
A911									
A769									
A375									
A901									
A252									
A918									
A824									
A379									
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A117									
A678									
A348									
A664									
A842									
A432									
A953									
A741									
A293									
A513									
A433									
A179									
A253									
A774									
A030									
A270									
A781									
A087									
A905									
A720									
A880									
A414									
A275									
A718									
A764									
A674									
A532									
A993									
A385									
A708									
A483									
A463									
A511									

A258									
A749									
A623									
A520									
A916									
A878									
A254									
A122									
A665									
A562									
A924									
A452									
A368									
A334									
A518									
A608									
A473									
A814									
A869									
A401									
A794									
A343									
A447									
A544									
A443									
A468									
A522									
A797									
A340									
A455									
A397									
A333									
A420									
A686									
A114									
A029									
A477									
A597									
A729									
A966									
A243									
A265									
A945									
A937									

A297									
A710									
A355									
A417									
A444									
A486									
A226									
A841									
A835									
A816									
A036									
A218									
A663									
A123									
A822									
A456									
A111									
A975									
A262									
A361									
A793									
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B829									
B564									
B739									
B771									
B899									
B177									
B858									
B888									
B084									
B694									
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B679									
B682									
B772									
B687									
B839									
B740									
B184									
B311									
B416									
B351									
B944									

B572									
B698									
B671									
B567									
B370									
B590									
B579									
B573									
C345									
C589									
C685									
C817									
C150									
C137									
C149									
C773									
C497									
C560									
C958									
C834									
C504									
C364									
C859									
C357									
C210									
C568									
C705									
C737									
C404									
C955									
C141									
C765									
C366									
C192									
C534									
C982									
C088									
C864									
C268									
C231									
C542									
C514									
C744									
C220									

C913									
C752									
C147									
C040									
C232									
C695									
C873									
C549									
C548									
C026									
C203									
C543									
C097									
C796									

Appendix VI – Example of Detailed Clinical Validation Sequencing Analysis

The following is an example of in depth analysis of detected mutant SNPs compared to wild type genome found during analysis. This example specifically covers mutations detected within the *embB* gene for all 392 blinded FIND samples. This type of table was used for resistance calling but was not supplied in the final summarized report for ease of interpretation.

Sample	Ethambutol Resistance SNP	Ethambutol Mutation	Ethambutol Wild Type Count (#)	Ethambutol Mutant Count (#)
A405	embB M306V	ATG -> GTG	9	365
A798	embB M306V	ATG -> GTG	24	471
A614	embB M306V	ATG -> GTG	5	369
A656	embB M306I	ATG -> ATA	61	793
A762	embB M306I	ATG -> ATA	61	866
A659	embB M306I	ATG -> ATA	57	749
A675	embB N296H	AAT -> CAT	31	1086
A202	embB N296H	AAT -> CAT	17	1043
A431	embB N296H	AAT -> CAT	21	1192
A521	embB G406D	GGC -> GAC	83	1847
A320	embB G406D	GGC -> GAC	106	1791
A284	embB G406D	GGC -> GAC	77	1762
A872	embB Q497R	CAG -> CGG	44	2275
A565	embB Q497R	CAG -> CGG	15	1265
A648	embB Q497R	CAG -> CGG	29	1702
A053	embB M306V	ATG -> GTG	18	1421
A581	embB M306V	ATG -> GTG	12	1382
A199	embB M306V	ATG -> GTG	19	1115
A045	embB M306V	ATG -> GTG	21	1272
A783	embB M306V	ATG -> GTG	49	1419
A847	embB M306V	ATG -> GTG	23	1556
A316	embB Y334H	TAC -> CAC	56	1466
A750	embB Y334H	TAC -> CAC	60	1232
A205	embB Y334H	TAC -> CAC	39	533
A267	embB M306V	ATG -> GTG	14	758
A743	embB M306V	ATG -> GTG	13	976
A152	embB M306V	ATG -> GTG	13	951
A246	embB M306I	ATG -> ATC	11	327
A921	embB M306I	ATG -> ATC	37	1256
A726	embB M306I	ATG -> ATC	35	1103
A554	embB Q497R	CAG -> CGG	41	993
A371	embB Q497R	CAG -> CGG	31	865
A056	embB Q497R	CAG -> CGG	24	617

A107	embB M306V	ATG -> GTG	21	862
A418	embB M306V	ATG -> GTG	19	1112
A167	embB M306V	ATG -> GTG	10	804
A537	embB M306V	ATG -> GTG	9	998
A519	embB M306V	ATG -> GTG	13	1517
A187	embB M306V	ATG -> GTG	17	1073
A062	embB Q497R	CAG -> CGG	17	929
A818	embB Q497R	CAG -> CGG	17	608
A217	embB Q497R	CAG -> CGG	24	719
A605	embB G406S	GGC -> AGC	54	868
A273	embB G406S	GGC -> AGC	38	664
A298	embB G406S	GGC -> AGC	41	492
A119	embB M306V	ATG -> GTG	16	765
A877	embB M306V	ATG -> GTG	9	665
A827	embB M306V	ATG -> GTG	6	974
A759				
A701				
A524				
A067				
A971				
A886				
A998	embB M306I	ATG -> ATA	31	419
A128	embB M306I	ATG -> ATA	85	1011
A809	embB M306I	ATG -> ATA	33	389
A310	embB M306V	ATG -> GTG	9	649
A240	embB M306V	ATG -> GTG	7	461
A430	embB M306V	ATG -> GTG	8	521
A661	embB M306V	ATG -> GTG	13	645
A274	embB M306V	ATG -> GTG	10	677
A929	embB M306V	ATG -> GTG	16	717
A249	embB S297A	TCG -> GCG	13	471
A490	embB S297A	TCG -> GCG	13	701
A922	embB S297A	TCG -> GCG	19	706
A410	embB M306I	ATG -> ATA	35	488
A139	embB M206I	ATG -> ATA	11	143
A349	embB M306I	ATG -> ATA	44	685
A197				
A670				
A832				
A594	embB M306V	ATG -> GTG	16	630
A312	embB M306V	ATG -> GTG	11	632
A820	embB M306V	ATG -> GTG	8	537
A082				
A391				

A362				
A277	embB G406S	GGC -> AGC	88	354
A423	embB G406S	GGC -> AGC	117	430
A582	embB G406S	GGC -> AGC	135	561
A908	embB M306V	ATG -> GTG	53	1379
A222	embB M306V	ATG -> GTG	5	425
A174	embB M306V	ATG -> GTG	18	483
A305	embB M306V	ATG -> GTG	27	528
A844	embB M306V	ATG -> GTG	26	731
A458	embB M306V	ATG -> GTG	33	549
A807	embB D354A	GAC -> GCC	40	753
A242	embB D354A	GAC -> GCC	57	2043
A563	embB D354A	GAC -> GCC	18	747
A394	embB G406A	GGC -> GCC	118	406
A871	embB G406A	GGC -> GCC	151	807
A338	embB G406A	GGC -> GCC	70	336
A596				
A512				
A980				
A505	embB M306V	ATG -> GTG	617	489
A662	embB M306V	ATG -> GTG	590	480
A399	embB M306V	ATG -> GTG	995	768
A299	embB Y334H	TAC -> CAC	41	447
A057	embB Y334H	TAC -> CAC	105	1962
A992	embB Y334H	TAC -> CAC	83	1031
A125	embB Q497R	CAG -> CGG	46	1013
A984	embB Q497R	CAG -> CGG	60	2074
A471	embB Q497R	CAG -> CGG	42	1371
A272	embB E405D	GAG -> GAT	68	1112
A474	embB E405D	GAG -> GAT	81	1279
A558	embB E405D	GAG -> GAT	59	930
A528	embB M306V	ATG -> GTG	48	1170
A555	embB M306V	ATG -> GTG	22	1021
A440	embB M306V	ATG -> GTG	34	881
A547	embB M306V	ATG -> GTG	103	2475
A808	embB M306V	ATG -> GTG	63	1594
A439	embB M306V	ATG -> GTG	23	677
A104	embB N296H	AAT -> CAT	24	950
A244	embB N296H	AAT -> CAT	32	938
A459	embB N296H	AAT -> CAT	28	1068
A191	embB M306V	ATG -> GTG	29	1184
A940	embB M306V	ATG -> GTG	19	577
A768	embB M306V	ATG -> GTG	22	1132
A276	embB M306V	ATG -> GTG	33	1355

A791	embB M306V	ATG -> GTG	24	1000
A539	embB M306V	ATG -> GTG	49	1541
A730	embB M306I	ATG -> ATA	54	477
A894	embB M306I	ATG -> ATA	60	825
A870	embB M306I	ATG -> ATA	100	749
A727	embB M306I	ATG -> ATC	37	591
A172	embB M306I	ATG -> ATC	50	1326
A496	embB M306I	ATG -> ATC	60	889
A891	embB M306V	ATG -> GTG	34	1009
A071	embB M306V	ATG -> GTG	29	777
A852	embB M306V	ATG -> GTG	25	1024
A830				
A690				
A928				
A422	embB M306I	ATG -> ATC	56	1180
A347	embB M306I	ATG -> ATC	63	1136
A785	embB M306I	ATG -> ATC	57	961
A034	embB M306I	ATG -> ATA	76	969
A156	embB M306I	ATG -> ATA	79	783
A988	embB M306I	ATG -> ATA	74	895
A213	embB M306I	ATG -> ATC	26	532
A700	embB M306I	ATG -> ATC	29	590
A453	embB M306I	ATG -> ATC	88	1945
A510	embB M306V	ATG -> GTG	34	1130
A134	embB M306V	ATG -> GTG	21	529
A158	embB M306V	ATG -> GTG	37	871
A861	embB M306I	ATG -> ATA	86	676
A879	embB M306I	ATG -> ATA	93	716
A271	embB M306I	ATG -> ATA	83	524
A723	embB M306I	ATG -> ATA	62	786
A245	embB M306I	ATG -> ATA	105	729
A479	embB M306I	ATG -> ATA	61	682
A845	embB M306I	ATG -> ATC	43	725
A991	embB M306I	ATG -> ATC	33	506
A421	embB M306I	ATG -> ATC	15	300
A186	embB M306I	ATG -> ATA	52	737
A556	embB M306I	ATG -> ATA	68	660
A890	embB M306I	ATG -> ATA	59	634
A229	embB M306V	ATG -> GTG	32	1232
A337	embB M306V	ATG -> GTG	21	1104
A383	embB M306V	ATG -> GTG	21	1100
A515	embB M306I	ATG -> ATC	31	944
A001	embB M306I	ATG -> ATC	28	959
A007	embB M306I	ATG -> ATC	40	1009

A035	embB M306I	ATG -> ATC	39	1182
A234	embB M306I	ATG -> ATC	22	960
A446	embB M306I	ATG -> ATC	46	967
A286				
A838				
A570				
A754	embB M306I	ATG -> ATA	46	353
A022	embB M306I	ATG -> ATA	54	766
A342	embB M306I	ATG -> ATA	40	385
A223	embB M306I	ATG -> ATA	46	559
A883	embB M306I	ATG -> ATA	49	572
A412	embB M306I	ATG -> ATA	48	594
A972	embB G406D	GGC -> GAC	64	236
A983	embB G406D	GGC -> GAC	145	662
A257	embB G406D	GGC -> GAC	134	661
A215	embB M306V	ATG -> GTG	17	845
A428	embB M306V	ATG -> GTG	22	746
A742	embB M306V	ATG -> GTG	20	879
A967	embB M306I	ATG -> ATA	14	170
A396	embB M306I	ATG -> ATA	58	619
A606	embB M306I	ATG -> ATA	30	523
A777	embB M306I	ATG -> ATA	54	657
A066	embB M306I	ATG -> ATA	76	573
A193	embB M306I	ATG -> ATA	38	511
A171	embB M306I	ATG -> ATT	22	761
A553	embB M306I	ATG -> ATT	28	827
A073	embB M306I	ATG -> ATT	32	1258
A164	embB M306I	ATG -> ATA	51	535
A335	embB M306I	ATG -> ATA	70	788
A261	embB M306I	ATG -> ATA	42	531
A046	embB Q497R	CAG -> CGG	12	942
A196	embB Q497R	CAG -> CGG	12	721
A962	embB Q497R	CAG -> CGG	22	1045
A332	embB M306V	ATG -> GTG	19	1089
A860	embB M306V	ATG -> GTG	31	917
A684	embB M306V	ATG -> GTG	25	891
A642	embB E405D	GAG -> GAC	48	796
A198	embB E405D	GAG -> GAC	41	710
A502	embB E405D	GAG -> GAC	28	357
A911	embB M306I	ATG -> ATC	43	1032
A769	embB M306I	ATG -> ATC	34	1068
A375	embB M306I	ATG -> ATC	23	941
A901	embB M306V, embB Q497P	ATG -> GTG, CAG -> CCG	9, 7	745, 485
A252	embB M306V, embB Q497P	ATG -> GTG, CAG -> CCG	32, 15	1140, 776

A918	embB M306V, embB Q497P	ATG -> GTG, CAG -> CCG	24, 19	1004, 685
A824	embB M306I	ATG -> ATA	19	230
A379	embB M306I	ATG -> ATA	54	574
A309	embB M306I	ATG -> ATA	60	641
A117				
A678				
A348				
A664	embB M306V	ATG -> GTG	25	861
A842	embB M306V	ATG -> GTG	23	909
A432	embB M306V	ATG -> GTG	17	811
A953				
A741				
A293				
A513				
A433				
A179				
A253				
A774				
A030				
A270	embB S297A	TCG -> GCG	16	848
A781	embB S297A	TCG -> GCG	14	885
A087	embB S297A	TCG -> GCG	24	805
A905				
A720				
A880				
A414	embB E378A, embB M306V	GAG -> GCG, ATG -> GTG	14, 32	620, 711
A275	embB E378A, embB M306V	GAG -> GCG, ATG -> GTG	22, 24	566, 660
A718	embB E378A, embB M306V	GAG -> GCG, ATG -> GTG	17, 26	214, 255
A764	embB D354A, embB M306I	GAC -> GCC, ATG -> ATA	26, 39	429, 233
A674	embB D354A, embB M306I	GAC -> GCC, ATG -> ATA	19, 31	256, 146
A532	embB D354A, embB M306I	GAC -> GCC, ATG -> ATA	80, 107	1034, 505
A993	embB E378A, embB G406D	GAG -> GCG, GGC -> GAC	12, 73	327, 304
A385	embB E378A, embB G406D	GAG -> GCG, GGC -> GAC	28, 65	143, 126
A708	embB E378A, embB G406D	GAG -> GCG, GGC -> GAC	23, 115	379, 303
A483	embB M306V	ATG -> GTG	22	555
A463	embB M306V	ATG -> GTG	39	688
A511	embB M306V	ATG -> GTG	21	665
A258	embB M306I	ATG -> ATA	31	219
A749	embB M306I	ATG -> ATA	47	296
A623	embB M306I	ATG -> ATA	59	360

A520	embB Q497R	CAG -> CGG	18	784
A916	embB Q497R	CAG -> CGG	41	986
A878	embB Q497R	CAG -> CGG	33	771
A254	embB M306I	ATG -> ATA	68	761
A122	embB M306I	ATG -> ATA	76	632
A665	embB M306I	ATG -> ATA	54	320
A562	embB G406S	GGC -> AGC	148	509
A924	embB G406S	GGC -> AGC	123	344
A452	embB G406S	GGC -> AGC	147	437
A368				
A334				
A518				
A608	embB M306I	ATG -> ATC	60	1082
A473	embB M306I	ATG -> ATC	44	896
A814	embB M306I	ATG -> ATC	40	674
A869	embB M306I	ATG -> ATA	69	686
A401	embB M306I	ATG -> ATA	53	442
A794	embB M306I	ATG -> ATA	92	651
A343	embB M306V	ATG -> GTG	40	964
A447	embB M306V	ATG -> GTG	27	780
A544	embB M306V	ATG -> GTG	32	580
A443	embB M306V	ATG -> GTG	47	907
A468	embB M306V	ATG -> GTG	28	829
A522	embB M306V	ATG -> GTG	23	523
A797	embB M306V	ATG -> GTG	29	639
A340	embB M306V	ATG -> GTG	40	997
A455	embB M306V	ATG -> GTG	20	455
A397	embB M306V	ATG -> GTG	26	928
A333	embB M306V	ATG -> GTG	21	623
A420	embB M306V	ATG -> GTG	27	717
A686	embB M306V	ATG -> GTG	32	1199
A114	embB M306V	ATG -> GTG	21	578
A029	embB M306V	ATG -> GTG	35	978
A477	embB M306V	ATG -> GTG	54	1000
A597	embB M306V	ATG -> GTG	36	830
A729	embB M306V	ATG -> GTG	56	535
A966				
A243				
A265				
A945	embB M306V	ATG -> GTG	36	816
A937	embB M306V	ATG -> GTG	31	850
A297	embB M306V	ATG -> GTG	33	963
A710	embB G406A	GGC -> GCC	155	575
A355	embB G406A	GGC -> GCC	174	878

A417	embB G406A	GGC -> GCC	174	660
A444	embB G406A	GGC -> GCC	152	718
A486	embB G406A	GGC -> GCC	42	169
A226	embB G406A	GGC -> GCC	90	425
A841	embB M306I	ATG -> ATA	49	606
A835	embB M306I	ATG -> ATA	130	463
A816	embB M306I	ATG -> ATA	32	523
A036	embB M306V	ATG -> GTG	41	954
A218	embB M306V	ATG -> GTG	19	610
A663	embB M306V	ATG -> GTG	29	1091
A123	embB M306V	ATG -> GTG	32	839
A822	embB M306V	ATG -> GTG	29	1176
A456	embB M306V	ATG -> GTG	37	878
A111	embB M306V	ATG -> GTG	31	630
A975	embB M306V	ATG -> GTG	39	903
A262				
A361	embB M306I	ATG -> ATA	45	662
A793	embB M306I	ATG -> ATA	45	720
A247	embB M306I	ATG -> ATA	54	664
B829	embB Y334H	TAC -> CAC	337	814
B564	embB Y334H	TAC -> CAC	253	700
B739	embB Y334H	TAC -> CAC	77	226
B771	embB Y334H	TAC -> CAC	474	321
B899	embB Y334H	TAC -> CAC	562	415
B177	embB Y334H	TAC -> CAC	614	416
B858	embB Y334H	TAC -> CAC	702	244
B888	embB Y334H	TAC -> CAC	617	226
B084	embB Y334H	TAC -> CAC	782	284
B694				
B583				
B679				
B682				
B772				
B687				
B839	embB Y334H	TAC -> CAC	358	746
B740	embB Y334H	TAC -> CAC	273	600
B184	embB Y334H	TAC -> CAC	315	640
B311	embB Y334H	TAC -> CAC	601	375
B416	embB Y334H	TAC -> CAC	275	165
B351	embB Y334H	TAC -> CAC	630	371
B944	embB Y334H	TAC -> CAC	497	129
B572	embB Y334H	TAC -> CAC	1047	291
B698	embB Y334H	TAC -> CAC	2830	914
B671				

B567				
B370				
B590				
B579				
B573				
C345				
C589				
C685				
C817				
C150				
C137				
C149				
C773				
C497				
C560				
C958				
C834				
C504				
C364				
C859				
C357				
C210				
C568				
C705				
C737				
C404				
C955				
C141				
C765				
C366				
C192	embB M306I	ATG -> ATC	31	915
C534	embB M306I	ATG -> ATC	39	1029
C982	embB M306I	ATG -> ATC	44	1361
C088	embB M306I	ATG -> ATC	13	725
C864	embB M306I	ATG -> ATC	22	418
C268	embB M306I	ATG -> ATC	31	639
C231	embB M306I	ATG -> ATC	5	154
C542	embB M306I	ATG -> ATC	30	668
C514	embB M306I	ATG -> ATC	37	653
C744	embB M306I	ATG -> ATC	48	1101
C220	embB M306I	ATG -> ATC	70	1280
C913	embB M306I	ATG -> ATC	90	1783
C752	embB M306I	ATG -> ATC	66	1315
C147	embB M306I	ATG -> ATC	94	1451

C040	embB M306I	ATG -> ATC	61	1127
C232	embB M306I	ATG -> ATC	2467	2730
C695	embB M306I	ATG -> ATC	582	587
C873	embB M306I	ATG -> ATC	486	505
C549	embB M306I	ATG -> ATC	405	430
C548	embB M306I	ATG -> ATC	551	499
C026	embB M306I	ATG -> ATC	51	888
C203	embB M306I	ATG -> ATC	44	1163
C543	embB M306I	ATG -> ATC	75	1158
C097	embB M306I	ATG -> ATC	66	1788
C796	embB M306I	ATG -> ATC	81	1455

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