Perceived Ethionamide Resistance in Isoniazid Susceptible Isolates of *Mycobacterium tuberculosis*

by

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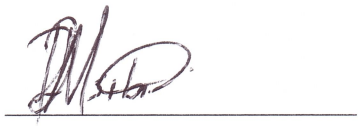
Submitted in partial fulfillment of the requirements for the degree of Master of Medical Science (Medical Microbiology) in the School of Laboratory Medicine and Medical Science (Infection Prevention and Control)

Declaration

This work has not been previously accepted for any degree and is not being currently considered for any other degree at any other university.

I declare that this dissertation contains my own work except where specifically acknowledged.



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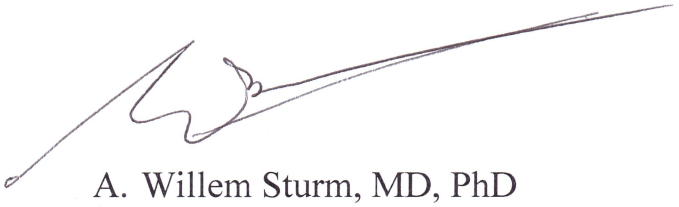
I, Prof A.Willem Sturm, has supervised the work presented here. I am satisfied with the contents of the dissertation which, to the best of my knowledge, is free of plagiarism.



A. Willem Sturm, MD, PhD

27 February 2015

Publications and Presentations

Contents of this work have been orally presented at the University of KwaZulu Natal, College of Health Sciences Symposium 2013. A poster presentation has arisen from this work, presented at the Federation of Infectious Diseases Society of Southern Africa (FIDSSA) Congress 2013. A manuscript will be submitted to the Journal of Clinical Microbiology for publication.

Acknowledgements

I would like to express my earnest gratitude to Professor A W Sturm for his impeccable supervision throughout this study, his contribution and guidance has proved invaluable towards this work, I could not have asked for a better supervisor. I would like to thank Bill Jacobs for inviting me to his lab at the Albert Einstein College of Medicine, his mentoring and training has not just been educational, but truly inspiring. Catherine Vilchèze who mentored me at Jacob's Lab, it was most pleasant working with her. I would like to thank Catherine for allowing me to use her primers for PCR and DNA sequencing as well as for her continued support and guidance. I thank Michelle Larsen for her words of encouragement, support and advice. Bisi Ashiru for her constructive advice, support and encouragement throughout this study, I am blessed to have come across a God fearing scientist such as her. I would like to thank Inga Elson for sharing her expertise in RFLPs and her encouragement. I would like to thank my colleague students for their assistance on various aspects of my work: Navisha Dookie, Lavania Joseph, Bronwyn Joubert and Refilwe Mzizi. My sincere appreciation to my friends and family; my mother Dudu Msibi who has provided constant support and encouragement, I hope I have made her proud; my friend Sethu Ngubane who has been there for me in good and difficult times; Snenhlanhla Zuma for being the sister I never had; Umbilo SDA Youth, Sandra Mendes, Dolly Seipone and my lovely daughter Nqobile Msibi for being my inspiration. I would like to express my humblest appreciation to NRF, CHS and TATA for financial sponsorship for the duration of my studies. Last but not least, I would like to thank YAHWEH my Father, through Whom everything was made possible for me. Ringrazio!

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Abstract

In *Mycobacterium tuberculosis*, resistance to ethionamide (ETH) is usually combined with isoniazid (INH) resistance due to a number of mutations in genes that are involved in the biosynthesis of mycolic acids. ETH resistance in INH susceptible isolates is rare. Ten such isolates were identified from patients participating in other studies.

Genotyping by means of IS6110 was performed to compare the relatedness of these isolates to each other. In attempts to identify the molecular basis for the resistance to ETH, the *eth*A*,* *msh*A and *msh*C genes were amplified and the amplicons sequenced using an ABI 3730 DNA Analyser. INH and ETH minimum inhibitory concentrations (MICs) were determined alone and in combination by means of checkerboard titrations in Middlebrook 7H9 broth, using the 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and microplate alamarblue assays (MABA) for detection of growth.

Seven isolates were not related to each other and their INH susceptibility was confirmed. No mutations were observed in all the sequenced genes. One out of seven isolates was found to be co-resistant to INH nad ETH. The MIC for the remaining isolates was 1µg/ml for ETH. MABA revealed a paradoxical susceptibility of the isolates to ETH, where mycobacterial growth was observed in ETH concentrations higher than the MIC for the six isolates. For combination of the two drugs, MABA revealed an antagonism between INH and ETH, where the isolates grew in high ETH concentrations regardless of the concentration of INH.

The paradoxical effect of ETH and antagonism between ETH and INH in our isolates does not result from mutations in *eth*A*, msh*A or *msh*C.

Chapter 1: Introduction

Since its primary discovery by Robert Koch in the second half of the nineteenth century [1], *Mycobacterium tuberculosis* has evolved into multi-drug resistant (MDR) and extensive drug resistant (XDR) strains. In the first half of the twentieth century chemotherapy for tuberculosis (TB) was deemed unachievable as a result of the lipid-rich cell wall [2]. This changed with the discovery of the first anti-tuberculosis drugs streptomycin [3] and para-aminosalicylic acid [4]. This was followed by the development of isoniazid [5], pyrazinamide [6], rifampicin [7] and ethambutol [8]. Shortly after the introduction of the first anti-tuberculosis drugs, resistance to those drugs was observed [9]. This led to the need for accurate and easy to perform drug susceptibility assays [10].

For several decades, treatment with the combination of isoniazid, rifampicin, pyrazinamde and ethambutol allowed for effective tuberculosis control in the clinical setting [11]. Although the TB epidemic was not completely under control in most developing countries, the prevalence was declining. The Human Immunodeficiency Virus (HIV) epidemic changed this picture and the number of new cases of TB spun beyond the point of control [12]. This lead to the declaration of TB as a worldwide health emergency by the World Health Organization (WHO) in 1993. This was followed by intense efforts to advance TB care and control nationally and globally [13]. In spite of the accessibility of effective short-course chemotherapy (DOTS) and the Bacilli-Calmette-Guerin (BCG) vaccine, tuberculosis is still responsible for more deaths than any other infectious agent [14]. The increase of drug resistance in clinical isolates of *M. tuberculosis* has been a major impediment in fighting the incidence and spread of TB, and consequently amplified universal labors to comprehend the molecular mechanisms resulting in drug resistance [15].

WHO estimated 8.7 million new TB cases in 2011 worldwide with 1.4 million TB related deaths [13]. This is almost 20 years after TB was declared a global health emergency [13]. This enduring crisis has resulted from disregard of TB by governments, insufficient access to health care and infrastructure, unsatisfactory adherence to medication by patients, reduced efficiency of TB management programmes, poverty, increase of populace and resettlement, and a major increase in the amount of TB cases in HIV infected persons [11].

MDR-TB is TB caused by bacilli that are resistant to isoniazid (INH) and rifampicin (RIF), which are the two most potent first-line drugs used in TB treatment [16]. XDR-TB results from bacilli that are resistant to fluoroquinolones, and any one of the injectable second-line drugs such as capreomycin, kanamycin or amikacin, on top of the two first-line drugs, consequently resulting in an elevated death rate especially in individuals co-infected with HIV [17 – 19].

The distinguishing traits of *M. tuberculosis* comprise dormancy, intricate cell envelope and intracellular survival and growth [20], which makes it intrinsically resistant to the action of many drugs that kill other pathogenic bacteria. Not many organisms generate such a varied range of lipid molecules as *M. tuberculosis*. These vary from a number of simple fatty acids, to very long-chain, extremely composite molecules. These molecules are known as mycolic acids [20].

The general understanding of tuberculosis drug resistance has significantly improved through the use of molecular techniques [10]. *M. tuberculosis* uses a number of strategies to oppose the action of anti-bacterial drugs [21]. One of these is reduced cell wall permeability,

resulting from the highly hydrophobic mycobacterial cell envelope [22, 23]. Genes that are linked with active drug efflux systems and degrading inactivating enzymes have been found in *M. tuberculosis*, which also aid in drug resistance [20, 24].

Exogenous agents, DNA polymerase errors, deletions, insertions and duplications are common means of mediating mutations in any prokaryotic genome through regular base changes, and these result in drug resistance in many prokaryotes [11]. This includes resistance of *M. tuberculosis* to anti-TB agents. Resistance to anti-TB drugs has been previously shown to be predominantly the effect of innate mutations in genes encoding drug targets, or enzymes implicated in drug activation [21]. Such mechanisms of resistance have been identified for all first-line and for various second-line drugs, including ethionamide [25 – 28].

ETH monoresitance in clinical isolates is rare. In this study, eleven *M. tuberculosis* clinical isolates were identified which, based on breakpoint testing, are phenotypically resistant to ETH, while showing full susceptibility to INH. To ensure that these isolates were not the same strain, these isolates were genotyped by means of IS6110 fingerprinting to compare their relatedness to each other. In an attempt to determine the molecular basis for ETH resistance in these isolates, *ethA, mshA* and *mshC* genes were sequenced, which are commonly implicated in high level resistance to ETH [33 – 35]. Finally, cell viability detection assays were performed to determine the precise minimum inhibitory concentrations (MICs) of the isolates to INH and ETH.

Chapter 2: Isoniazid and Ethionamide

2.1 Isoniazid

Before the discovery of isoniazid (INH), the treatment of TB was not very effective, as it was restricted to a few antimicrobial agents and synthetic drugs [29]. Broad-spectrum antimicrobial agents were not effective against TB [29]. The process leading to the discovery of isoniazid in 1952 began with the finding in the mid 1940s, by researchers in Europe, that nicotinamide was effective against TB [36]. Pyrazinamide was discovered as a result of the production of new synthetic molecules based on nicotinamide [37]. However, *M. tuberculosis* rapidly became resistant to pyrazinamide [37], hence the continued search for more effective antituberculosis agents. The β- and γ-pyridylaldehyde thiosemicarbazones, which demonstrated better activity against the tubercle bacilli, were synthesized in an effort to improve the activity of nicotinamide and other related compounds [29]. A breakthrough was achieved when one of the intermediates in the production of γ-pyridylaldehyde thiosemicarbazone , isonicotinic acid hydrazide (INH) had an unexpected increased effect against TB [38; 39], significantly higher than other antimicrobial agents used at the time [38; 39]. INH is still one of the main drugs used in TB chemotherapy [29].

2.2 Ethionamide

Ethionamide (2-ethylthioisonicotinamide, ETH) was first discovered in 1956 [40]; like isoniazid, ETH is a prodrug which requires metabolic activation [32] by the mycobacterial monooxygenase EthA [41]. It has been previously shown that ETH has a strong bacteriostatic effect against mycobacteria, more especially against isoniazid resistant mutants [33]. Resistance of mycobacterial strains to ethionamide is often coupled with resistance to

isoniazid, although there have been cases where resistance to isoniazid in mycobacteria has been observed with no co-resistance to ethionamide [42 – 43]. Treatment of MDR-TB still greatly relies on the use of the second line drug ETH [44]. ETH is a narrow-spectrum molecule, and like many other drugs used in the treatment of tuberculosis, the activity of ETH is mostly limited to mycobacteria, most likely as a result of the specificity of its interaction with essential components unique to mycobacteria [45]. On the other hand, the specificity may be due to the buildup or activation of ETH by uptake systems or converting enzymes only found in mycobacteria [45]. As a result of its high toxicity levels, the use of ETH in the treatment of infections caused by agents in the mycobacteria family is limited [46].

2.3 Inhibition of Mycolic Acids

INH and ETH are generally active against mycobacterial bacilli [29] by inhibiting mycolic acid biosynthesis [47]. Mycolic acids are long-chain α-alkyl-β-hydroxy fatty acids [29], and these substances are uniquely found in mycobacteria and related genera [48]. Mycolic acids play a critical function in mycobacterial-envelope structural design, forming an intersection between the capsule and the cell-wall frame, where they are covalently bound [49]. Winder & Collin in 1970 first demonstrated the inhibition of mycolic acids by INH, where they observed an inhibition of mycolic acid synthesis in *M. tuberculosis* H37Ra and *M. bovis* BCG following treatment with INH; similar inhibition patterns were not observed in INH resistant strains [47]. Two years later, Takayama and colleagues confirmed the inhibition of mycolic acid synthesis in *M. tuberculosis* treated with INH [50]; further, they demonstrated that this inhibition was linked to cell death [50] as well as the accretion of long chain fatty acids [51]. Previous research shows that INH treatment results in major damage to the envelope

organization, such as loss of the acid-fastness [52], discharge of irregular quantities of proteins into the culture medium and distorted ultrastructure [53].

2.4 Mechanism of Action of Isoniazid

INH is a pro-drug which requires activation by the mycobacterial enzyme catalase-peroxidase (KatG) [54] in combination with NADH oxidase [55]. NADPH oxidase-derived peroxidase activity of KatG seems essential in activation of INH [56]. Treatment with INH induces a powerful selection for INH-resistant mutants, where most mutations are found in the *katG* gene [54; 55; 58]. KatG activates INH, in concurrence with its peroxidase activity, by peroxidation to generate intracellular, reactive, INH-derived toxic species [59]. A variety of oxidants support KatG oxidation of INH, including superoxide [60], hydrogen peroxide [61] and simple alkyl hydroperoxides [62]. The *in vitro* auto-oxidation of INH [63] and NADH, when utilised [61], can supply adequate oxidants to permit INH activation by KatG, even if there is a deficiency of supplementary oxidants [63]. It is possible that the oxidant *in vivo* is a low flux of hydrogen peroxide that may occur inside the bacteria as a by-product of aerobic metabolism [61]. Both these superoxide- and low-flux hydrogen peroxide-oxidizing systems demonstrate the anticipated decline in the capacity of mutant S315T KatG to activate INH *in vitro,* in comparison to alkyl hydroperoxides which do not [63], however, of these the *in vitro* oxidant species has not been established [59]. Horseradish peroxidase [64; 65] or even inorganic manganese ions [66; 67] have been previously postulated to be possible model-oxidising systems which may be capable of activating INH. Nevertheless, it has not been decided if these representation oxidants precisely imitate the accurate species produced by KatG, since even greatly associated KatG enzymes can vary in the products they generate [68]. The crystal structures of KatG [69; 70] and recently the S315T variant [61] give an

improved understanding of the mechanisms by which INH is activated. The contraction of the haem access channel from 6 Å ′ in the wild type to 4.7 Å ′ in the S315T variant implies that diminished INH access to the oxidizing site of KatG may be the means to resistance, this is in agreement with prior spectroscopic analyses [71 – 73]. In the NAD+ or NADP+ deficiency, no considerable constant anti-tubercular products resulting from INH metabolism are generated during *in vitro* activation [59]. Consequently, reactive intermediates resulting from INH metabolism are essential to the efficacy of INH [59]. KatG oxidatively activates INH through the formation of a variety of carbon-, oxygen- and nitrogen-centered free radical species, with the formation of acyl, acylperoxo and pyridyl radical adducts of phenylbutylnitrone (PBN) projected from outcomes of spin trapping experiments [63]. Nevertheless, the differentiation of dissimilar adducts of PBN by hyperfine coupling constant alone is convoluted, thus these allocations remain rather uncertain [74]. The spin trap 5,5-dimethyl-1-pyrolline-*N*-oxide (DMPO) frequently permits improved allocations than PBN, since the variety of hyperfine coupling constants of its spin adducts are wider [59]. Consequently, species formed from INH allocated as carbon-centred and alkoxyl adducts of DMPO have been observed upon KatG oxidation of INH [75], with an extra peroxyl radical species observed in the presence of molecular oxygen, due to a reaction of one of these radicals with O_2 [59]. The nitric oxide radical ($NO\cdot$) has also been trapped from KatG oxidation of INH, with 15N isotopic labelling of INH hydrazide resulting in $^{15}NO\cdot$ [75; 76]. The outcomes of other oxidizing systems imply that the hydrazyl radical [77] can be easily formed by INH oxidation and is possibly a preliminary product, although verification with a proper KatG enzyme is necessary [59]. In spite of these advances, the necessity to describe the nature of these radical intermediates remains, and in particular, to show ultimately the formation of the isonicotinoyl radical [78], since this is the crucial intermediate that adds to NAD+ and NADP+ to generate a variety of potent inhibitors [59]. While it took long to

establish the mechanisms, it had been known for ages that INH disturbs production of both mycolic acids [47; 50] and nucleic acids [80]. The discovery that oxidation of INH in the presence of NADH and InhA led to covalent INH-NADH adducts that are potent inhibitors of InhA [80] was a major advance [59]. InhA mediates the production of mycolic acids [81], which are exclusive and essential mycobacterial cell wall lipids, and so its inhibition is in harmony with the characteristic susceptibility of mycobacteria to INH [59]. The metabolic effects of NADH/NAD+ ratios on INH susceptibility in mycobacteria supports the function of NAD+ in production of INH-NAD adducts [82], in which decreased NAD+ levels caused INH resistance, and also by mutations in the NADH oxidase gene *ndh* in some isolates resistant to INH [83]. INH-NAD adducts proficient in InhA inhibition are formed both in simple Mn/INH/NADH mixtures [66; 78; 84] and in KatG-mediated reactions. The *S* isomer binds to InhA when the stereochemical centre is produced by the addition of the isonicotinoyl radical to NAD+ in the INH-NAD adduct [80]. This *S* isomer is generated as a tight-binding (*K*i = 0.75 nM) inhibitor [85]. The two primary INH-NAD adducts generates a variety of diastereoisomers upon subsequent cyclization [66; 67; 84]; however, these do not seem to have the inhibitory effect which the acyclic *S* isomer has on InhA [59]. The coupling of activated INH with NADP+ may possibly occur, *in vitro* these INH-NADP adducts are a potent inhibitor of MabA, an NADPH dependent b-ketoacyl-ACP reductase which is also vital in the synthesis of mycolic acids [86]. Consequently, INH adducts of both NAD+ and NADP+ may well inhibit various steps in cell wall lipid synthesis, though an *in vivo* role for MabA inhibition has not been demonstrated yet [59]. Additionally, previous studies have revealed that the acyclic 4*R* isomer of the INH-NADP adduct binds *M. tuberculosis* dihydrofolate reductase (DHFR), which is essential in nucleic acid biosynthesis to generate nucleotide pools, with a *K*i less than 1 nM [87]. Even though it seemed as if an INH adduct of KasA, another mycolic acid synthetic enzyme, may arise by means of an INH-induced 80

kDa covalent complex consisting of KasA, AcpM and an INH-derived isonicotinic acyl fragment [88], it appears probable that effects on KasA are mediated through INH-NAD adduct inhibition of InhA [89] as an outcome of its identified close regulation with InhA [90]. The comparative importance of InhA and KasA as targets has been further elucidated by inducing mutations seen in clinical isolates, such as the S94A mutation found on the *inhA* gene in wild-type mycobacteria [91]. The induced S94A mutation amplified INH resistance, whereas *kasA* mutants G269S as well as F413L were unable to produce a similar effect [91]. Accordingly, a variety of strong INH-NAD/NADP inhibitors have been described, and in general, the essential genetic alterations of target mycobacteria have resulted in the expected alteration in INH sensitivity [59]. Nonetheless, an increased concentration of NAD+/NADP+ and a very low concentration of other molecules with the ability to react with the isonicotinic acyl radical was used in these *in vitro* systems , in so doing compelling the isonicotinoyl radical to react with NAD+ or NADP+ [59]. The isonicotinoyl radical will be highly reactive with a broad variety of reactants as for other acyl radicals [92; 93] such as proteins, mycothiol and unsaturated lipids [94]. Thus, when generated within the tubercle bacilli, the isonicotinoyl radical will react mostly with other molecules rather than NAD+ or NADP+, that are present at only ~0.4 mM and 0.2 mM, respectively, in *M. tuberculosis* [79]. While the gathered data is persuasive, final verification of the function of these INH-NAD and INH-NADP species will necessitate their isolation from INH-treated mycobacteria at concentrations concurring with a considerable inhibitory effect from their known *K*is [59].

2.5 Mechanism of action of ethionamide

The mode of action of ETH upon activation is through the inhibition of the enoyl-ACP reductase [30; 95] enzyme which is encoded by the *inhA* gene and forms part of the mycolic acid biosynthesis system [96]. The activation of ETH is via the *ethA* gene encoded mono-

oxygenase (EthA), which results in the formation of an S-oxide metabolite (ETH-SO) [33; 97], possibly a sulfinate, which demonstrates superior bactericidal action against *M. tuberculosis* compared to the inactivated form of ETH *in vitro* [33]. Previous studies have implied that ETH-SO maintains the biological activity of the inactivated form of the drug [98] which correlates with the fact that a number of drugs used in TB treatment, including ETH, need some kind of metabollic activation parallel to the oxidative, reductive, or hydrolytic unmasking of active groups [99 – 101].

2.6 Killing by isoniazid

Passive diffusion is the mode of entry of INH into the mycobacterial cell interior [102] which then kills only dividing bacteria; no killing of mycobacterial cells is seen during stationary phase or when the bacteria are growing under anaerobic conditions [103]. During the first 24 hours of INH administration, the action of INH against *M. tuberculosis* is bacteriostatic, followed by the killing of the bacteria [29]. It has also been reported in the past that there is a postponement from 1 to 4 days before INH begins its bacteriocidal action [52; 103 – 105]; in the meanwhile, the mycobacterial cells lose their acid fastness [52; 105]. INH also brings about physical alterations in mycobacterial cells, such as the loss of inner structure and the emergence of surface wrinkles and bulging [106 – 108].

2.7 Genes associated with ETH resistance

In the year 2000, Baulard *et al* and DeBarber *et al* separately identified the *EthA* and *EthR* genes which are implicated in the activation of ETH [33; 34]. EthA is a FAD-containing enzyme that mediates two steps in the activation of ETH [41]. The resistance of *M.*

tuberculosis to ETH has been ascribed to the transcriptional suppression of ethA applied by the bacterial regulator EthR, encoded by the *ethA* neighboring gene *ethR* [45]. EthR was shown to repress the expression of *ethA*, the binding of EthR upstream of *ethA* results in the suppression of *ethA* expression [45]. In wild-type mycobacteria, some of the ETH molecules remain unactivated [45]. This has been signified by the linking of the chromosomal inactivation of *ethR* with ETH hypersensitivity, and as a result, EthR adds to the resistance of *M. tuberculosis* to ETH [96]. EthR was proposed and confirmed as a drug target to boost the bioactivation of ethionamide and increase the efficacy of ETH *in vivo* [109]. Flipo and colleagues observed and documented structure−activity relationships of a progression of druglike 1,2,4-oxadiazole EthR inhibitors identified via a judicious drug design approach [110; 111]. In 2008, Vilcheze *et al* showed that mutations in the glycosytranferase MshA encoding gene, *mshA*, resulted in high level resistance of spontaneous mutants of *M. tuberculosis* [35]. MshA mediates the first step in mycothiol biosynthesis [112; 113], which had been previously reported to be crucial in the survival of *M. tuberculosis* [114]. However, Vilcheze and colleagues showed that some *M. tuberculosis* mutants deficient of mycothiol were viable [35]. Most mutations in genes that mediate mycothiol biosynthesis were found to confer ETH and INH resistance. Vilcheze and colleagues later showed that *in vitro* induced *mshA* and *mshC* mutations were responsible for high level resistance to ETH and low level resistance to INH [99]. Mutations in the *mshA* gene, however, must be carefully examined because some clinical isolates showing phenotypic susceptibility to ETH and INH have been observed to have some mutations in this gene [99].

2.8 Common INH Resistance Conferring Mutations

Mutations in *katG*, a gene encoding the INH activator, are the most common cause of INH resistance in *M. tuberculosis*. This results in a decline in or loss of catalase-peroxidase activity [57; 115; 116]. There have been observed insertions, deletions, truncation, missense and nonsense mutations, and less frequently, full gene deletion [55; 117; 118]. S315T mutation, which harbours a product which has a highly decreased capacity to form the INH-NAD adduct, is most common in *katG* [116 – 122]. Consequently, *M. tuberculosis* strains with a mutation in *katG* are most probably resistant to INH as a result of a lowered capacity to form the INH-NAD adduct, which is the InhA inhibitor. The subsequent mutation detected in an INH resistant strain was the mutation in *inhA* [32] which ecodes the enzyme enoyl-acyl carrier protein reductase (InhA). This mutation decreases the attraction of InhA for its cofactor NADH and is located in the NADH binding pocket of InhA [32]. Consequently, the InhA (S94A) protein has a greater *K*i for the INH-NAD adduct and is more resistant to the inhibition by the INH-NAD adduct than the wild-type enzyme [91]. This mutation disrupts the hydrogen bonding network, as indicated by X-ray crystallographic structural determinations [91]. Consequently, an INH-NAD adduct with reduced binding abilities to InhA is formed [91]. Other INH resistance conferring mutations in *inhA,* such as I16T, I21V, I47T, and I95P, are also situated in the NADH binding pocket and diminish the affinity for NADH [124]. When a *M. tuberculosis* isolate is resistant to INH, mutations in the *inh*A gene are not always found but if they are found, these typically coexist with mutations in the promoter region of *kat*G or *inh*A mutations [116 – 119; 121 – 124]. Overexpression of *inh*A is another way in which *M. tuberculosis* can become resistant to INH. This resistance results from dilution of INH by the InhA enzyme [29]. The C-15T *inh*A promoter mutation is the second most frequent mutation in INH-resistant *M. tuberculosis* clinical isolates [116 – 122]. This mutation results in InhA overexpression by increasing the *inh*A mRNA level 20 times as

compared to the wild-type, resulting in a much higher resistance of mycobacteria to INH [91]. Mutations in the NADH dehydrogenase NdhII were initially found in INH-resistant *M. smegmatis* laboratory strains [82]. INH-resistant *M. bovis* BCG laboratory strains were later shown to also harbour *ndh* mutations [99]. The *ndh* mutants had low NdhII activity, which resulted in an accrual of NADH, the substrate for NdhII [99]. This NADH accumulation played the role of a competitive inhibitor for binding to InhA and shielded InhA from the inhibitory effect of the INH-NAD adduct [99]. R268H is the only mutation in *ndh* that has been reported to occur in clinical isolates of INH-resistant *M. tuberculosis* [122; 125]. The inactivation of INH by the *nat*-encoded arylamine *N*-acetyltransferase (Nat) is another means of resistance [29]. The acetylation of the nitrogen group of INH by *M. tuberculosis* Nat prevents the activation of INH by KatG [126; 127]. Following transformation with the mycobacterial expression vector pACE-1, overexpression of *M. tuberculosis nat* results in an increased level of INH tolerance in *M. smegmatis* [126]. However, mutations in *nat* identified in clinical isolates of INH resistant *M. tuberculosis* were constantly interrelated with *kat*G mutations and also observed in INH-sensitive clinical isolates [116].

2.9 Other Mutations Conferring INH Resistance

INH resistance has also been observed in *M. smegmatis* strains lacking in mycothiol [29]. The genes, *msh*A, *msh*B, *msh*C, and *msh*D, mediate the biosynthesis of mycothiol with *M. smegmatis* mutants having a transposon in either *msh*A [129], *msh*C [129], or *msh*D [130]. This renders the organisms extremely resistant to INH [29]. A *M. smegmatis msh*B mutant was surprisingly found to be sensitive to INH [131], while a twofold increase in INH resistance was seen in a *M. tuberculosis msh*B mutant [131]. The reasons for INH-resistance in mycothiol deficient mutants are not yet understood although it has been hypothesized that

mycothiol is necessary for the activation of INH [129; 131]. Other mutations found to facilitate INH resistance in clinical strains of *M. tuberculosis* include mutations found *in fur*A, *afp*C, *fad*E24 and *kas*A [116; 117; 120; 122; 132 – 137]. However, these mutations were often related to katG and/or inhA mutations in promoter region, or were also present in INH-susceptible clinical isolates [29].

2.10 INH Tolerance Conferring Mutations

Almost all of the *M. tuberculosis* cells die within 3 to 4 days after treatment with INH [29]. The surviving tubercle bacilli signifies a heterogeneous population made up of bacilli that have either acquired their INH resistance through spontaneous mutations or are genetically sensitive to INH but maintaining tolerance to the drug [29]. This drug tolerance phenotype was characterized by testing the INH-inducible *iniBAC* operon, which encodes an unidentified membrane transporter, for its capability to shield *M. tuberculosis* against cell wall synthesis inhibitors like INH and ethambutol [138]. This research led to the finding that the *iniA* gene in *M. tuberculosis,* upon overexpression, conferred a multidrug-tolerant phenotype to both INH and ethambutol [138]. However, Colangeli and co-workers [138] showed that the IniA protein on its own does not work as a drug transporter and hypothesized that its function is to eradicate toxic compounds from the cells [29].

Chapter 3: Methodology

Ethical clearance to conduct this study was obtained from the University of KwaZulu Natal Biomedical Research Ethics Committee (BREC), reference number BE067/12.

3.1 Bacterial strains and media

Mycobacterium tuberculosis clinical isolates from patients admitted to the Church of Scotland Hospital in Tugella Ferry in 2007 were obtained from storage. The susceptibility of these isolates was identified using the agar incorporation method on Middlebrook 7H10 agar (Difco, Becton and Dickenson, USA). The concentrations used were 1 mg/L for isoniazid (INH) (Sigma-Aldrich (Pty) LTD, SA) and 2.5 mg/L for ethionamide (ETH) (Sigma-Aldrich (Pty) LTD, SA). Susceptibility was defined as the absence of growth and resistance was defined as growth of any number of colonies. The isolates were grown in Middlebrook 7H9 liquid medium (Difco, Becton and Dickenson, USA) supplemented with 10% (v/v) OADC (BD, USA) enrichment (Difco) and 0.2% (v/v) glycerol.

DNA was extracted from these isolates to perform *IS1660* fingerprinting as well as sequencing of predefined genes. Quantitative susceptibility to isoniazid and ethionamide alone and in combination was also performed.

3.2 DNA Extraction

Once an OD_{600nm} of 1 has been reached, 2 ml of the culture was transferred into an Eppendorf tube (Merck, USA) and centrifuged for 10 min at 3000 x g. The supernatant was discarded and the deposit was resuspended in 500 µl triple distilled water and killed by exposure to 80ºC for 30 min. This was followed by the addition of 70 µl of 10% sodium dodecyl sulphate

(SDS) (Sigma-Aldrich (Pty) LTD, SA) and 50 µl proteinase K (10 mg/ml) (Roche, SA) and incubation at 60ºC for 1 hour. A solution of 5M NaCl and CTAB (10% CTAB, 0.7M NaCl) was pre-heated to 60ºC and 100 µl 5M NaCl was added. The tube's contents were mixed by inverting repeatedly. The mixtures were incubated at 60ºC for 15 min then frozen at -70ºC for another 15 min. The tubes were removed and defrosted at 60ºC, 700 µl chloroform-isoamyl alcohol (24:1) was added and the contents were mixed by inversion by hand 20-25 times followed by centrifugation at 13000 x g for 10 min. The upper aqueous phase was transferred to a tube with 700 µl cold isopropanol and mixed by tilting the tubes up and down several times. These mixtures were incubated at -20ºC for 30 min. The tubes were centrifuged for 10 min at room temperature (25ºC). The supernatants were drained and the pellets were washed with 80% ethanol and centrifuged for 5-10 min. The liquid was drained and the pellets were dried in speed vacuum for about 5 minutes. Following this 55 µl 1x TE was added. The DNA quality was checked by electrophoresis in a 1% agarose gel.

3.3 Gel Electrophoresis

Seakem agarose gel powder (Whitehead Scientific, SA) was dissolved in 1X TBE buffer (0.3 g in 30 ml) by heating in a microwave oven for 30 sec. Ethidium bromide (Sigma-Aldrich (Pty) LTD, SA) was added to a final concentration of 0.5 mg/L. The mixture was poured onto a Hoefer gel electrophoresis tray (7 cm X 10 cm) and was left to solidify at room temperature (25ºC). The gel was placed on a Hoefer electrophoresis machine containing 1XTBE buffer. Each well was loaded with 1 µl DNA mixed with 5 µl of gel loading buffer. The gel was run for 30 min at 100 V and then viewed under UV light to check the quality and the approximate quantity of the extracted DNA.

3.4 IS6110 Restriction Fragment Length Polymorphisms (RFLPs)

Endonuclease restriction

Similar amount of DNA of each isolate were diluted with distilled water to reach a final volume of 22 µl. This was mixed with 2.5 µl PvuII buffer, and 1.5 µl of 5000 U PvuII enzyme and incubated for three and half hours in a water bath at 37°C. The digested samples were run in a 1% agarose gel (15 cm X 20 cm) for 30 min at 90 V and then overnight at 36 V on a Hoefer electrophoresis machine.

Southern blot

A Hybond-N+ membrane (Amersham-GE Health, USA) was briefly submerged in water and then soaked in 10X SSC (Annexure 4) for 5 min. The gel was placed on the prepared membrane. A vacuum of -55 cm mbar was created by means of a GE-Health Vacugen-XL vacuum pump. The gel was flooded with 1/100 HCl for 20 min after which the liquid was removed. The gel was flooded with Soak I (Annexure 4) for 20 min and, after removal of the fluid, again with Soak II for 20 min. The gel was once more submerged in 10X SSC for 1.5 hours after which the fluids were removed by vacuum. After removal of the gel, the membrane was allowed to dry for 5 min. To optimize the cross links, the membrane was briefly irradiated under UV (UVP-CL1000).

Hybridization

The probes used were manufactured by PCR (Annexure 3) according to van Embden *et al* (1993). The membrane was emersed in 20 ml of hybridization buffer while rotating at ~5

RPM at 42ºC for 30 min. A mixture of equal volumes of 10 µl of probe and 10 µl of water was boiled for 5 min and then placed on ice for 10 min. To this, 20 µl of labeling agent and 20 µl of glutaraldehyde (Amersham ECL Direct Nucleic Acid Labeling and Detection System – RPN 3000) were added followed by incubation in a 37ºC water bath for 15 min. The probe was added to the hybridization buffer at the required concentration. Hybridization was allowed overnight in a rotating incubator at 6 RPM at 42ºC.

Detection of banding pattern

Following hybridisation, the membrane was briefly rinsed with primary wash buffer. The tube containing the membrane in fresh primary wash buffer was rotated for 30 min at ~5 RPM at 42ºC. The membrane was removed from the tube and soaked in secondary wash buffer on a Stuart Orbital SSL1 shaking platform for 10 min. The liquid was discarded and the above step was repeated.

The membrane was incubated (1 min at $\pm$ 25^{o}C) in a mixture of 5 ml of Soak I and 5 ml of Soak II, wrapped in plastic and placed with the Amersham Hyperfilm ECL in a cassette (Amersham, USA) and developed by chemiluminescence in a dark room till bands with the required density were observed.

3.5 Minimum Inhibitory Concentrations (MICs)

Minimum inhibitory concentrations (MIC) to INH and ETH were determined for the *M. tuberculosis* isolates using the 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Include ref) and the microplate alamar blue assays (MABA) (ref: fransblau, ref# 141). Cultures were incubated till the log phase of growth was reached, indicated by an

OD_{600nm} of 1. This corresponds with a growth density of 10^8 cfu/mL. A test inoculum of 10^3 cfu/mL for each isolate was prepared by serially diluting the cultures in Middlebrook 7H9 broth. Stock solutions of INH and ETH were prepared in distilled water and dimethyl sulfoxide (DMSO) respectively. Both solutions were filter sterilized. Serial 2-fold dilutions were made in the wells of a microtiterplate with 7H9 broth as diluent. To each test well, containing 50 µl of drug solution, 50 µl of the test inoculum was added. The final concentrations for INH were 16, 8, 4, 2, 1, 0.5 and 0.25 µg/ml and for ETH 64, 32, 16, 8, 4, 2 and 1 µg/ml. The plates were incubated for seven days in a 37ºC O_2 incubator before the respective colorimetric reagents were added. Both the MTT assay and MABA was done in triplicate to test for intra-test variation and both were repeated in triplicate on different days to test for inter-test variation.

The MTT solution was prepared by dissolving 0.00513 g MTT powder (Sigma-Aldrich (Pty) LTD, SA) in 1 ml 7H9 broth and filter sterilized. Of the MTT solution 7.5 µl was added to each well of the MIC microtiter plate. After a further incubation period of 24 hours, 25 µl of 20% sodium dodecyl sulphate (SDS) solution with *N*$_1$*N*-dimethylformamide (DMF) (Sigma-Aldrich (Pty) LTD, SA) [1:1 vol/vol] was added to each well. This was followed by an additional 24 hour incubation step to allow for colour development. A purple colour and/or precipitate indicates bacterial growth.

For the MABA, at the end of the 7-day incubation period of the MIC microtiterplates, 15 µl of AlamarBlue® reagent (Life Technologies, SA) was added to each well. The plates were further incubated for 24 hours. A colour change from blue to pink indicates bacterial growth. Wells showing colour change were subcultured on Middlebrook 7H11 agar plates to confirm mycobacterial growth.

3.6 Checkerboard Titrations

Plates for the checkerboard titrations were prepared as shown in (figure 3.6.1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	E64	I16	18	I4	I2	I1	I0.5	I0.25	I0.125	I0.0625	+ C	-C
B	E32	E64 I16	E32 I16	E16 I16	E8 I16	E4 I16	E2 I16	E1 I16	E0.5 I16	E0.25 I16	+ C	-C
C	E16	E64 I8	E32 I8	E16 I8	E8 I8	E4 I8	E2 I8	E1 I8	E0.5 I8	E0.25 I8	+ C	-C
D	E8	E64 I4	E32 I4	E16 I4	E8 I4	E4 I4	E2 I4	E1 I4	E0.5 I4	E0.25 I4	+ C	-C
E	E4	E64 I2	E32 I2	E16 I2	E8 I2	E4 12	E2 I2	E1 12	E0.5 I2	E0.25 I2	+ C	-C
F	E2	E64 I1	E32 I1	E16 I1	E8 I1	E4 I1	E2 I1	E1 I1	E0.5 I1	E0.25 I1	+ C	-C
G	E1	E64 I0.5	E32 I0.5	E16 I0.5	E8 I0.5	E4 I0.5	E2 I0.5	E1 I0.5	E0.5 I0.5	E0.25 I0.5	+ C	-C
H	E0.5	E64 I0.25	E32 I0.25	E16 I0.25	E8 I0.25	E4 I0.25	E2 I0.25	E1 I0.25	E0.5 I0.25	E0.25 I0.25	+ C	-C
I	E0.25	E64 I0.125	E32 I0.125	E16 I0.125	E8 I0.125	E4 I0.125	E2 I0.125	E1 I0.125	E0.5 I0.125	E0.25 I0.125	+ C	-C
J		E64 I0.0625	E32 I0.0625	E16 I0.0625	E8 I0.0625	E4 I0.0625	E2 I0.0625	E1 I0.0625	E0.5 I0.0625	E0.25 I0.0625	+ C	-C

Figure 3.6.1: Representation of checkerboard titration assays.

E = ethionamide; I = isoniazid; numerical values = drug concentrations.

3.7 DNA sequencing and analysis

The extracted DNA samples were shipped to Jacobs Lab at the Albert Einstein College of Medicine (New York) where PCR amplification of the *msh*A, *msh*C and *eth*A genes was performed followed by amplicon sequencing and analysis of the data obtained.

Polymerase Chain Reaction (PCR) Amplification

The following primers were used for PCR amplication: *msh*A, *msh*C and *eth*A genes

mshAF: GGCAGCTGGAGTCCACTGT

mshAR: GCAGCAGGAACCGGTATACG

mshCF: ACAACCAACTGGACCCCTAC

mshCR: TCACCGCCAGCTCTGATTTG

ethAF: AGGCGGACGGTCCTCGAGAA

ethAR: GGGCGGGGTGACATTCGTTC

A PCR kit from Applied Biosystems (Life Technologies, USA) was used. The composition of the master mix is shown in table 3.7.1. Of this master mix, 49 µl volumes were aliquoted into PCR tubes and 1 µl of extracted DNA was added. The PCR final reagent concentrations and cycling steps are summarized in table 3.7.1 and table 3.7.2 respectively.

Table 3.7.1 Final concentrations of the PCR components

Master Mix component for a 100 µl reaction	**Concentration**
dNTPs	40 nmol
PCR Buffer II, 10X	10 µl
$MgCl_2$	300 nM
Reverse primer	40 pmol
Forward primer	40 pmol
Enzyme (taq) AmpliTaq DNA polymerase	0.5 µl

Table 3.7.2 PCR cycling steps and parameters

	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Temperature (ºC)	94	94	55	72	72
Duration (min)	5	30 sec	1	1.30	7
Number of PCR cycles: 40					

Purification of PCR products

To each PCR product, 250 µl of buffer PB provided with the QIAquick PCR purification kit was added in a QIAquick spin column. The mixture was centrifuged for 60 sec. The flow-through was discarded and 0.75 ml of buffer PE was added to the column and centrifuged for an additional 60 sec. The column was placed in a clean 1.5 ml microcentrifuge tube. To elute the DNA, 30 µl of elution buffer was added to the centre of the QIAqiuck membrane of the column. The column was allowed to stand for 2 min and then centrifuged for 2 min.

DNA Sequencing

DNA sequencing was performed at the Albert Einstein College of Medicine DNA sequencing facility using an ABI 3730 DNA Analyzer. To prepare the sample, 3.2 µl of each primer was aliquoted in separate tubes and 2 µl of purified PCR product was added to each tube. The samples were sent to the sequencing facility for DNA sequencing. The sequences were analyzed using clone manager sequence alignment program.

Chapter 4: Results

4.1 Restriction Fragment Length Polymorphism Genotyping

The result of the DNA extraction of all 10 isoniazid susceptible but ethionamide resistant *Mycobacterium tuberculosis* isolates was checked by means of elecrophoresis. The results are shown in Fig. 4.1.1 One isolate (TF2232, lane 9) was resistant to both drugs.

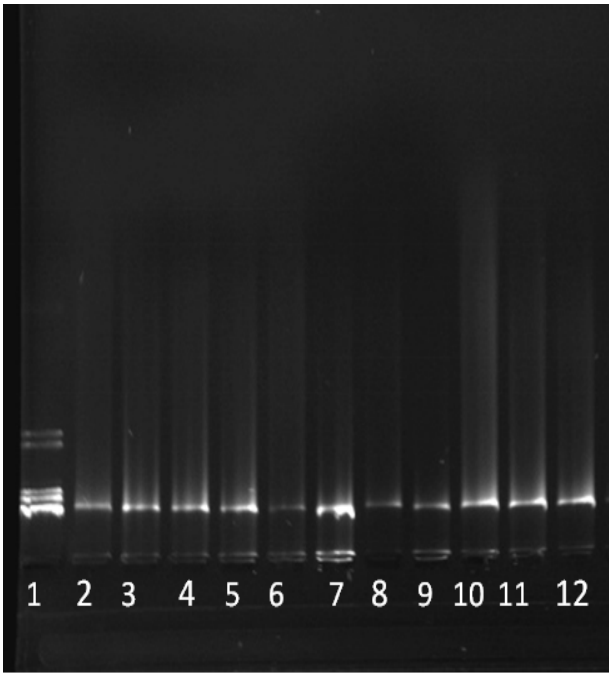

Figure 4.1.1: DNA gel image of INH susceptible ETH resistant isolates of *M. tuberculosis.* 1: marker 2, 2: TF727, 3: TF1701, 4: TF1719, 5: TF1734, 6: TF2205, 7: TF2204, 8: TF2226, 9: TF2232, 10: TF1400, 11: TF1735 and 12: TF1091.

Seven out of ten isolates had distinct restriction patterns. Four isolates (TF1701, TF1719, TF1734 and TF1735) showed identical restriction patterns. Therefore only one (TF1719) of these was included in the experiments.

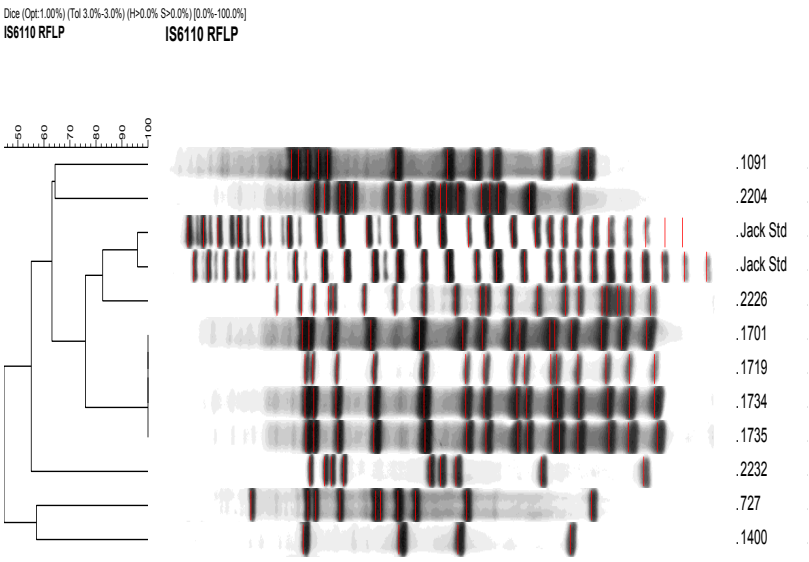

Figure 4.1.2: I*S6110* RFLP dendogram showing restriction patterns of *M. tuberculosis* isolates. The TF isolate numbers are shown on the right side of the dendogram.

4.2 Checkerboard Titrations

4.2.1 Tetrazolium Microplate Assay

The results of the checkerboard titration using 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) microplate assay is shown in figure 4.2.1.1. The lay-out of the plate is as shown in figure 3.6.1. As mentioned in chapter 3.5 bacteria were exposed to the drugs for 7 days followed by overnight incubation with the reagent. The MIC for INH was >.0.0625 mg/L (A2 – A10) and 2 mg/L for ETH. However, the MTT reagent also showed a reaction with the 4 to 5 highest ETH concentrations (A1-D1/E1).

The assay was repeated without bacteria. After 7 days of incubation a colour change was observed in all wells, independent of the ETH concentration (not shown). Therefore, the microplate alamar blue assay (MABA) was employed for the detection of bacterial growth.

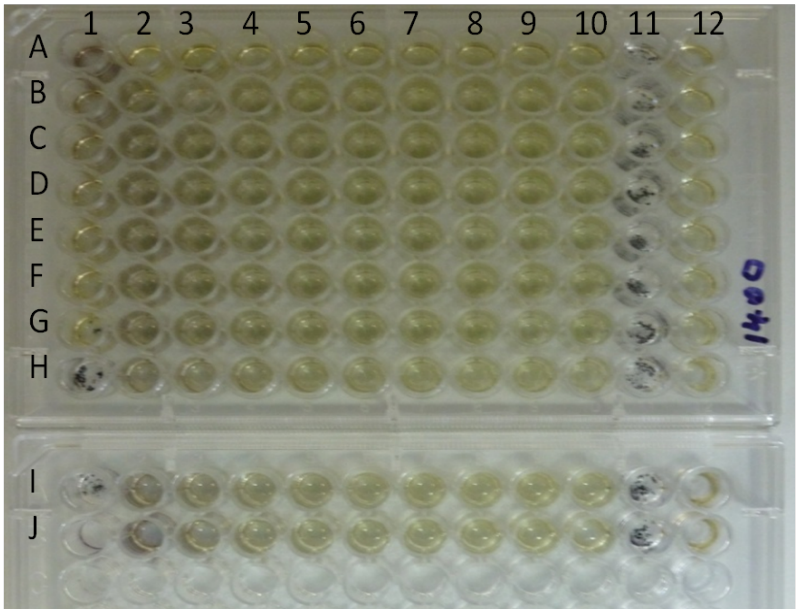

Figure 4.2.1.1: Checkerboard MTT assay

Blue-black: bacterial growth,

Row 11: positive control

Row 12 negative control.

4.2.2 Microplate Alamar Blue Assay

The results of the checkerboard titration of INH and ETH using the Microplate Alamar Blue Assay (MABA) as shown in figure 4.2.2.1. The lay-out of the plate is as shown in 3.6.1. No interaction of INH or ETH with the AlamarBlue reagent was observed in the absence of bacteria.

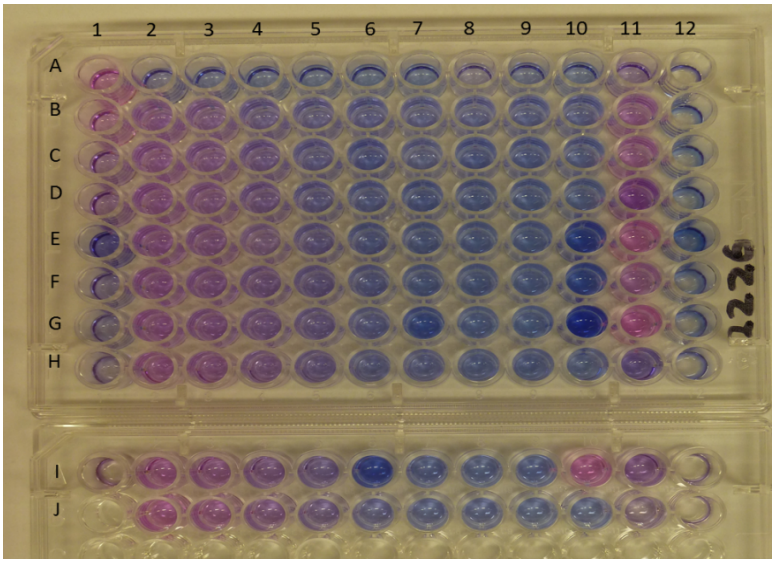

Figure 4.2.2.1: Checkerboard titration with Microplate Alamar Blue Assay

Pink= bacterial growth,

Row 11: positive control

Row 12 negative control

With INH only (wells A2 – A10), growth inhibition was observed in all wells, resulting in an MIC ≤ 0.0625 mg/L. The dilution series with ETH only (wells A1 – I1) showed growth at concentrations 64, 32, 16 and 8 mg/L, no growth from 4 mg/L till 1 mg/L and growth again at a concentration of 0.5 mg/L and lower. This indicates an MIC of 1 mg/L. Growth at the high concentrations is in keeping with paradoxical susceptibility or Eagle's phenomenon [166]. This was observed in six isolates: TF727, TF1091, TF1400, TF1719, TF2204 and TF2226. TF2232 was found to be co-resistant to both INH and ETH, with an MIC of 8 mg/L for INH and an MIC ≤ 64 mg/L for ETH.

This paradoxical effect of ETH was seen with all six isolates that, based on the proposed WHO breakpoints (WHO, 4th Edition), had been identified as ETH resisitant while susceptible to INH. All the six isolates showed complete susceptibility to INH, where their MICs were less than or equal to 0.0625 µg/ml (lowest concentration used).

When the growth in wells with the highest concentrations of ETH was subcultured in 7H11 agar plates, growth with colony morphology compatible with *M. tuberculosis* was observed (Figure 4.2.1.2). Ziehl Neelsen staining confirmed the cultured organims to be acid fast.

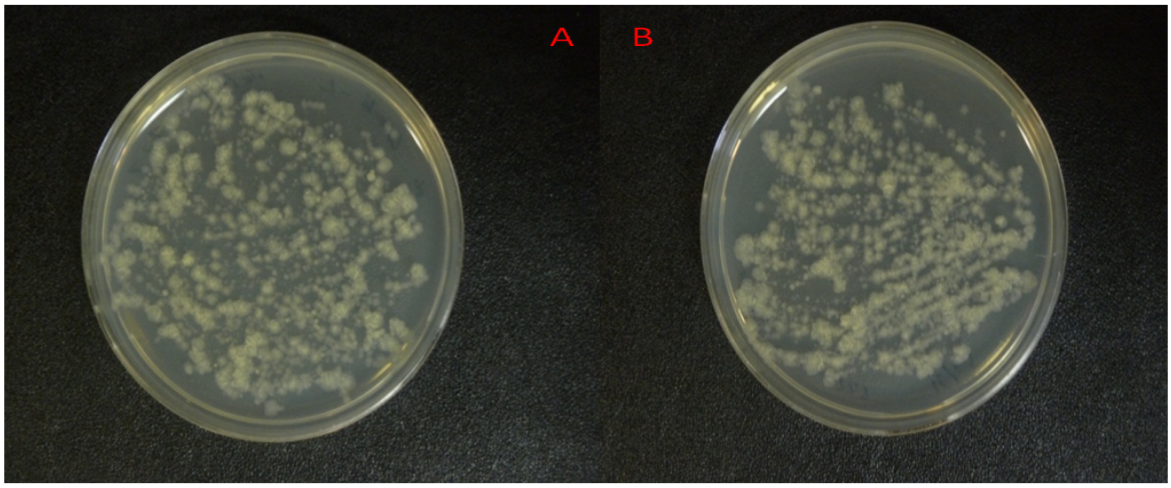

Figure 4.2.2.2: 7H11 agar plates with subcultures from a representative wells of the checkerboard titration.

A: INH 16mg/L and ETH 64 mg/L

B: ETH 64 mg/L

To confirm the observed paradoxical susceptibility pattern for ETH with those isolates that were selected based on the proposed WHO breakpoints (WHO, 4th Edition) MICs were performed with two of the paradoxically susceptible isolates, two *M. tuberculosis* isolates susceptible to INH and ETH, as well as two *M. tuberculosis* isolates resistant to both INH and ETH. The paradoxical effect of both isolates (TF2226 and TF727) was confirmed as shown in figure 4.2.2.3. showing an MIC of 1 mg/L and growth in wells 1 to 4.

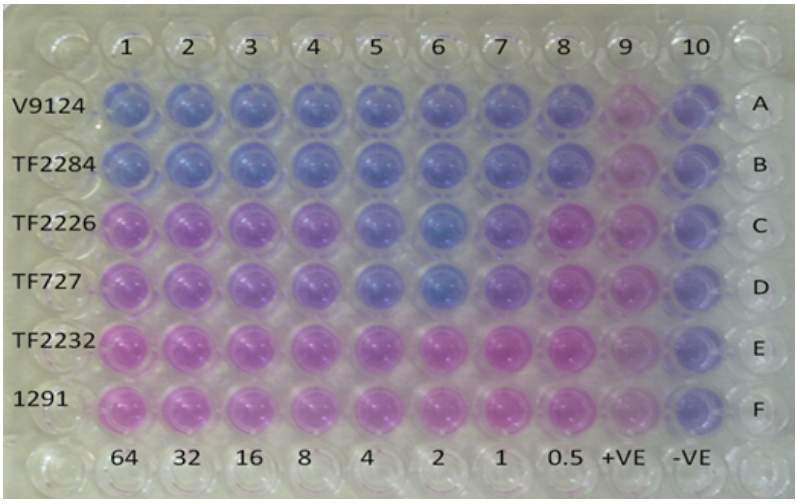

Figure 4.2.2.3: *M. tuberculosis* isolates tested for ETH susceptibility alone.

V9124 and TF2284: susceptible to both INH and ETH

TF2226 and TF727: susceptible to INH; resistant to ETH

TF2232 and 1291: resistant to INH and ETH.

Rows 9 positive control Row 10: negative control

4.3 PCR and DNA Sequencing

4.3.1 PCR of *eth*A, *msh*A and *msh*C genes

No mutations were observed on *eth*A, *msh*A and *msh*C genes. All isolates showing PCR product were sequenced, PCR was repeated for those isolates that did not show a PCR product, where the DNA concentration was adjusted.

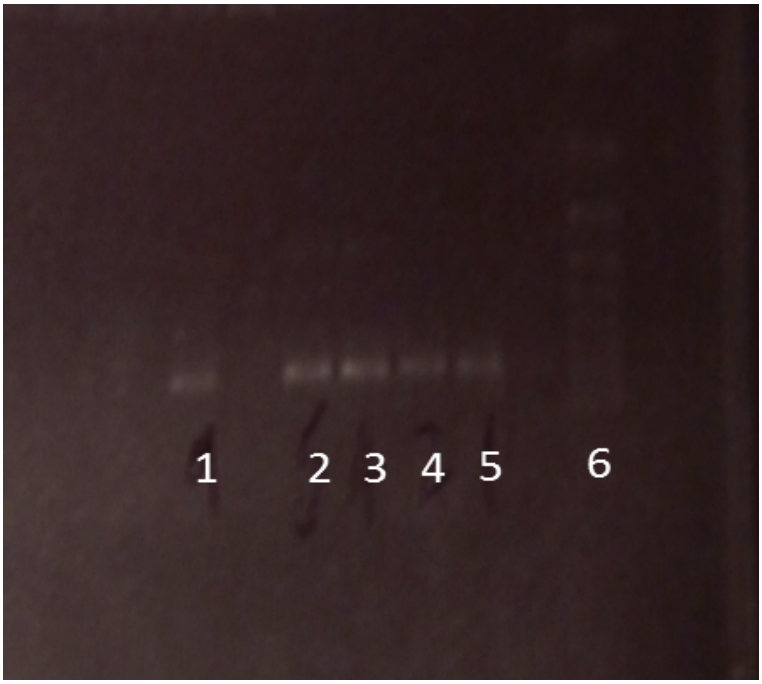

Figure 4.3.1.1: PCR products of the *eth*A gene of *M. tuberculosis* paradoxically susceptible isolates.

Lanes: 1-TF2204, 2-TF1719, 3-TF1091, 4-TF2226, 5- H37Rv (positive control) and 6-Marker 2.

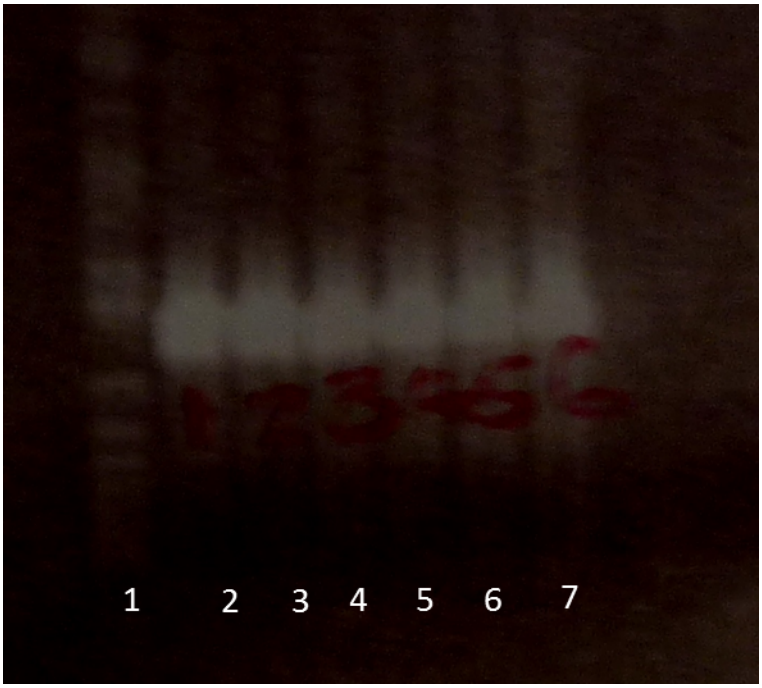

Figure 4.3.1.2: PCR products of the *msh*A gene of *M. tuberculosis* paradoxically susceptible isolates.

Lanes: 1-Marker 2, 2-TF727, 3-TF1091, 4-TF1719, 5-2204, 6-TF2226 and 7-H37Rv (positive control).

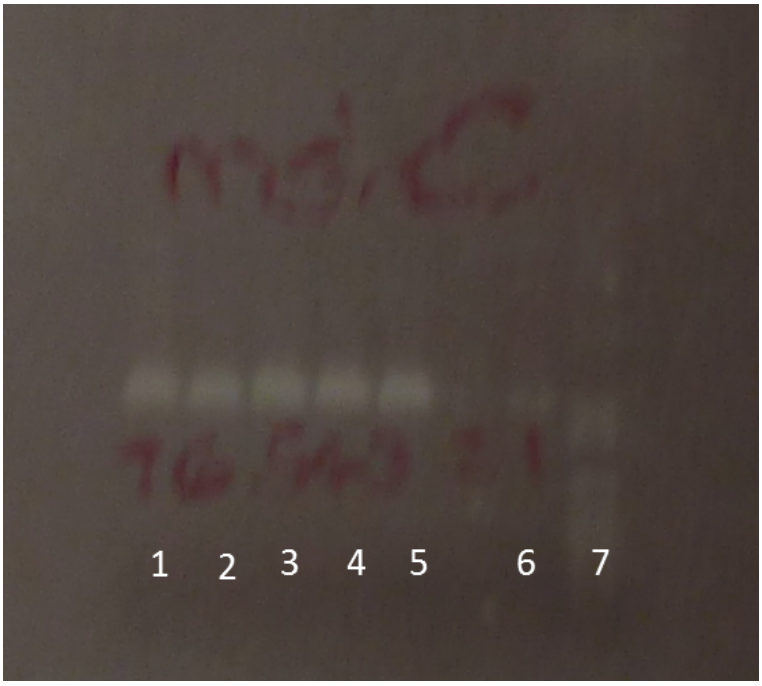

Figure 4.3.1.3: PCR products of the *msh*C gene of *M. tuberculosis* paradoxically susceptible isolates.

Lanes: 1-H37Rv (positive control), 2-TF1400, 3-TF2204, 4-TF1719, 5-TF2226, 6-TF727 and 7-marker 2.

4.3.2 DNA Sequencing

The sequences of TF727 for *eth*A, *msh*C and *msh*C aligned with a wildtype reference sequences (H37Rv) for each of the genes are attached in Annexture 1. The other 6 sequences are similar. Annexture 2 contains the chromatograms generated with the DNA sequences. For DNA sequence analysis, the generated DNA sequences were examined by alignment with reference sequences using Clone Manager 9 Professional Edition software (serial number 873-081-7617), these were examined in conjunction with the generated chromatograms to check if a putative mutation seen from the alignment is a true or false mutation. All sequenced genes for all isolates were found to be wildtype.

Chapter 5: Discussion

Mono-resistance to ethionamide (ETH) in *M. tuberculosis* is rarely reported. This may be due to the fact that ETH resistance is often accompanied with isoniazid (INH) resistance and/or resistance to other drugs [99; 139]. Combined resistance to INH and ETH has a molecular basis in mutations in *inh*A. Resistance of *M. tuberculosis* to ETH is increasing, especially in those African countries where use of this drug is part of the standard regimen for treatment of multi-drug resistant tuberculosis (MDR-TB) [139]. In this study, we determined the MICs for INH and ETH of the selected group of isolates. The selection criteria were susceptibility to 0.1 mg/L of INH and resistance to 2.5 mg/L of ETH. Both drugs were tested in combination to establish whether inhibiting both related drug targets leads to synergism or antagonism. This was done by means of a checkerboard titration.

The MIC determination as well as the checkerboard titration was done by means of broth micro-dilution using a colorimetric growth detection system. The detection methods employed were the MTT and the Alamar Blue (MABA) assays . Although the MTT assay has shown to give good results with other anti-TB drugs, it was deemed to be unsuitable for our study since the reagent reacted with ETH in the absence of bacteria. This may be on the account of oxidative activity of EthA. The MABA has also been shown to be an efficient method of determining MICs for a number of compounds against mycobacteria [3; 140]. This was therefore chosen as an alternative for the MTT assay. In this assay, no interaction with the reagent and any of the two drugs was observed.

Previous studies have shown MICs to be generally lower when tested in liquid media compared to MICs tested on solid media [139; 141; 142]. However, there is not much information on which of the two media used for drug susceptibility testing best represents what takes place *in vivo*.

Combinations of drugs have long been used for the treatment of infections. Such combinations provide a broad spectrum of activity, delay or suppress the emergence of drug resistance and in some situations allow the use of a decreased dose of individual drugs to avoid toxicity [143]. Drug combination regimens are of vital importance in the treatment of a number of infections like malaria, HIV infection and TB, where the use of monotherapy has been observed to select for resistant mutants [3; 144], ultimately resulting in treatment failure. Although combination regimens are the cornerstone of control programmes for susceptible as well as drug resistant TB worldwide, studies on the pharmacodynamics of these drugs in combination are rare.

In contrast, studies on pharmacokinetic interactions between INH as well as ETH with other compounds, including anti-TB drugs, are numerous. INH has been previously reported to negatively interact with other compounds [145]. Among the clinically significant interactions were those with phenytoin [146 – 150], carbamazepine [151; 152] and certain foods [153 – 155]. These interactions may result in elevated levels of drug toxicity and insufficient INH absorption, if special attention is not given to patients taking INH concomitantly with these compounds. INH has been also documented to have pharmacokinetic interactions with other anti-tuberculosis drugs such as para-aminosalicylic acid [156] and rifampicin [157], but these are not of clinical significance [3]. There also were instances where INH was reported to have interactions with antacids [159] anticoagulants [159], benzodiazepines [160] and vitamin D [3], which also poses no significant threat to the patient although precautions must be taken when INH is being co-administered with these compounds.

Absorption of ETH from intestinal tract is almost complete [146], leaving a very small proportion of the drug to be excreted in an unchanged form in the stool [161]. Food and antacids, unlike in INH absorption, appear to have little effect on the absorption of ETH [146] and it has been previously reported that ETH is distributed with ease throughout the body [162].

ETH is given in combination with rifampicin in drug susceptible tuberculous meningitis, although the effect of this combination, as well as that of co-administration of ETH with other drugs is unknown [161]. Thee and colleagues [161] recently conducted a study of ETH serum levels in children given ETH concomitantly with rifampicin over a period of four months. This study revealed no significant pharmacokinetic alterations in ETH, although high variability was documented in children receiving a South African standard oral dose of 15 – 20 mg/kg body weight [161]. This was consistent with studies in adults [146; 147; 163]. Little is known regarding the accumulation of ETH during continuous therapy in children, but no significant clinical interactions between the second-line ETH and first-line drug rifampicin were observed [161]. However this does not exclude the possibility of such reactions.

We report on a pharmacodynamic study on the interaction between ETH and INH *in vitro*. We found concentration dependent antagonism between the two drugs in all our INH susceptible isolates that showed resistance to 2.5 mg/L of ETH. No such antagonistic effect was observed between INH and ETH in the susceptible control strains of *M. tuberculosis*. This suggests that the basis for this phenomenon is not in the composition of the drugs themselves, but may be the result of changes in mycolic acid synthesis pathways in our isolates, suggesting a new mechanism for drug resistance.

Since the standard drug combination of INH, rifampicin, pyrazinamide and ethambutol has shown to be highly effective in the treatment of susceptible TB, it is unlikely that any of these drugs will have antagonistic effect on the others. However, drug combinations for treatment of drug resistant TB are known to be less effective than standard first line treatment. In South Africa, the standard treatment regimen for MDR-TB includes ETH but not INH. Therefore, our findings do not provide an explanation for the observed poorer response to treatment with that regimen as compared to first line treatment. However, the results presented here indicate that testing for antagonistic effect between drugs used in combination for treatment of drug resistant TB may be useful to improve treatment outcomes.

Henderson and colleagues (2008) proposed a model of ETH metabolism (figure 5.1) [164]. ETH is actively metabolized by human FMO2 and mycobacterial EthA to its more reactive species, the S-oxide, and the non-reactive 2-etthyl-4-amidopyridine decomposition product.

Concerns were raised by Palmer *et al* in 2012, following the outcome of their study, where they measured the metabolism and pharmacokinetics of ETH in wild type (WT) mice and compared these with the flavin-containing mono-oxygenase deficient knock-out (KO *fmo1/2/3* null) mice [165]. They found that concentrations of the S-oxide, which is the primary metabolite in ETH metabolism, was greater than that of the parent drug in WT mice. In KO mice, they observed greater ETH concentrations than S-oxide concentrations. Extrapolating their finding to clinical settings, one of their concerns in administering clinically relevant doses of ETH to healthy individuals with normal flavin mono-oxygenase activity (represented by the WT mice), would be chronic oxidative stress due to the S-oxide mediated depletion of glutathione (figure 5.1). On the other hand, accumulation of ETH over

multiple doses in individuals with the *FMO2*2* genotype (represented by the KO mice) was thought to be clinically more relevant. The authors suggest that interactions of ETH with co-administered drugs through competitive inhibition may occur [166]. Such competitive inhibition could aggravate the observed antagonistic effect of ETH on INH in patients. This needs further investigation.

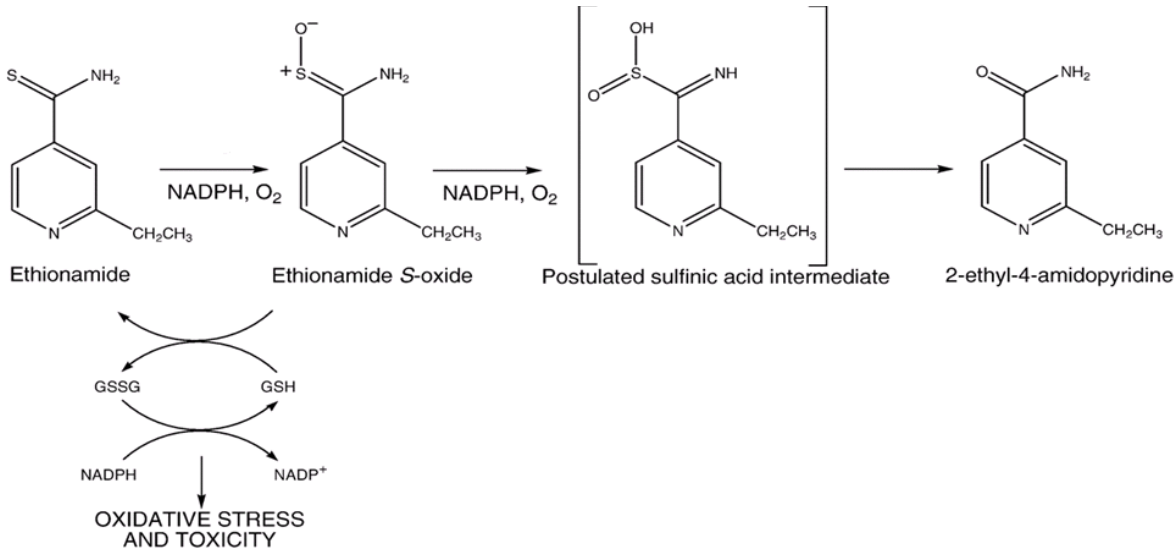

Figure 5.1: Representation of oxidative stress and toxicity in ETH metabolism pathway [165].

Our study revealed a paradoxical effect of ETH on our selected isolates, where growth was observed at high concentrations of ETH but not with lower concentrations. Growth reappeared at drug concentrations below the MIC of the isolate. The growth intensity, as depicted by the colour signal, decreased with decreasing ETH concentrations. This phenomenon was first described by Harry Eagle in 1948, where he observed a decreased activity of penicillin against strains of certain bacterial species at high concentrations [166]. Eagle argued the previous hypothesis posed by Garrod (1945) who reported similar observations. Garrod attributed this retarded activity of penicillin to impurities in the drug

[167]. Eagle found this interpretation inconsistent with his findings and described this zonal inhibition as a 'paradoxical effect' of penicillin. Until a decade ago, such reactions have not been reported in *in vitro* susceptibility testing of anti-tuberculosis drugs. A study by Abate and colleagues in 2002 showed thiacetazone to have a paradoxical effect on certain strains of *M. tuberculosis.* They observed this drug to have greater inhibiting activity at lower concentrations as opposed to higher concentrations [168]. Thiacetazone is not used in South Africa in first line treatment of TB, however it may still be considered for the control of drug resistant TB [168].

The paradoxical effect of ETH and antagonism between ETH and INH was confirmed by the presence of growth on Middlebrook 7H11 plates, following sub-culturing of our isolates from the wells with the highest concentrations of ETH alone and in combination with INH, at the highest concentrations. This eliminates the possibility that a reaction between ETH and alamar blue is the cause of the observed colour change. It can be concluded that both the paradoxical effect of ETH and the antagonistic reaction between ETH and INH in our isolates does not result from mutations found in *eth*A*, msh*A or *msh*C genes, which are commonly implicated in high level resistance of isolates of *M. tuberculosis* to ETH [41; 169]. Further investigations have to be done to elucidate the mechanism by which both these phenomena occur in our isolates.

The proposed WHO resistance breakpoint for ETH in liquid media is 2.5 to 5 mg/L and on solid agar media 5 to 10 mg/L. We selected isolates that showed mono-resistance to ETH using these breakpoints. However, this resulted in a wrong selection since the true MIC in of these isolates in liquid media was shown to be 1 mg/L. Therefore these isolates are fully

susceptible which is corroborated by the absence of mutations in the *msh* genes and *eth*A. This error is based on the paradoxical phenomenon. To avoid false reporting of ETH resistance, tests using lower concentrations need to be included. Whether this paradoxical effect has clinical significance also needs further exploration.

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Annexure 1

DNA sequences of *eth*A, *msh*A and *msh*C genes, aligned with reference sequences, for TF727

***Eth*A: TF727**

Region 1

```
Homology Block:  Percent Matches 95   Score 784   Length 880
                 Mol 1  10 to 889,  Mol 2  1400 to 2280

8919_G02_BJ-6136     10 cgcgcggtgcgccggcccctaggcagcgaa-cctgactggccgcggaggtggtcaccctg
i-ethA region      1400 ............................g............................

8919_G02_BJ-6136     69 gcagcttactacgtgtcgatagtgtcgacatctcgttgacggcctcaacattacgttgat
i-ethA region      1460 .............................................g............

8919_G02_BJ-6136    129 agcgtggatccatgaccgagcacctcgacgttgtcatcgtgggcgctggaatctccggtg
i-ethA region      1520 ............................................................

8919_G02_BJ-6136    189 tcagcgcggcctggcacctgcaggaccgttgcccgaccaagagctacgccatcctggaaa
i-ethA region      1580 ............................................................

8919_G02_BJ-6136    249 agcgggaatccatgggcggcacctgggatttgttccgttatcccggaattcgctccgact
i-ethA region      1640 ............................................................

8919_G02_BJ-6136    309 ccgacatgtacacgctaggtttccgattccgtccctggaccggacggcaggcgatcgccg
i-ethA region      1700 ............................................................

8919_G02_BJ-6136    369 acggcaagcccatcctcgagtacctcaagagcaccgcggccatgtatggaatcaacaggc
i-ethA region      1760 ......................g.............................g......

8919_G02_BJ-6136    429 atatccggttccaccacaaggtgatcagtgccgattggtctaccgcggaaaaccgctgga
i-ethA region      1820 ........................................g...................

8919_G02_BJ-6136    489 ccgttcacatccaaagccacggcacgctcaccgccctcacctgcgaattcctctttctgt
i-ethA region      1880 ..............................g.............................

8919_G02_BJ-6136    549 gcagcggctactacaactacgaatagggctactcgccgagattccccggctcggaggatt
i-ethA region      1940 ......................cg.....................g..............

8919_G02_BJ-6136    609 tcctccggcctatcatccatccgcggcactggcccgaggacctcgactaccacgctaaaa
i-ethA region      2000 ..g..g....g.............a.........................g......g.

8919_G02_BJ-6136    669 acatcgtcgtgatcggtagtggcgcaacggcagtcacgctcgtgccggcgctggctgact
i-ethA region      2060 ................c..............g........................g....

8919_G02_BJ-6136    729 caggtggcaagcacgtcaggatgctgccatgctcacccagctacattggtgtc-ttccca
i-ethA region      2120 .g..c.c...........c........agc.........c.....-.c.....gcag...

8919_G02_BJ-6136    788 gaccgggacggcatcgccgagaggctcaaccgctggctgcccgagaccatggtctacacc
i-ethA region      2179 ......................a..................g..........c.......

8919_G02_BJ-6136    848 gcggtacgggggaacaacgtgctgagcttcgcggccatgtac
i-ethA region      2239 .........t....g.........c..cag......g.....
```

Region 2

```
Homology Block:  Percent Matches 99   Score 963   Length 970
                 Mol 1  4 to 973,  Mol 2  1787 to 2759

8919_G03_BJ-6136      4 agag-a-cgcgg-catgtatggaatcgacaggcatatccggttccaccacaaggtgatca
i-ethA region      1787 ....c.c.....c.............................................

8919_G03_BJ-6136     61 gtgccgattggtcgaccgcggaaaaccgctggaccgttcacatccaaagccacggcacgc
i-ethA region      1847 ............................................................

8919_G03_BJ-6136    121 tcagcgccctcacctgcgaattcctctttctgtgcagcggctactacaactacgacgagg
i-ethA region      1907 ............................................................

8919_G03_BJ-6136    181 gctactcgccgagattcgccggctcggaggatttcgtcgggccgatcatccatccgcagc
i-ethA region      1967 ............................................................

8919_G03_BJ-6136    241 actggcccgaggacctcgactacgacgctaagaacatcgtcgtgatcggcagtggcgcaa
i-ethA region      2027 ............................................................

8919_G03_BJ-6136    301 cggcggtcacgctcgtgccggcgctggcggactcgggcgccaagcacgtcacgatgctgc
i-ethA region      2087 ............................................................

8919_G03_BJ-6136    361 agcgctcacccacctacatcgtgtcgcagccagaccgggacggcatcgccgagaagctca
i-ethA region      2147 ............................................................

8919_G03_BJ-6136    421 accgctggctgccggagaccatggcctacaccgcggtacggtggaagaacgtgctgcgcc
i-ethA region      2207 ............................................................

8919_G03_BJ-6136    481 aggcggccgtgtacagcgcctgccagaagtggccacggcgcatgcggaagatgttcctga
i-ethA region      2267 ............................................................

8919_G03_BJ-6136    541 gcctgatccagcgccagctacccgaggggtacgacgtgcgaaagcacttcggcccgcact
i-ethA region      2327 ............................................................

8919_G03_BJ-6136    601 acaacccctgggaccagcgattgtgcttggtgcccaacggcgacctgttccgggccattc
i-ethA region      2387 ............................................................

8919_G03_BJ-6136    661 gtcacgggaaggtcgaggtggtgaccgacaccattgaacggttcaccgcgaccggaatcc
i-ethA region      2447 ............................................................

8919_G03_BJ-6136    721 ggctgaactcaggtcgcgaactgccggctgacatcatcattaccgcaacggggttgaacc
i-ethA region      2507 ............................................................

8919_G03_BJ-6136    781 tgcagctttttggtggggcgacggcgactatcgacggacaacaagtggacatcaccacga
i-ethA region      2567 ............................................................

8919_G03_BJ-6136    841 cgatggcctacaagggcatgatgctttccggcatccccaacatggcctacacggttggct
i-ethA region      2627 ............................................................

8919_G03_BJ-6136    901 acaccaatgcctcctggacgctgaacgccgacctggtgtcggagtttgtctgtcgcttgt
i-ethA region      2687 ........................g...................................
```

Region 3

```
Homology Block:  Percent Matches 99   Score 889   Length 893
                 Mol 1  7 to 899,  Mol 2  2196 to 3092


8919_G04_BJ-6136      7 cgag-agctc-accgctggctgccggagaccatggcctacaccgcggtacggtggaagaa
i-ethA region      2196 ....a.....a.............................................

8919_G04_BJ-6136     65 cgtgctgcgccaggcggccgtgtacagcgcctgccagaagtggccacggcgcatgcggaa
i-ethA region      2256 ............................................................

8919_G04_BJ-6136    125 gatgttcctgagcctgatccagcgccagctacccgaggggtacgacgtgcgaaagcactt
i-ethA region      2316 ............................................................

8919_G04_BJ-6136    185 cggcccgcactacaacccctgggaccagcgattgtgcttggtgcccaacggcgacctgtt
i-ethA region      2376 ............................................................

8919_G04_BJ-6136    245 ccgggccattcgtcacgggaaggtcgaggtggtgaccgacaccattgaacggttcaccgc
i-ethA region      2436 ............................................................

8919_G04_BJ-6136    305 gaccggaatccggctgaactcaggtcgcgaactgccggctgacatcatcattaccgcaac
i-ethA region      2496 ............................................................

8919_G04_BJ-6136    365 ggggttgaacctgcagctttttggtggggcgacggcgactatcgacggacaacaagtgga
i-ethA region      2556 ............................................................

8919_G04_BJ-6136    425 catcaccacgacgatggcctacaagggcatgatgctttccggcatccccaacatggccta
i-ethA region      2616 ............................................................

8919_G04_BJ-6136    485 cacggttggctacaccaatgcctcctggacgctgaaggccgacctggtgtcggagtttgt
i-ethA region      2676 ............................................................

8919_G04_BJ-6136    545 ctgtcgcttgttgaattacatggacgacaacggttttgacaccgtggtcgtcgagcgacc
i-ethA region      2736 ............................................................

8919_G04_BJ-6136    605 gggctcagatgtcgaagagcggcccttcatggagttcaccccaggttacgtgctgcgctc
i-ethA region      2796 ............................................................

8919_G04_BJ-6136    665 gctggacgagctgcccaagcagggttcgcgtacaccgtggcgcctgaatcagaactacct
i-ethA region      2856 ............................................................

8919_G04_BJ-6136    725 acgtgacatccggctcatccggcgcggcaagatcgacgacgagggtctgcggttcgccaa
i-ethA region      2916 ............................................................

8919_G04_BJ-6136    785 aaggcctgccccggtgggggtttagctttagcgacggtttagcgccggtttaggccatag
i-ethA region      2976 ............................................................

8919_G04_BJ-6136    845 tcagacgacgatgatgccgtcgtcgtcgctgtaggcgatatcgcccgg-acg-atgt
i-ethA region      3036 ..............................................a...a....
```

Region 4

```
Homology Block:  Percent Matches 99   Score 482   Length 485
                 Mol 1  8 to 492,  Mol 2  2618 to 3102


8919_G05_BJ-6136      8 tcaccacgacgatggcctacaagggcatgatgctttccggcatccccaacatggcctaca
i-ethA region      2618 ............................................................

8919_G05_BJ-6136     68 cggttggctacaccaatgcctcctggacgctgaaggccgacctggtgtcggagtttgtct
i-ethA region      2678 ............................................................

8919_G05_BJ-6136    128 gtcgcttgttgaattacatggacgacaacggttttgacaccgtggtcgtcgagcgaccgg
i-ethA region      2738 ............................................................

8919_G05_BJ-6136    188 gctcagatgtcgaagagcggcccttcatggagttcaccccaggttacgtgctgcgctcgc
i-ethA region      2798 ............................................................

8919_G05_BJ-6136    248 tggacgagctgcccaagcagggttcgcgtacaccgtggcgcctgaatcagaactacctac
i-ethA region      2858 ............................................................

8919_G05_BJ-6136    308 gtgacatccggctcatccggcgcggcaagatcgacgacgagggtctgcggttcgccaaaa
i-ethA region      2918 ............................................................

8919_G05_BJ-6136    368 ggcctgccccggtgggggtttagctttagcgacggtttagcgccggtttaggccatagtc
i-ethA region      2978 ............................................................

8919_G05_BJ-6136    428 agacgacgatgatgccgtcgtcgtcgctgtaggcgatatcgcccggaacgaatgtacccc
i-ethA region      3038 ..........................................................ca...

8919_G05_BJ-6136    488 cgccc
i-ethA region      3098 .....
```

***msh*A: TF727**

Region 1

```
Homology Block:  Percent Matches 96   Score 867   Length 952
                 Mol 1  6 to 957,  Mol 2  575801 to 576751


8944_B01_BJ-6159      6 ctcgcactactggctgtcgggtcaggtcggctggctggcgcgcgaccgctgggcggtgcc
MTBH37RV         575801 ..........................................................

8944_B01_BJ-6159     66 gttggtgcacaccgcacacacgctggccgccgtgaagaacgcggcactggccgacggcga
MTBH37RV         575861 ..........................................................

8944_B01_BJ-6159    126 cggacccgagccgccgctgcgtacggtcggggagcagcaggtcgtcgacgaggcggatcg
MTBH37RV         575921 ..........................................................

8944_B01_BJ-6159    186 gttgatcgtcaacaccgacgatgaagccaggcaagtgatttcgcttcatggtgccgatcc
MTBH37RV         575981 ..........................................................

8944_B01_BJ-6159    246 ggcacgaatcgacgtggtccatcccggtgtcgatctggacgtgttccgcccgggtgatcg
MTBH37RV         576041 ..........................................................

8944_B01_BJ-6159    306 gcgcgcggcccgggccgcgctaggactaccagttgacgagcgcgtggtggccttcgtcgg
MTBH37RV         576101 ..........................................................

8944_B01_BJ-6159    366 acgcatccagccgctgaaggcacccgacattgtgctgcgtgcggccgccaagttgcccgg
MTBH37RV         576161 ..........................................................

8944_B01_BJ-6159    426 ggtgcgcatcatcgtggccggcggaccgtcgggcagcggtctggcttcaccggacggact
MTBH37RV         576221 ..........................................................

8944_B01_BJ-6159    486 ggtccggctcgccgacgaactgggcatctctgcacgggtgacgtttctgccgccgcagtc
MTBH37RV         576281 ..........................................................

8944_B01_BJ-6159    546 ccacacggatctggccaccttgtttcgggcggcggacctggttgcggtgccgagctactc
MTBH37RV         576341 ..........................................................

8944_B01_BJ-6159    606 cgagtcgttcggcctggttgctgtggaggcccaagcgtgcggcacaccggtggtggccgc
MTBH37RV         576401 ..........................................................

8944_B01_BJ-6159    666 ggcggtgggcgggctgcccgtcgcggtgcgcgacgggatcaccggcaccctggtgtccgg
MTBH37RV         576461 ..........................................................

8944_B01_BJ-6159    726 gcacgaggtcggtcagtgggccgacgccatcgatcacctgctgcggttgtgtgccgggcc
MTBH37RV         576521 ..........................................................

8944_B01_BJ-6159    786 acggggacgggtgatgagccggggcggccgcacggcacgccgccacgttctcgtgggaga
MTBH37RV         576581 ....................-.......g.............................

8944_B01_BJ-6159    846 accccaccgaggctctgatgg-caaatatcgggcgtgcgatcgttgagtac-accccgag
MTBH37RV         576640 ..a.......c..g...t...c..gt....-............gc......a..g....-

8944_B01_BJ-6159    904 gggctagtgtcgggcgagcgaggtgataaagacgttggtatcgatgggcaagcc
MTBH37RV         576698 .c..c..c.c....gcg...........tc.gacc.....g..g..........
```

Region 2

```
Homology Block:  Percent Matches 99   Score 953   Length 958
                 Mol 1  3 to 960,  Mol 2  576285 to 575325 C


8944_B02_BJ-6159      3 ggacgagt-cgt-cggtg-agccagaccgctgcccgacggtccgccggccacgatgatgc
MTBH37RV         576285 ....c...c...c.....a.....................................

8944_B02_BJ-6159     60 gcaccccgggcaacttggcggccgcacgcagcacaatgtcgggtgccttcagcggctgga
MTBH37RV         576225 ............................................................

8944_B02_BJ-6159    120 tgcgtccgacgaaggccaccacgcgctcgtcaactggtagtcctagcgcggcccgggccg
MTBH37RV         576165 ............................................................

8944_B02_BJ-6159    180 cgcgccgatcacccgggcggaacacgtccagatcgacaccgggatggaccacgtcgattc
MTBH37RV         576105 ............................................................

8944_B02_BJ-6159    240 gtgccggatcggcaccatgaagcgaaatcacttgcctggcttcatcgtcggtgttgacga
MTBH37RV         576045 ............................................................

8944_B02_BJ-6159    300 tcaaccgatccgcctcgtcgacgacctgctgctccccgaccgtacgcagcggcggctcgg
MTBH37RV         575985 ............................................................

8944_B02_BJ-6159    360 gtccgtcgccgtcggccagtgccgcgttcttcacggcggccagcgtgtgtgcggtgtgca
MTBH37RV         575925 ............................................................

8944_B02_BJ-6159    420 ccaacggcaccgcccagcggtcgcgcgccagccagccgacctgacccgacagccagtagt
MTBH37RV         575865 ............................................................

8944_B02_BJ-6159    480 gcgagtgcacgatgtcgtagtaacccggttcgtggaccgcctcggcgcgcagcaccccgg
MTBH37RV         575805 ............................................................

8944_B02_BJ-6159    540 cggcgaacgcacaaagctgggtgggcaggtcgtacttgtccaaaccctcgaagggccccg
MTBH37RV         575745 ............................................................

8944_B02_BJ-6159    600 ccaccacgttgcgcaccagcaccccgggtgccacccgcaccaccggtggatctgccgatg
MTBH37RV         575685 ............................................................

8944_B02_BJ-6159    660 cggtggcccgggtgaagatctccacctcgatgccccgacgggccaggtgcagcgcacttt
MTBH37RV         575625 ............................................................

8944_B02_BJ-6159    720 gcagcatgtagacgttcatgccgccggcgtcaccggtgcccggctgtgccagcggtgagg
MTBH37RV         575565 ............................................................

8944_B02_BJ-6159    780 tgtgcaccgccagcagcgcaacccggcgcgggtcgtctgctgccgaaacattccgattgg
MTBH37RV         575505 ............................................................

8944_B02_BJ-6159    840 atggcccggatgggccgcgcgagcgggtggcaccctcgccgcggaccggacggcgctggg
MTBH37RV         575445 ............................................................

8944_B02_BJ-6159    900 cgatcaaccctgaaccgtcatcgtgccgcacacctgccatccttgcaggaaccgaagtga
MTBH37RV         575385 ............................................................
```

Region 3

```
Homology Block:  Percent Matches 99   Score 842   Length 861
                 Mol 1  11 to 871,  Mol 2  576168 to 577028


8944_B03_BJ-6159     11 cagccgctg-atgcacccgacattgtgctgcgtgcggccgccaagttgcccggggtgcgc
MTBH37RV         576168 .........a.g.....................................................

8944_B03_BJ-6159     70 atcatcgtggccggcggaccgtcgggcagcggtctggcttcaccggacggactggtccgg
MTBH37RV         576228 .........a.g.....................................................

8944_B03_BJ-6159    130 ctcgccgacgaactgggcatctctgcacgggtgacgtttctgccgccgcagtcccacacg
MTBH37RV         576288 .........a.g.....................................................

8944_B03_BJ-6159    190 gatctggccaccttgtttcgggcggcggacctggttgcggtgccgagctactccgagtcg
MTBH37RV         576348 .........a.g.....................................................

8944_B03_BJ-6159    250 ttcggcctggttgctgtggaggcccaagcgtgcggcacaccggtggtggccgcggcggtg
MTBH37RV         576408 .........a.g.....................................................

8944_B03_BJ-6159    310 ggcgggctgcccgtcgcggtgcgcgacgggatcaccggcaccctggtgtccgggcacgag
MTBH37RV         576468 .........a.g.....................................................

8944_B03_BJ-6159    370 gtcggtcagtgggccgacgccatcgatcacctgctgcggttgtgtgccgggccacgggga
MTBH37RV         576528 .........a.g.....................................................

8944_B03_BJ-6159    430 cgggtgatgagccgggcggcggcacggcacgccgccacgttctcgtgggagaacaccacc
MTBH37RV         576588 .........a.g.....................................................

8944_B03_BJ-6159    490 gacgcgctgttggccagttatcggcgtgcgatcggcgagtacaacgccgagcgccagcgc
MTBH37RV         576648 .........a.g.....................................................

8944_B03_BJ-6159    550 cggggcggcgaggtgatatcggacctggtagcggtgggcaagccccgccactggacgccg
MTBH37RV         576708 .........a.g.....................................................

8944_B03_BJ-6159    610 cgtcgcggggtgggcgcgtgacttcctccttgccgaccgtgcaacgtgtgatccagaatg
MTBH37RV         576768 .........a.g.....................................................

8944_B03_BJ-6159    670 cgctcgaggtcagccagctgaagtactcccaacacccccgcccgggcggggcgccgcccg
MTBH37RV         576828 .........a.g.....................................................

8944_B03_BJ-6159    730 cgctgatcgtcgagctgccgggcgaacgcaagctcaagatcaacagcatcctgagcgtcg
MTBH37RV         576888 .........a.g.....................................c.............

8944_B03_BJ-6159    790 gcgagcattcggtgcgtgtcgaggcgttcgtgtgtcgcaagcctgacgagaaccgcgaac
MTBH37RV         576948 .........a.g.....................................................g

8944_B03_BJ-6159    850 acgtatacctgtttactgctgc
MTBH37RV         577008 .........-.g..c.......
```

*msh*C: TF727

Region 1

```
Homology Block:  Percent Matches 99   Score 907   Length 910
                 Mol 1  8 to 917,  Mol 2  2391177 to 2392089


8953_G03_BJ-6164      8 agcgatgt-atggccg--aacgtaccgccgcgccggctacaggtccaccccgagcagggc
MTBH37RV         239117 ........c.......aa.........................................

8953_G03_BJ-6164     65 atcgatcgccgtcgccaccaacttcggcgccccggcatcgtggccgccgtactccaccgc
MTBH37RV         239123 ............................................................

8953_G03_BJ-6164    125 atcggtgacccaaccatccagtgcggcaatcgctttgggcgtatcgagatcgtcggccag
MTBH37RV         239129 ............................................................

8953_G03_BJ-6164    185 gtagcggcgcacccgagcgacaacgtcaactgcggccggaccggcgggaagtgcggttgc
MTBH37RV         239135 ............................................................

8953_G03_BJ-6164    245 ggtgcgccaacggtgcagccgggcggtcgcctcgtcaagcacctgctggctccagaaccg
MTBH37RV         239141 ............................................................

8953_G03_BJ-6164    305 atcggctcggtagtgtccggcgagcaaacccagccgaaccgccgatggctcaacgtcctg
MTBH37RV         239147 ............................................................

8953_G03_BJ-6164    365 cgcacgcagcgccgacaccagcacgaggttgccgcggctctttgacatcttgtgcccgtc
MTBH37RV         239153 ............................................................

8953_G03_BJ-6164    425 ccagccgatcatcccggcatgcacgtagtgccgcgcgaatcgccgttcgccgctgacaca
MTBH37RV         239159 ............................................................

8953_G03_BJ-6164    485 ttcggcgtgcgcagcggtgaactcgtggtgcggaaagatcagatcgctaccaccgccctg
MTBH37RV         239165 ............................................................

8953_G03_BJ-6164    545 gatgtcgaggccgcttccgatacgactgagcgcgatggctgcgcactcgacatgccagcc
MTBH37RV         239171 ............................................................

8953_G03_BJ-6164    605 tggccggccaggcccgaacggggacggccagctgggctcaccgggccgcgcggcccgcca
MTBH37RV         239177 ............................................................

8953_G03_BJ-6164    665 caacaacgcgtcgagttcgtcgctcttgccggggcgccgcggatcgccgccacgttcctc
MTBH37RV         239183 ............................................................

8953_G03_BJ-6164    725 gcacagccgcagcatggtgtcacggtcataccctgactcgtagccgaactgcagggtggc
MTBH37RV         239189 ............................................................

8953_G03_BJ-6164    785 gtcagcgcggaagtagatgtcctggtactctcccatttcccggtctatgacataggccgc
MTBH37RV         239195 ............................................................

8953_G03_BJ-6164    845 cccgcacgccagcatttttttcgatgagctcgaccatttcagcaatcgcttcggtggcccc
MTBH37RV         239201 ............................................................

8953_G03_BJ-6164    905 cacgtagtcttgc
MTBH37RV         239207 .............
```

Region 2

```
Homology Block:  Percent Matches 99   Score 937   Length 939
                 Mol 1  11 to 949,  Mol 2  2392080 to 2391140 C


8953_G04_BJ-6164     11 cgtggggg-caccgaagcgattgctg-aatggtcgagctcatcgaaaaaatgctggcgtg
MTBH37RV         239208 ........c..................a.................................

8953_G04_BJ-6164     69 cggggcggcctatgtcatagaccgggaaatgggagagtaccaggacatctacttccgcgc
MTBH37RV         239202 ............................................................

8953_G04_BJ-6164    129 tgacgccaccctgcagttcggctacgagtcagggtatgaccgtgacaccatgctgcggct
MTBH37RV         239196 ............................................................

8953_G04_BJ-6164    189 gtgcgaggaacgtggcggcgatccgcggcgccccggcaagagcgacgaactcgacgcgtt
MTBH37RV         239190 ............................................................

8953_G04_BJ-6164    249 gttgtggcgggccgcgcggcccggtgagcccagctggccgtccccgttcgggcctggccg
MTBH37RV         239184 ............................................................

8953_G04_BJ-6164    309 gccaggctggcatgtcgagtgcgcagccatcgcgctcagtcgtatcggaagcggcctcga
MTBH37RV         239178 ............................................................

8953_G04_BJ-6164    369 catccagggcggtggtagcgatctgatctttccgcaccacgagttcaccgctgcgcacgc
MTBH37RV         239172 ............................................................

8953_G04_BJ-6164    429 cgaatgtgtcagcggcgaacggcgattcgcgcggcactacgtgcatgccgggatgatcgg
MTBH37RV         239166 ............................................................

8953_G04_BJ-6164    489 ctgggacgggcacaagatgtcaaagagccgcggcaacctcgtgctggtgtcggcgctgcg
MTBH37RV         239160 ............................................................

8953_G04_BJ-6164    549 tgcgcaggacgttgagccatcggcggttcggctgggtttgctcgccggacactaccgagc
MTBH37RV         239154 ............................................................

8953_G04_BJ-6164    609 cgatcggttctggagccagcaggtgcttgacgaggcgaccgcccggctgcaccgttggcg
MTBH37RV         239148 ............................................................

8953_G04_BJ-6164    669 caccgcaaccgcacttcccgccggtccggccgcagttgacgttgtcgctcgggtgcgccg
MTBH37RV         239142 ............................................................

8953_G04_BJ-6164    729 ctacctggccgacgatctcgatacgcccaaagcgattgccgcactggatggttgggtcac
MTBH37RV         239136 ............................................................

8953_G04_BJ-6164    789 cgatgcggtggagtacggcggccacgatgccggggcgccgaagttggtggcgacggcgat
MTBH37RV         239130 ............................................................

8953_G04_BJ-6164    849 cgatgccctgctcggggtggacctgtagccggcgcggcggtacgttttcggccatgacat
MTBH37RV         239124 ............................................................

8953_G04_BJ-6164    909 cgctgcaaggcaaggtcgtcttcattaccggtgctgcccgg
MTBH37RV         239118 ....................................
```

Region 3

```
Homology Block:  Percent Matches 99   Score 603   Length 613
                 Mol 1  7 to 619,  Mol 2  2391663 to 2391052 C


8953_G05_BJ-6164      7 cgccgcatgtgtcagcggcg-acggcgattcgcgcggcactacgtgcatgccgggatgat
MTBH37RV         239166 .....a.............a.....................................

8953_G05_BJ-6164     66 cggctgggacgggcacaagatgtcaaagagccgcggcaacctcgtgctggtgtcggcgct
MTBH37RV         239160 ............................................................

8953_G05_BJ-6164    126 gcgtgcgcaggacgttgagccatcggcggttcggctgggtttgctcgccggacactaccg
MTBH37RV         239154 ............................................................

8953_G05_BJ-6164    186 agccgatcggttctggagccagcaggtgcttgacgaggcgaccgcccggctgcaccgttg
MTBH37RV         239148 ............................................................

8953_G05_BJ-6164    246 gcgcaccgcaaccgcacttcccgccggtccggccgcagttgacgttgtcgctcgggtgcg
MTBH37RV         239142 ............................................................

8953_G05_BJ-6164    306 ccgctacctggccgacgatctcgatacgcccaaagcgattgccgcactggatggttgggt
MTBH37RV         239136 ............................................................

8953_G05_BJ-6164    366 caccgatgcggtggagtacggcggccacgatgccggggcgccgaagttggtggcgacggc
MTBH37RV         239130 ............................................................

8953_G05_BJ-6164    426 gatcgatgccctgctcggggtggacctgtagccggcgcggcggtacgttttcggccatga
MTBH37RV         239124 ............................................................

8953_G05_BJ-6164    486 catcgctgcaaggcaaggtcgtcttcattaccggtgctgcccggggaatcggggctgagg
MTBH37RV         239118 ............................................................

8953_G05_BJ-6164    546 tcgcccgtcggctgcacaacaagggcgccaaactggtgctgaccgacctgagcaaatcag
MTBH37RV         239112 ............................................................

8953_G05_BJ-6164    606 actgggggcggtga
MTBH37RV         239106 .--.ct........
```

Annexure 2

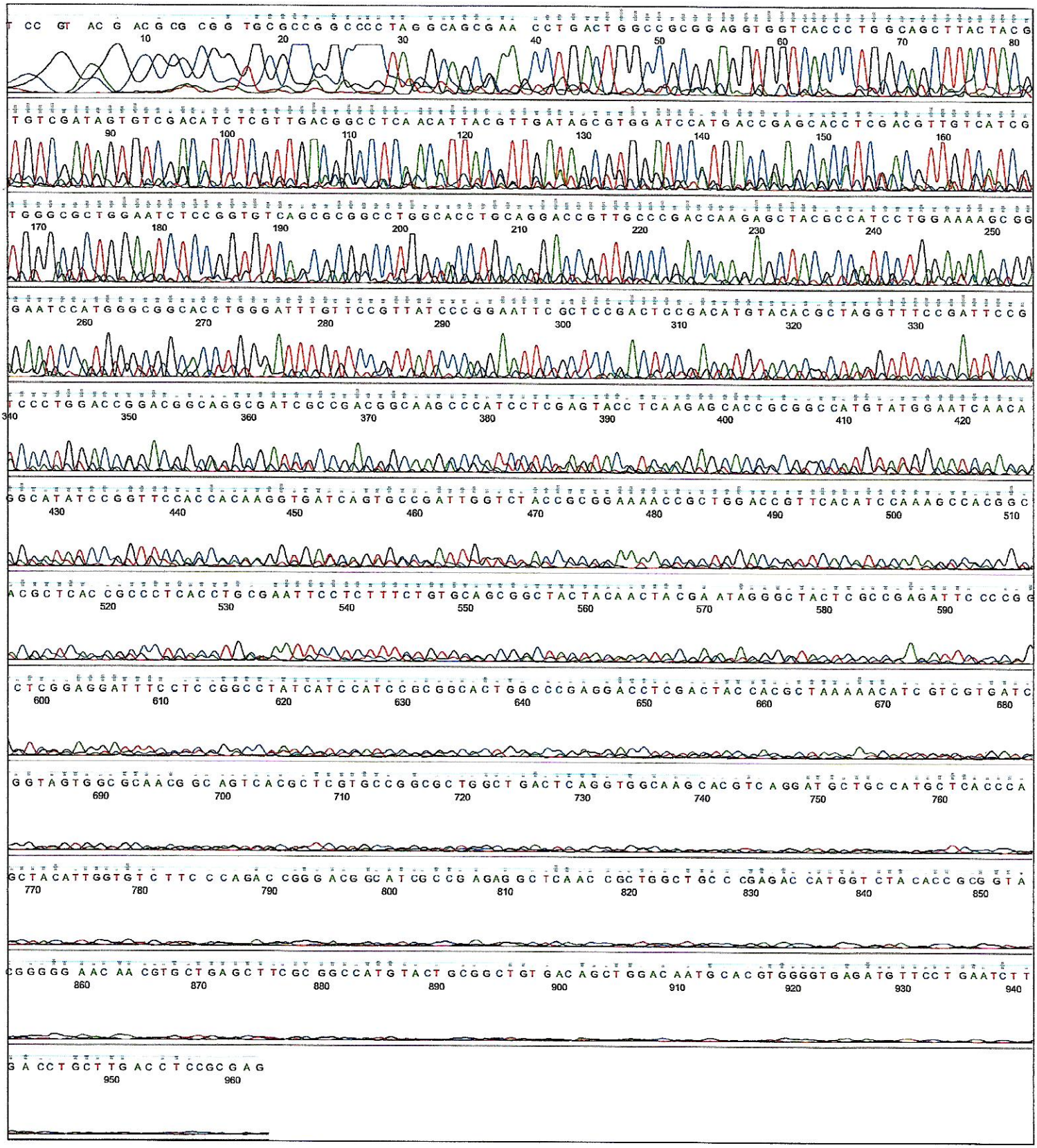
DNA sequence chromatograms of *Mycobacterium tuberculosis ethA*, *mshA* and *mshC* genes for TF727

File: TF727 EthA REGION1.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-613615
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.2686
Comment: VILCHEZE/1/EHTA11

Signal Strengths: A = 316, C = 410, G = 601, T = 300
Lane/Cap#: 2
Matrix: n/a
Direction: Native

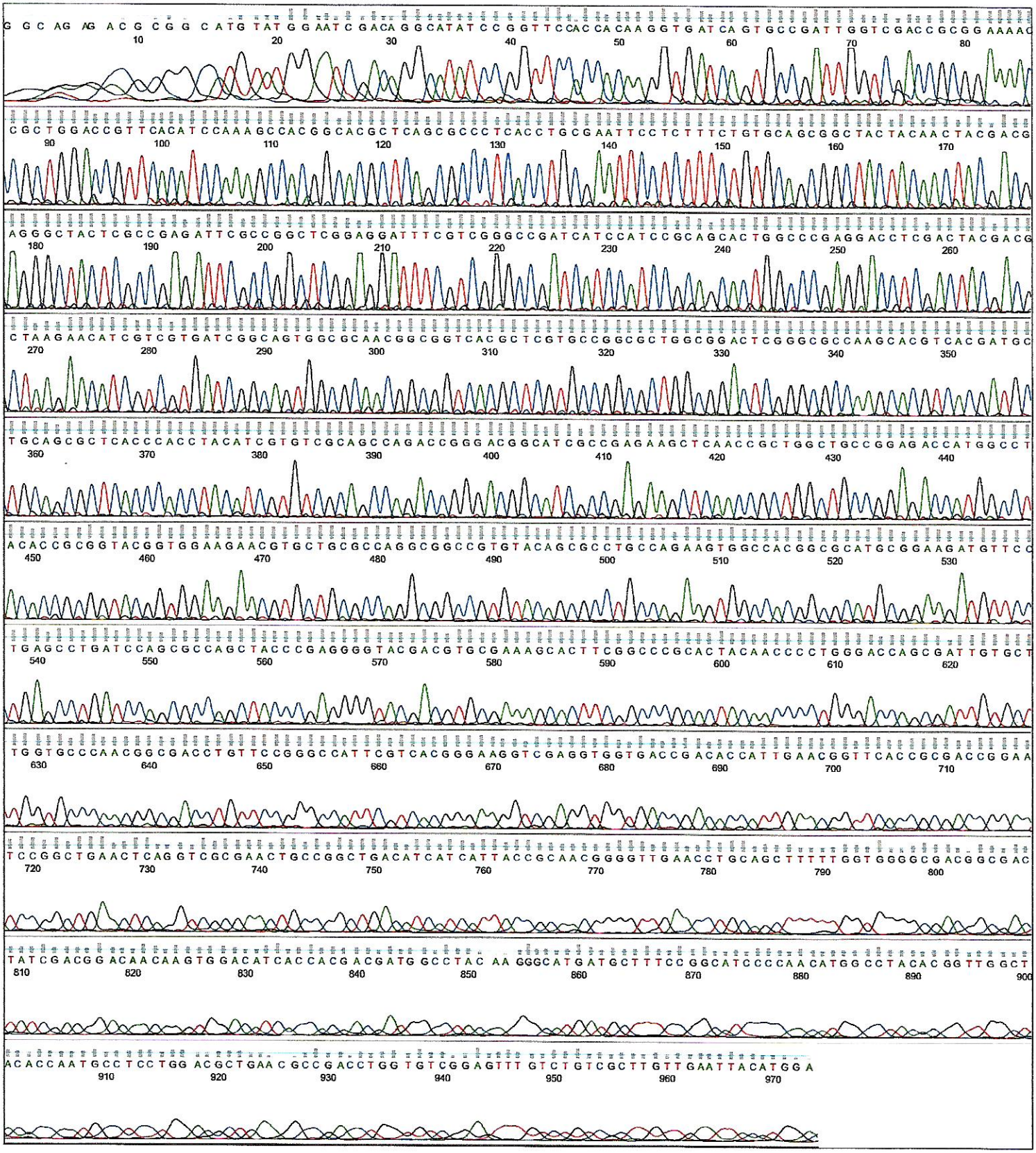


File: TF727 EthA REGION2.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-613616
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.2167
Comment: VILCHEZE/2/EHTA12

Signal Strengths: A = 1084, C = 1456, G = 1834, T = 881
Lane/Cap#: 10
Matrix: n/a
Direction: Native

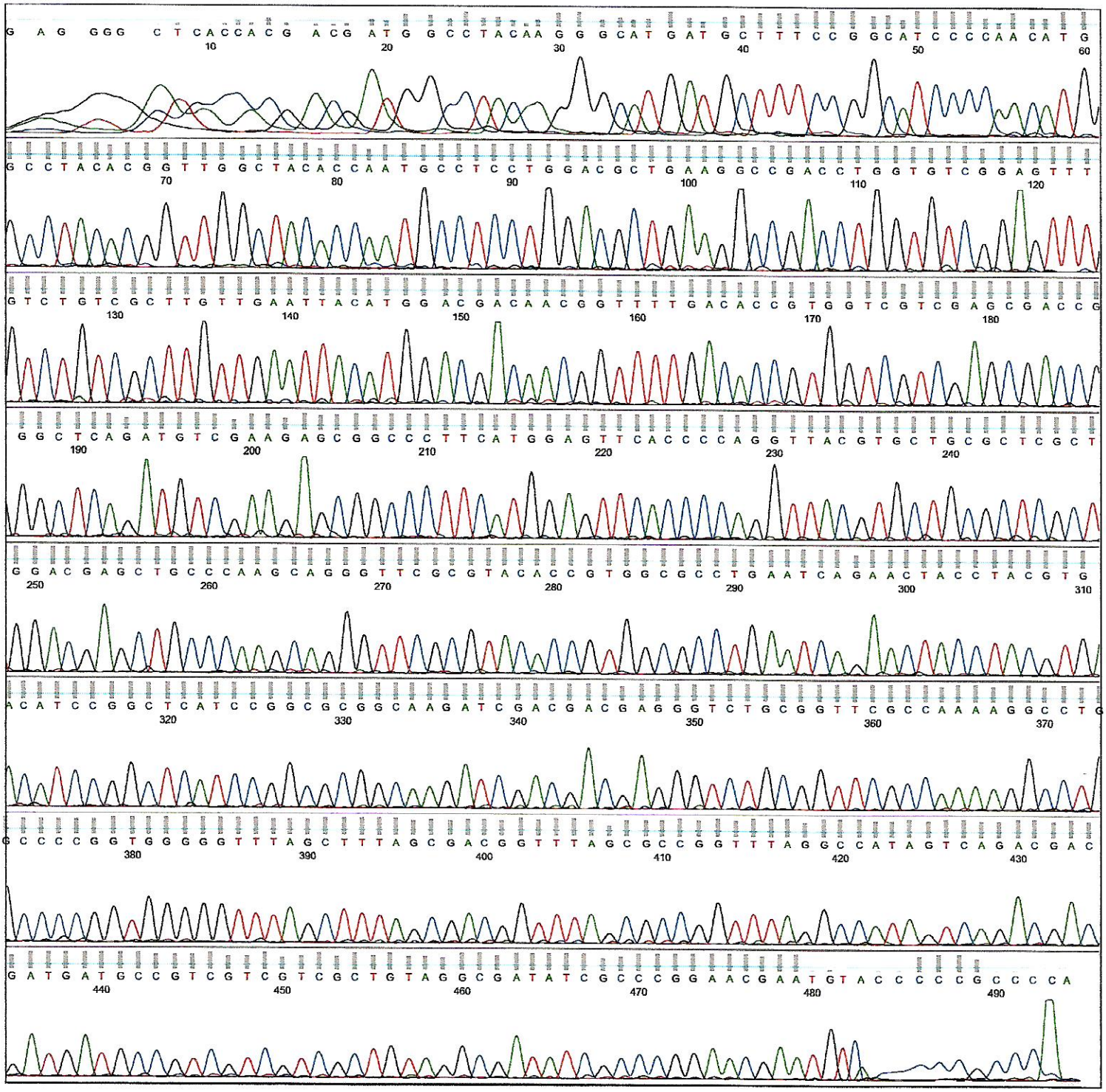


File: TF727 EthA REGION3.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-613618
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.2039
Comment: VILCHEZE/4/EHTA14

Signal Strengths: A = 822, C = 1056, G = 1717, T = 811
Lane/Cap#: 18
Matrix: n/a
Direction: Native

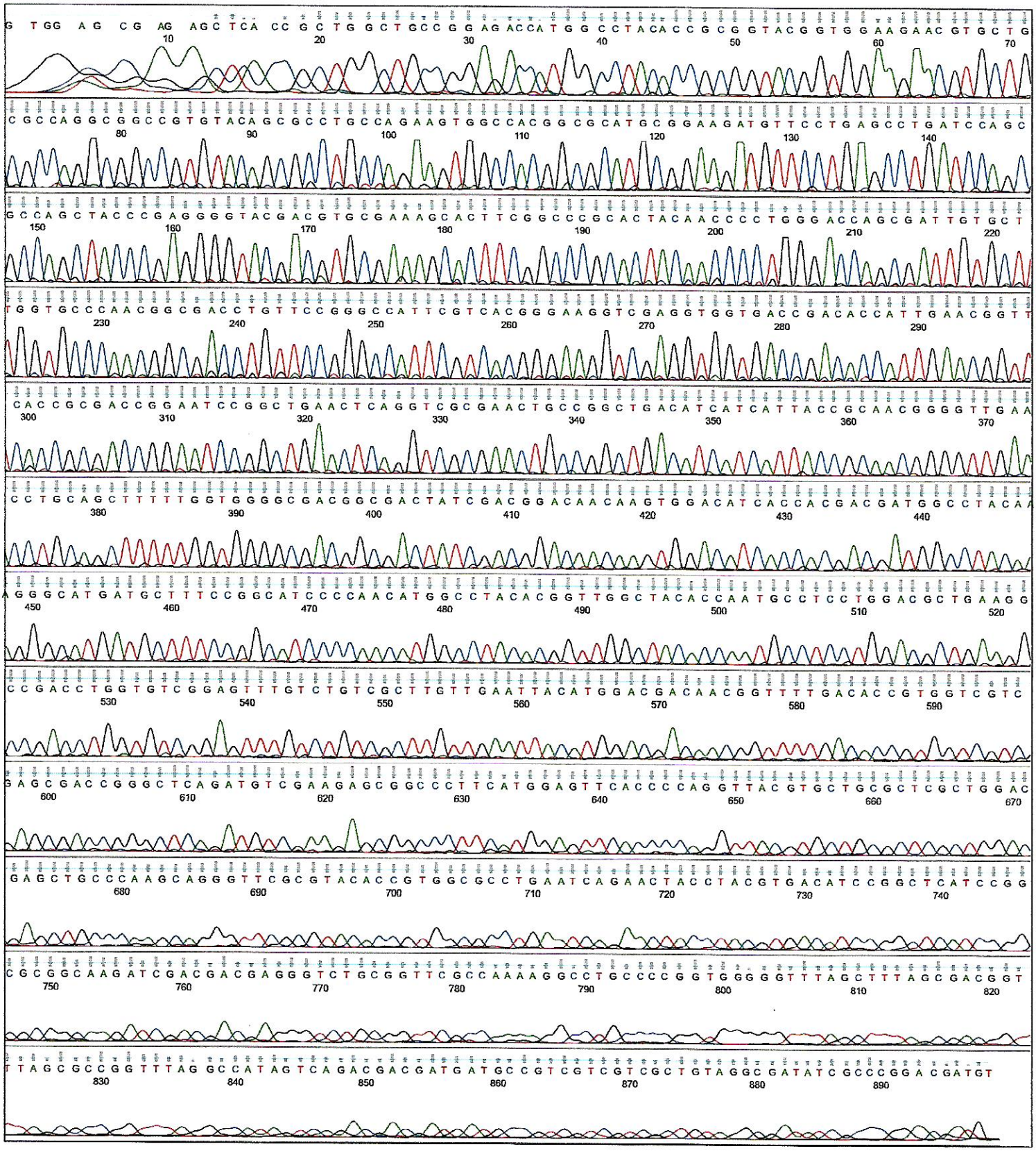


File: TF727 EthA REGION4.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-613617
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.2969
Comment: VILCHEZE/3/EHTA13

Signal Strengths: A = 1123, C = 1407, G = 2047, T = 898
Lane/Cap#: 10
Matrix: n/a
Direction: Native

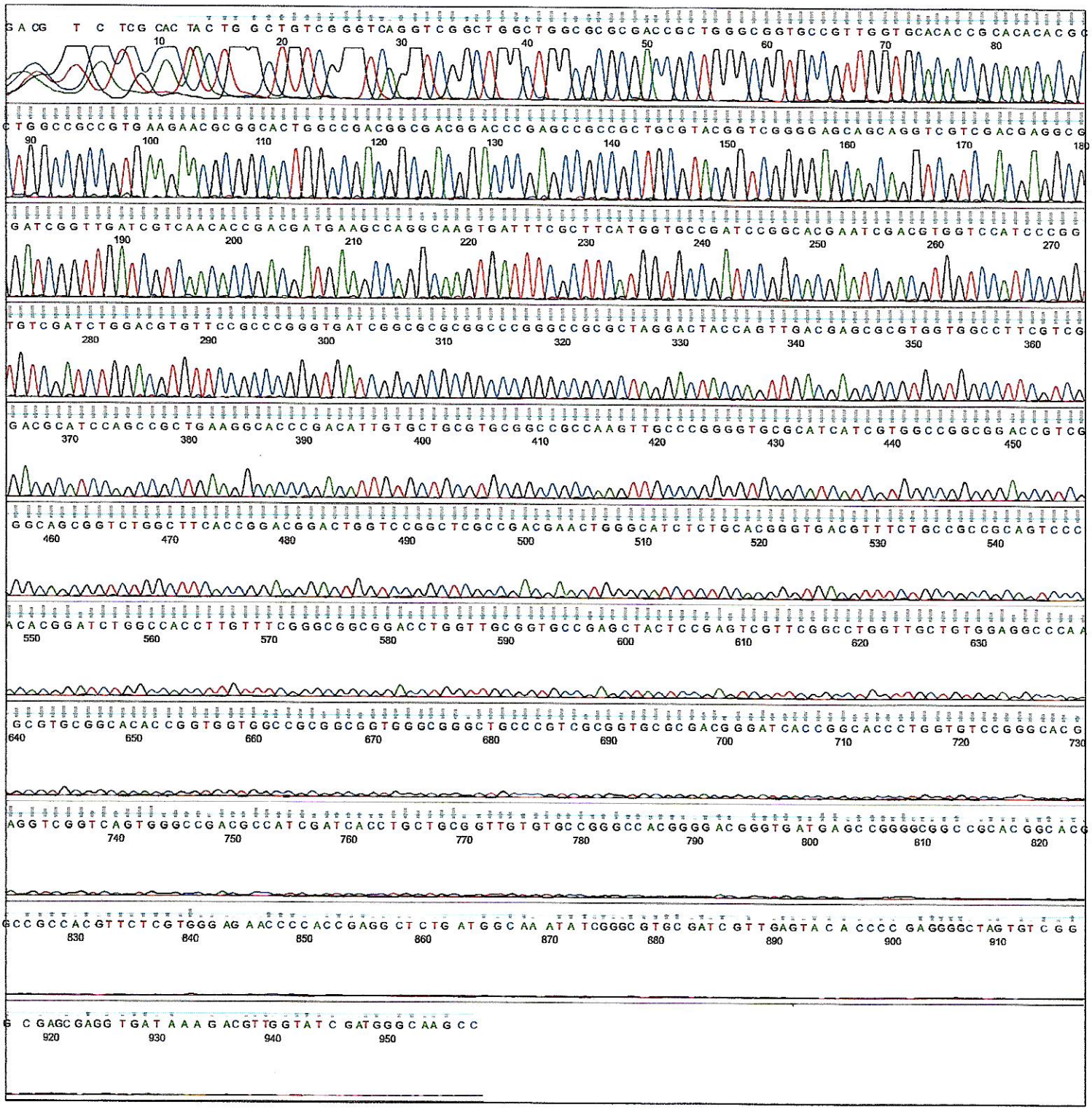


File: TF727 MshA REGION1.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-615905
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.3423
Comment: VILCHEZE/1/MSHA11

Signal Strengths: A = 393, C = 657, G = 767, T = 445
Lane/Cap#: 7
Matrix: n/a
Direction: Native

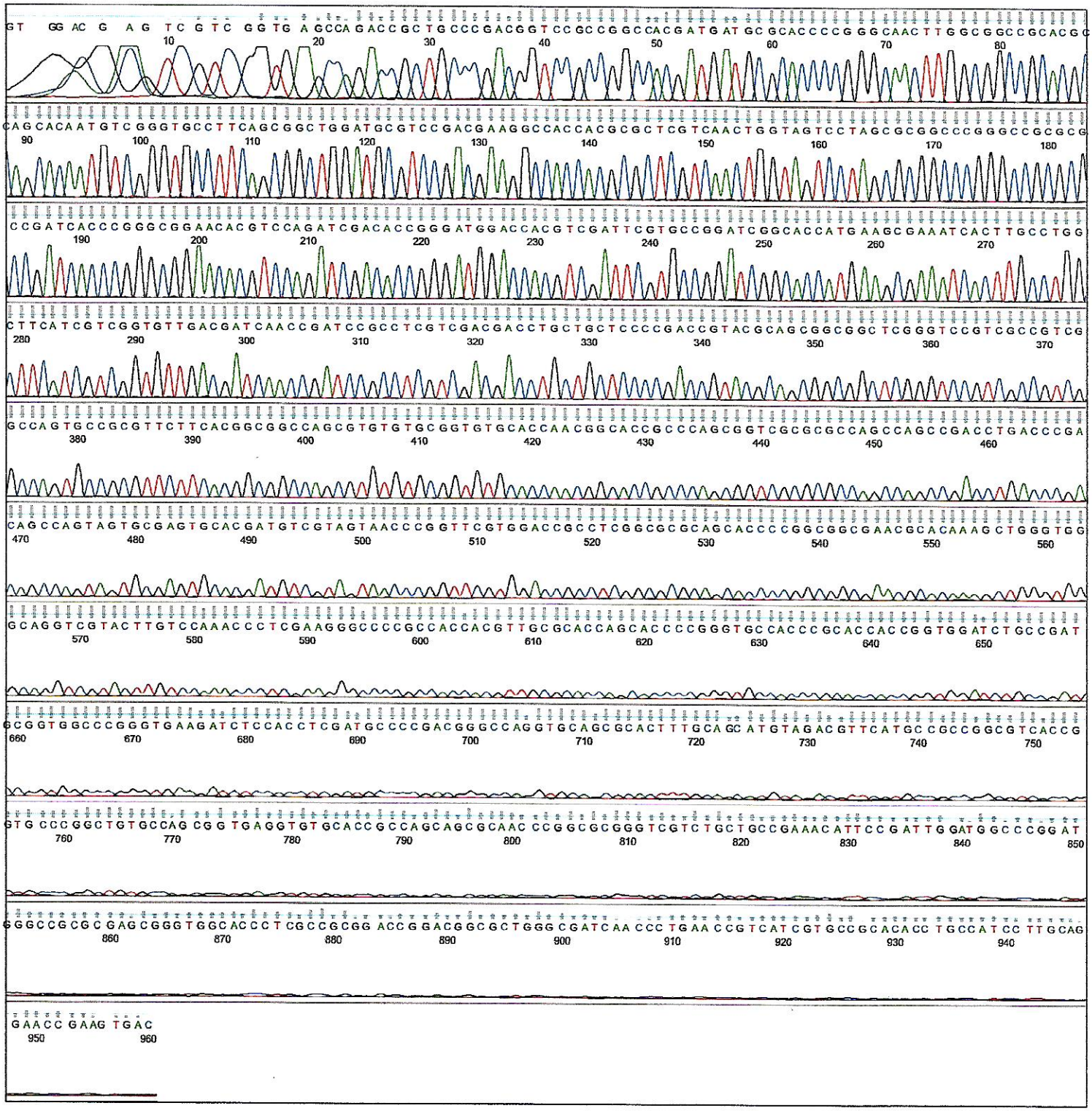


File: TF727 MshA REGION2.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-615906
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.3076
Comment: VILCHEZE/2/MSHA12

Signal Strengths: A = 1278, C = 2298, G = 2153, T = 1125
Lane/Cap#: 7
Matrix: n/a
Direction: Native

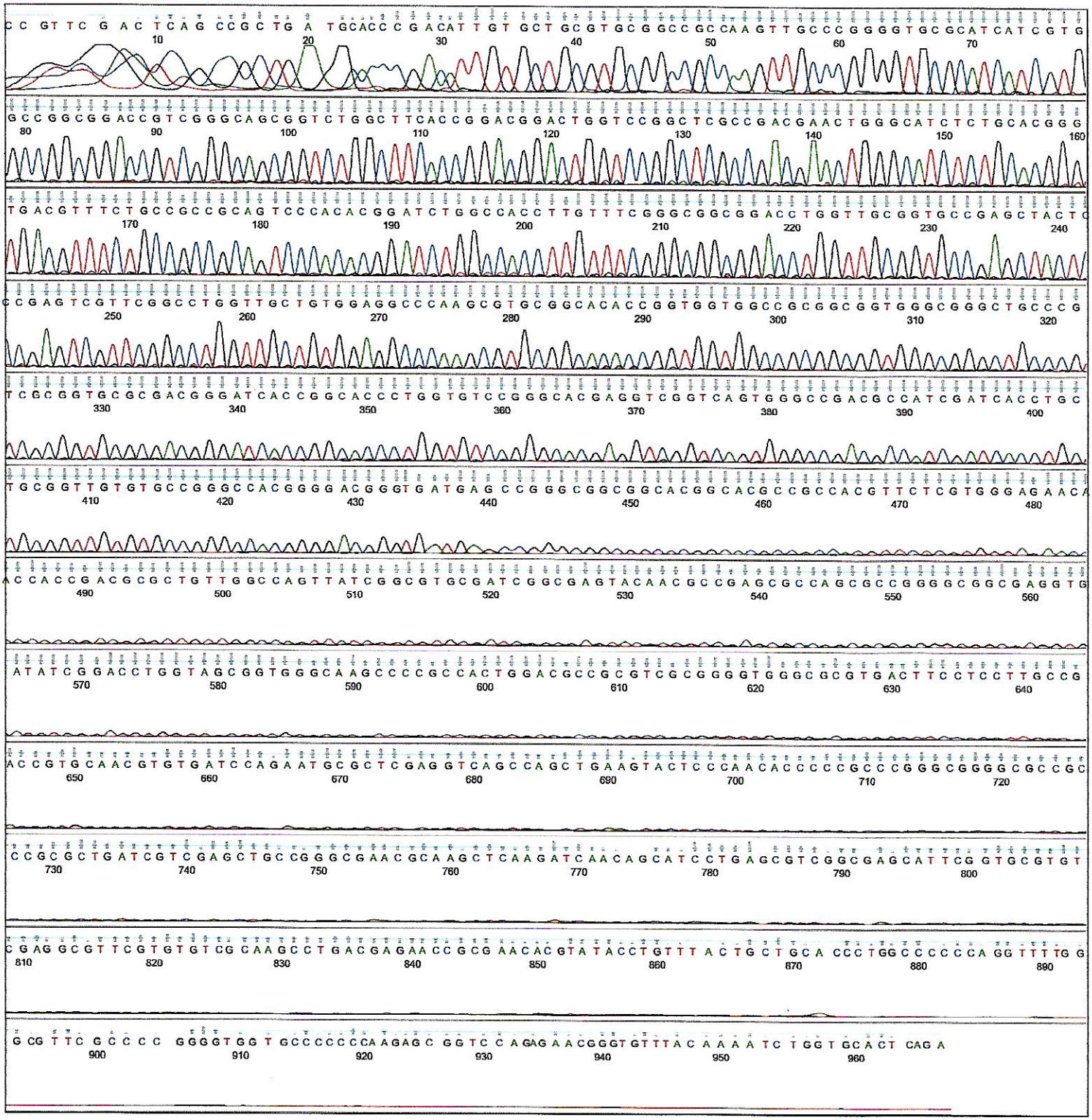


File: TF727 MshA REGION3.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-615907
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.306
Comment: VILCHEZE/3/MSHA13

Signal Strengths: A = 981, C = 2086, G = 2269, T = 1286
Lane/Cap#: 15
Matrix: n/a
Direction: Native

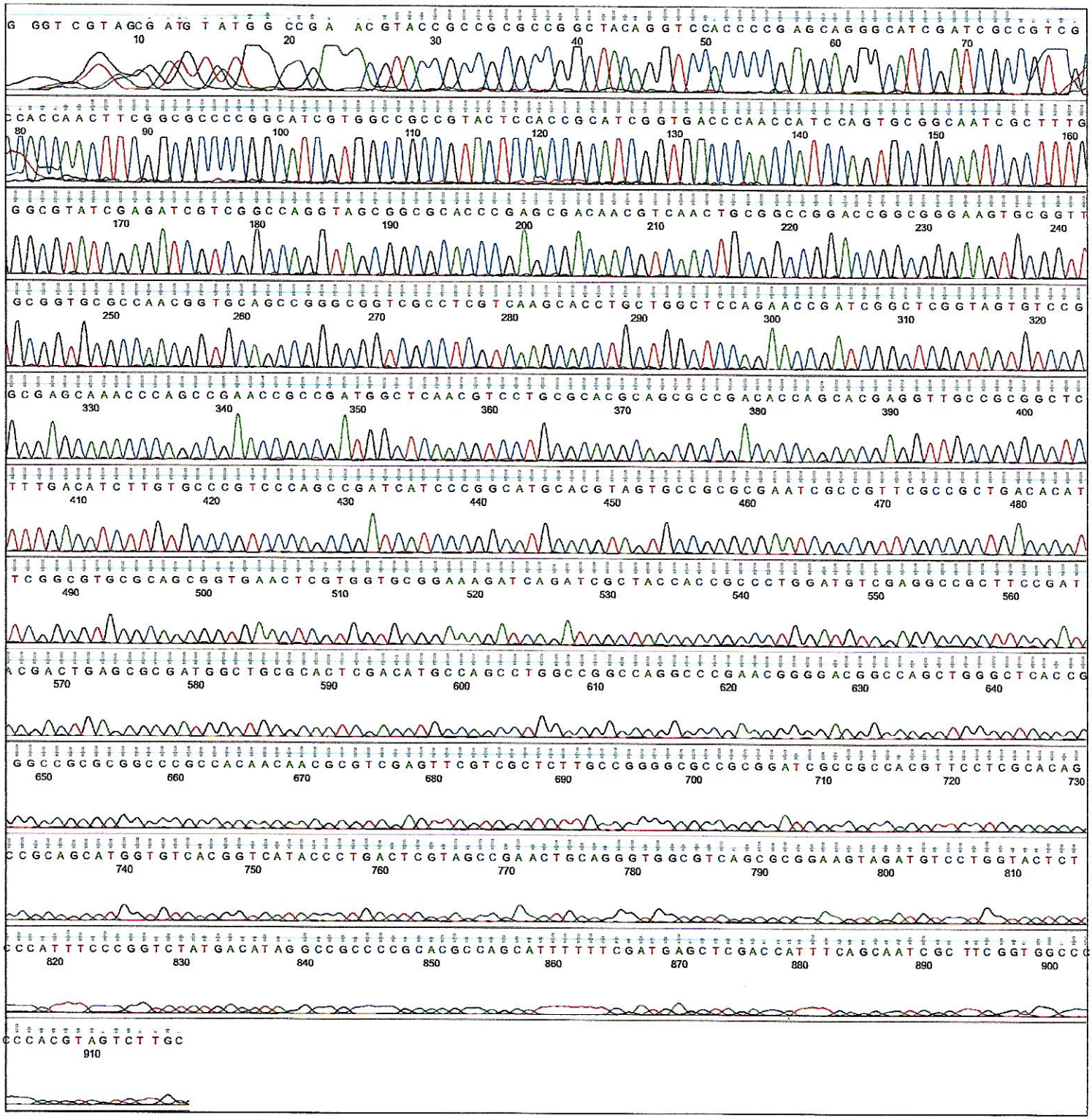


File: TF727 MshC REGION1.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-616446
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.9941
Comment: VILCHEZE/1/MSHC11

Signal Strengths: A = 228, C = 370, G = 549, T = 200
Lane/Cap#: 10
Matrix: n/a
Direction: Native

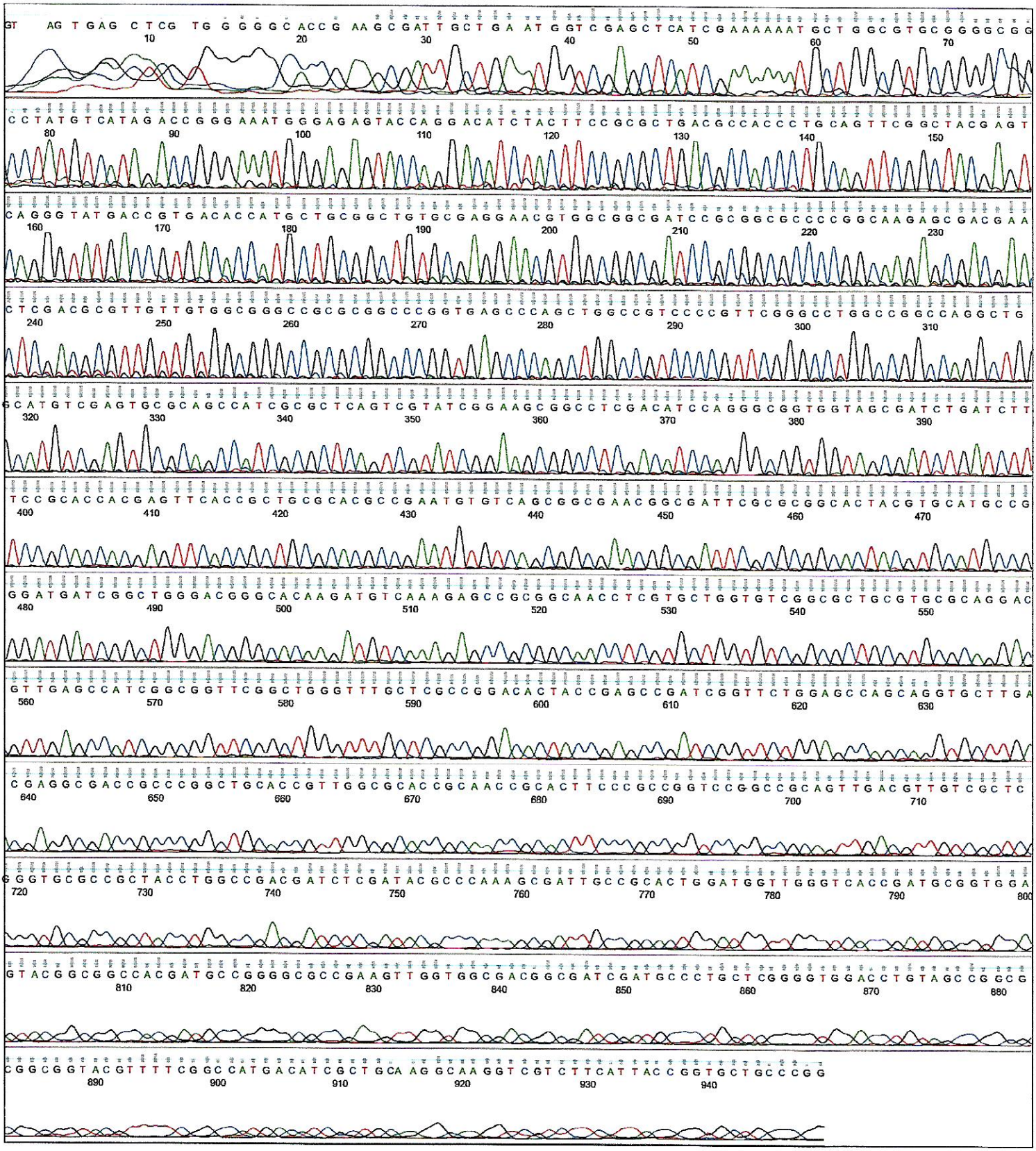


File: TF727 MshC REGION2.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-616447
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.4808
Comment: VILCHEZE/2/MSHC12

Signal Strengths: A = 293, C = 364, G = 707, T = 262
Lane/Cap#: 10
Matrix: n/a
Direction: Native

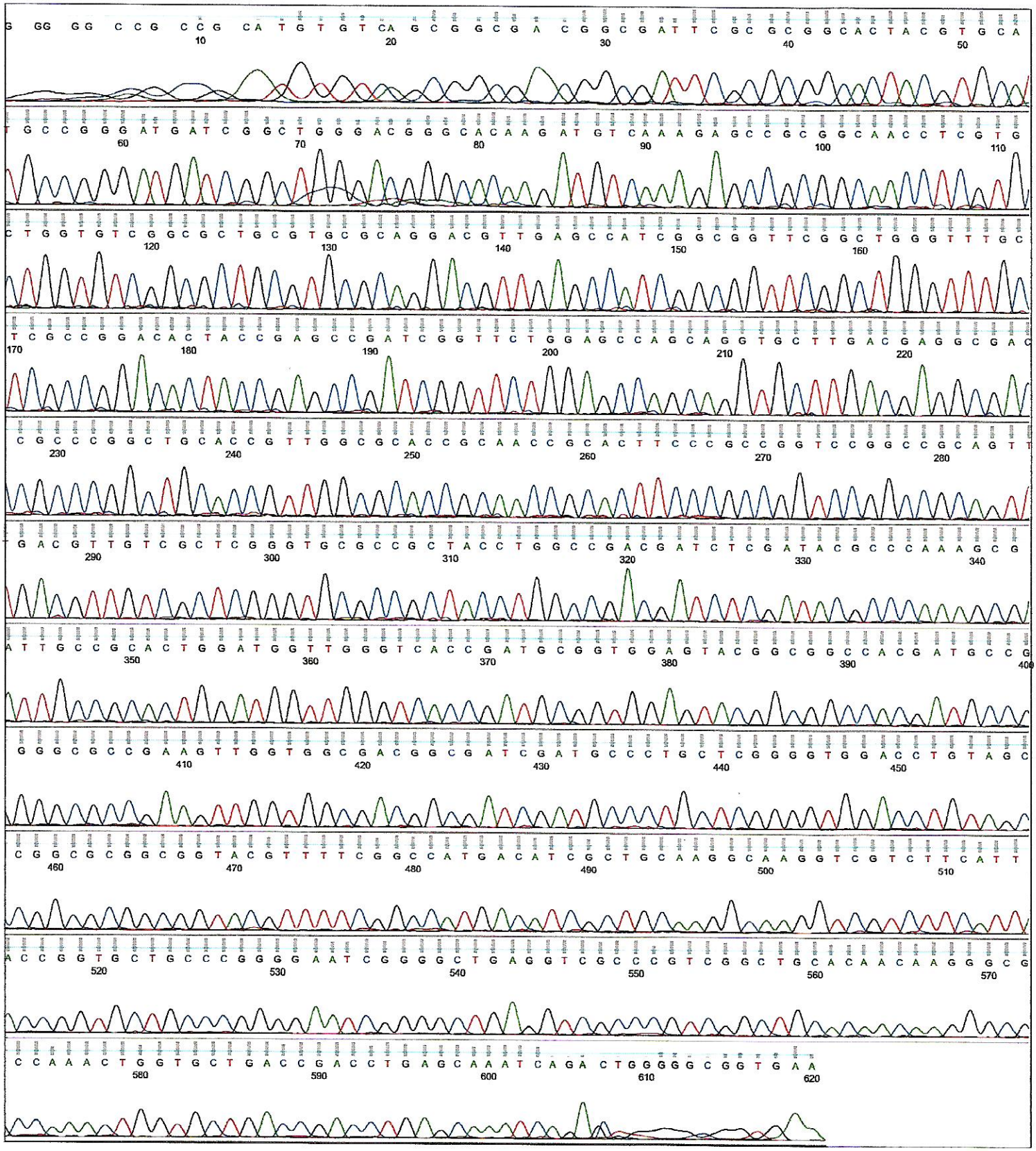


File: TF727 MshC REGION3.ab1

Geospiza
www.geospiza.com

Sample Name: BJ-616448
Mobility: KB_3730_POP7_BDTv3.mob
Spacing: 15.9147
Comment: VILCHEZE/3/MSHC13

Signal Strengths: A = 507, C = 690, G = 1470, T = 537
Lane/Cap#: 18
Matrix: n/a
Direction: Native



Annexure 3

Preparation of Probe DNA by PCR

Preparation of probe DNA by PCR [170]:

Preparation of PCR mix. Required per reaction;

10x PCR buffer (Saiki)	5µl
dNTP mix (each dNTP; 2.5 mM)	4µl
Primer 1 (50 ng/µl)	5µl
Primer 2 (50 ng/µl)	5µl

Primers:

INS-1 5' CGTGAGGGCATCGAGGTGGC 3'

INS-2 5' GCGTAGGCGTCGGTGACAAA 3'

To the PCR mix, 0.25 µl Taq polymerase (1.25 U) (Ampli-Taq) per reaction was added. The mix was vortexed and kept on ice. PCR mix was transferred to PCR tube in 20 ml aliquots. 30µl of the target DNA preparation (10 ng) isolated from *M. bovis* BCG was added to the PCR mix. The reaction mixture was overlayed with approximately 75 µl mineral oil (paraffin, Sigma), to prevent evaporation. This was mixed and centrifuged for 5 seconds in a microcentrifuge at 12 000 g. Program used for denaturing-annealing-synthesizing cycle:

3 min 94°C	once
1 min 94°C	25 cycles
1 min 65°C	25 cycles
2 min 72°C	25 cycles
4 min 72°C	once

The PCR products were examined by electrophoresis and staining with ethidium bromide. The PCR fragments should be 245 bp in length using primers INS-1 and INS-2 from IS*6110*. When the correctly sized fragments were obtained, purification on a Sephadex column was used. The PCR products were carefully collected by carefully pipetting the samples from under the oil in fresh microcentrifuge tubes. If necessary, the collected samples were centrifuged at 12000 g to be able to move the rest of the oil. A Quick Spin^{TM} Sephadex G-50 column (Boehringer Mannheim) was removed from its zip-lock bag and was gently inverted several times to re-suspend the medium. The top cap was removed from the column, then the bottom tip was removed. This sequence is necessary to avoid creating a vacuum and uneven flow of the buffer. The buffer was allowed to drain and was discarded. The column was placed in a collection tube (10 ml) and was centrifuged at 200g in a swing out rotor for 2 minutes. The collection tube was discarded with the eluted buffer. 100 µl of the sample was applied to the censer of the column bed. The column was placed in an upright position in fresh collection tube and was centrifuged for 4 minutes at 200 g in a swing out rotor. The eluate from the second collection tube contained the purified DNA. 100 µl of 1XTE and 500µl 96% ethanol was added to precipitate the DNA. This was stored at -20°C for 30 minutes. This was spun in a microcentrifuge for 15 minutes at room temperature at 12000g. The supernatant was discarded leaving the last 20 µl above the pellet. 1ml of 70% ethanol was added and the tube was carefully shaken. This was spun for 5 minutes in a microcentrifuge at room temperature and the supernatant was discarded leaving the last 20 µl above the pellet. This was again spun for 1 minute and the last 20 µl of ethanol was removed using a pipette and the pellet was permitted to air dry at room temperature for 10 minutes. The pellet was dissolved in 50 µl of 0.1XTE. The concentration of the PCR fragment was determined by measurement of the OD_{260} of a 1:50 dilution of the sample.

Annexure 4

Buffers and Solutions Components

Buffers and solutions

10X TBE:

108g Tris base

55g boric acid

9.3g EDTA

Add distilled water up to 1000ml

20X SSC:

173g NaCl

88.2g sodium citrate

Add distilled water up to 1000ml, pH=7

Soak I:

20g NaOH (0.5M)

87.66g NaCl (1.5M)

Add distilled water up to 1000ml

Soak II:

62.6g Tris base (0.5M)

87.6g NaCl (1.5M)

40ml HCl

Add distilled water up to 1000ml, pH=7.2

Hybridization buffer (500ml):

29.22g NaCl (0.3M)

20g blocking agent

10x PCR buffer:

30Mm $MgCl_2$

100Mm Tris-HCl pH 8.3

500Mm KCL

0.2 gelatine

Primary wash buffer:

360g urea

4g SDS (or 20ml of 20% SDS)

25ml 20X SSC

Add distilled water up to 1000ml

Secondary wash buffer:

2X SSC

Gel loading buffer:

5ml 10X TBE

25ml glycerol (Merck, Germany)

15ml distilled water

5ml 1% DD

1% DD:

1g bromophenol blue

1g xylene cyanole

100ml distilled water