The development of a novel fluorescent-marker phage technology system for the early diagnosis of tuberculosis disease

by
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Summary

Mycobacterium tuberculosis, the causative organism of tuberculosis (TB), is a major cause for mortality and morbidity world-wide with a death toll only second to HIV among infectious diseases. Drug resistance is wide-spread and cases of multiple drug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) have emerged in several countries. Drug treatment is problematic and new drugs are not developed rapidly enough to offset the rapid drug resistance mutation rate of *M. tuberculosis*. Simple and effective diagnostics are required to contain the spread of the disease as current routine diagnostics are not fulfilling this role. Additionally, current rapid TB diagnostics are out of reach to resource poor settings due to infrastructure, cost and skill requirements. Novel TB diagnostics are thus required that meet these requirements. Mycobacteriophages are phages that infect mycobacteria and could offer a viable and cost effective alternative rapid TB diagnostics. In this study, an affinity-tagged fluorescent reporter mycobacteriophage is described, which was engineered to act as a TB diagnostic. Its performance proved favourable and superior to current existing mycobacteriophage-based TB diagnostics.

Opsomming

Mycobacterium tuberculosis, die organisme verantwoordelik vir tuberkulose (TB), is `n groot bron van mortaliteit en morbiditeit wêreldwyd en slegs HIV is verantwoordelik vir groter getalle sterftes as gevolg van n aansteeklike siekte. Middelweerstandigheid is algemeen en gevalle van meervoudigemiddelweerstandige tuberkulose (MDR-TB) en uiters weerstandige tuberkulose (XDR-TB) kom in verskeie lande voor. Antibiotika behandeling is problematies en nuwe anti-TB middels word nie vinnig genoeg ontwikkel om die antibiotika weerstandigheid mutasie spoed van *M. tuberculosis* te bekamp nie. Doeltreffende diagnostiese toetse word benodig om die verspreiding van die siekte te beheer en bestaande roetine diagnostiese toetse voldoen tans nie aan hierdie vereiste nie. Behalwe hiervoor, is huidige vinnige TB diagnostiese toetse buite bereik van arm instansies weens vereistes aan infrastruktuur, meegaande kostes en werknemervaardigheid. Nuwe TB diagnostiese toetse is dus nodig om aan hierdie vereistes te voldoen. Mikobacteriofaage is fage wat mikobacteria infekteer en kan moontlik 'n lewensvatbare en koste-effektiewe alternatief bied vir vinnige TB diagnostiese toetse. In hierdie studie word 'n affiniteitgekoppelde fluoreserende rapporteringsmikobakteriofaag beskryf wat ontwerp is om op te tree as `n nuwe vinnige TB diagnostiese toets. Die werking hiervan vertoon gunstige en beter resultate as die huidige, mikobacteriofaag-gebaseerde TB-diagnostiese toetse.

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List of abbreviations

~	approximately
Ω	Ohm
µF	micro farad
%	percent
AK	adenylate kinase
ADP	adenosine-di-phosphate
AMP	adenosine-mono-phosphate
Amp^R	ampicillin resistance
ATP	adenosine-triphosphate
BCG	Bacilli de Calmette et Guerin
BIND	Bacterial Ice Nucleation Diagnostic
BLAST	basic local alignment search tool
bp	basepair
BSA	bovine serum albumin
℃	degrees Celsius
DNA	deoxyribonucleic acid
E.	*Escherichiae*
EDTA	ethylenediaminotetraacetic acid
FIGE	field inversion gel electrophoresis
g	gravity
GFP	green fluorescent protein
GST	Glutathione S-transferase
h	hour
His	histidine
Hyg^R	hygromycin resistance
Kan^R	kanamycin resistance

kbp	kilobase pairs
kDa	kilodalton
kV	kilovolt
L	liter
LAMP	loop-mediated isothermal amplification
LB	lysogeny broth
LO	lysis from without
M	molar
M.	*Mycobacterium*
Mb	megabases
min	minutes
msec	milliseconds
ml	milliliter
MP	mycobacteriophage
MWCO	molecular weight cut-off
Ni	nickel
µg	microgram
µM	micromolar
µl	microliter
ng	nanogram
NTM	non-tuberculous mycobacteria
nm	nanometer
OADC	oleic acid/albumin/dextrose/catalase
OD	optical density
ONPG	o-nitrophenyl-β-D-galactoside
ORF	open reading frame
pmol	picomolar
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PlyG	Phage-Lysin-Gamma
PPD	purified protein derivative
pfu	plaque forming units
^{R}	resistance
RE	restriction endonuclease
rpm	revolutions per minute
SAP	Shrimp Alkaline Phosphatase
sec	seconds
SDS	sodium dodecyl sulphate
SOB	super optimal broth
SOC	super optimal broth with catabolite repressor
TAE	tris/acetic acid/EDTA buffer
TB	tuberculosis
U	units
V	Volt
V/cm	Volts per centimeter
WCL	whole cell lysate
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3indolyl-β-galactopiranoside

Chapter 1: Introduction

1.1 *Mycobacterium tuberculosis* epidemic background

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M. tb*), is a major cause of death worldwide with a death toll of 1.7 million people in 2009 (World Health Organization, 2009a). In the same year, 9.4 million new TB cases were detected, exacerbating the state of the epidemic. Multiple drug resistant (MDR) (defined by resistance to isoniazid and rifampicin, with or without resistance to other first-line drugs) as well as extensively drug resistant (XDR) TB) (defined by resistance to isoniazid and rifampicin, and any fluoroquinolone as well as resistance to a second-line injectable such as capreomycin, amikacin, and kanamycin) have also emerged in recent years making treatment difficult and expensive. The WHO stated that 2010 had been the year in which the highest rates of MDR-TB and that XDR-TB had been detected in at least 58 countries (World Health Organization, 2010). Clearly, there is a drastic need to address the spread of the TB epidemic through rapid diagnostics and effective treatment regimens, as the current methodology is not suitable to effectively address and contain the disease.

1.2 Infection and treatment

TB is transmitted through inhalation of aerosolized respiratory droplets containing *M. tb* bacilli, commonly spread through coughing (Strull & Dym, 1995). Aerosolized *M. tb* particles can stay airborne for a long period of time, remaining infective and potentially exposing many people to the disease (Wells, 1955). The WHO estimates that one third of the world's population is infected with *M. tb* (Corbett et al., 2003, World Health Organization, 2009a). Exposure results in a 5-10% lifetime chance of developing active TB (Harries & Dye, 2006), a figure which rises dramatically to 10% per year if the individual is HIV infected (Decker & Lazarus, 2000).

As *M. tb* manifests itself in the lungs, the body fends off the disease by forming granulomas to contain the bacterium (Saunders & Britton, 2007). *M. tb* can stay dormant and viable for extensive periods of time inside granulomas. These granulomas can spontaneously rupture at a later period to expose viable bacteria into new areas of the lung, culminating in drastic disease progression (Saunders &

Britton, 2007). If the disease is allowed to persist, it can lead to disseminated tuberculosis during which the bacteria spread throughout the body causing further morbidity. As the body forms granulomas in order to contain the bacteria, it inadvertently shields the *M. tb* bacilli from the bacteriostatic and bactericidal effects of antibiotic treatment since current anti-TB drugs cannot permeate granulomas. A further complication in drug treatment of TB, is the slow replication rate of the organism of 12 to 18 hours (Strull & Dym, 1995), and its ability to enter a dormant non-replicating state. Almost all classes of antibiotics currently in use require replicating targets for effective drug action (Saunders & Britton, 2007). Drug treatment regimens thus span over extensively long periods in order for a patient to be considered as cured, 6-8 months for drug sensitive TB and 18-24 months for MDR-TB (which is also much more costly) according to the WHO recommendations (World Health Organization, 2009a). Despite the long drug treatment period, cured patients still have a risk of relapse after drug treatment due to reactivation of dormant *M. tb* (Saunders & Britton, 2007), which also carries an increased risk of drug resistance (World Health Organization, 2009b).

The WHO currently recommends a standard 6-8 month drug regimen for new, previously untreated, TB patients which consists of four drugs: isoniazid, rifampicin, pyrazinamide, and ethambutol. Isoniazid and rifampicin is used for two months followed by a continuation phase of 4-6 months consisting of isoniazid and rifampicin (ethambutol is added in high rifampicin resistance settings) (World Health Organization, 2009b). Such a combination therapy is given to avoid drug resistance associated with mono-therapy (David, 1970). If resistance to isoniazid and rifampicin is encountered, the patients are considered to have MDR-TB which is treatable only with second line drugs. If additional resistance to quinolones and an injectable aminoglycoside (kanamycin, capreomycin or amikacin) is encountered then the infection is considered XDR-TB, in which case the amount of remaining effective drugs is drastically reduced (World Health Organisation, 2006). The WHO recommends that patients that have been previously treated require drug susceptibility testing (DST) to confirm or exclude MDR-TB since MDR is prevalent in 15.3% of retreatment cases (World Health Organization, 2009b). However, most high burden TB countries lack the necessary DST facilities and funding to perform DST, which leads to incorrect drug regimens and poor patient outcomes (Parsons *et al*., 2011). In the case where DST is available, a specific drug regimen can be selected to best suit

the patient to improve patient outcome. A further problem with TB treatment is patient non-compliance or fallout where patients either take their drugs irregularly, or do not complete their treatment cycle (Parsons *et al*., 2011). There are several reasons why patient fallout occurs, such as loss of financial income, adverse side-effects of drugs and patients stopping due to the fact that they feel better etc (Parsons *et al*., 2011). The outcome of patient fallout is troublesome; if the patient has interrupted treatment for longer than 2 months, the WHO recommends DST to be performed, which is not readily available to low resource settings (World Health Organization, 2009b). The problematic TB treatment process stresses the importance of diagnostics in early detection and prevention of TB.

1.3 TB diagnostics

Routine TB diagnosis typically starts with clinical examination, followed by chest radiography if the patient presents with typical symptoms of TB such as persistent cough, night fever and weight loss (Zaman, 2010). If pulmonary TB is suspected, the tuberculin skin test (TST) is performed on the patient and a sputum sample is taken for smear microscopy as well as culture testing in most high burden TB countries (Parsons *et al*., 2011). This is however not the case in resource-limited settings which often use smear microscopy as definitive indicator for TB infection (Parsons *et al*., 2011). The following section contains a summary of existing routine diagnostics currently in use, as well as new emerging TB diagnostics.

1.3.1 Chest radiography

Chest radiography is widely used in the diagnosis of patients with active TB since infection typically leads to distinct patterns such as upper lobe infiltrates, cavities, hilar and mediastinal lymphadenopathy (Trinker *et al*., 1996). Chest radiography is typically used if a clinician determines that the patient presents a positive skin test and/or symptoms such as persistent cough, night swears, weight loss, fever or chills(). However, radiographs are only suggestive of TB and do not offer a definitive test. Radiographs may show abnormalities from previous TB infection as well as similar radiograph features due to other diseases, which require further testing to validate, and which means that radiographs are mostly useful to rule out TB. Due to the poor performance (reader bias, poor sensitivity and specificity) of chest radiographs, the WHO discouraged its use since 1974 for two

decades in favour of sputum microscopy (see Section 1.3.2) for symptomatic patients (World Health Organization, 1974). The WHO continued to discourage their use in 1994 with the implementation of DOTS (directly observed treatment short-course) (World Health Organization, 1994).

1.3.2 Microscopy-based TB diagnostics

Mycobacteria have a particularly thick lipid layer which causes them to retain certain chemical stains despite de-staining steps with acidic reagents (Brennan & Nikaido, 1995). They are thus called “acid-fast” bacteria. Smear microscopy involves spreading clinical samples onto a glass slide followed by staining the sample with one of several staining techniques followed by analysis using microscopy (Steingart *et al*., 2006). The three most widely used staining techniques are Ziehl–Neelsen, auramine–rhodamine fluorochrome and Kinyoun stains (Attorri *et al*., 2000, Gordin & Slutkin, 1990). Smear microscopy has several drawbacks. Firstly, it is not specific to *M. tb,* since it will detect all acid fast bacilli, which decrease the specificity of the test. The sensitivity of the test is also low due to its detection limit, as at least 5000 to 10 000 bacilli/ml of sputum is needed for detection (Hobby *et al*., 1973). This results in the test only detecting an estimated 45% of active pulmonary disease (Dinnes *et al*., 2007, Dye *et al*., 1999, Dye *et al*., 2005). Smear negative TB is higher in HIV positive patients, than in HIV negative patients which decreases sensitivity below the estimated 45% (Steingart *et al*., 2006) to as low as 10-30% (Vittor *et al*., 2011). Furthermore, the test cannot distinguish between viable and nonviable cells and also cannot provide drug resistance information (Dinnes *et al*., 2007). Despite the drawbacks of smear microscopy, it is still widely used, since the test is relatively rapid, inexpensive, does not require much equipment compared to culture and because it detects the patients with the highest bacillary load (i.e. the individuals who are the most infective) (Steingart *et al*., 2006). However, with the drawbacks of low sensitivity as well as the need for further validation for strain, species and drug resistance information, light microscopy is a less than ideal diagnostic.

Recent improvements in microscopy have led to an improved performance through alterations to sample processing and use of fluorescence microscopy and LED microscopy. Specimen processing has been shown to increase sensitivity in comparison to direct sputum smears (Steingart *et al*., 2006). It has also been shown that using bleach and centrifugation on HIV positive patient samples results in

a 15% (5% for HIV negative) increase in sensitivity for smear microscopy and a 17% (2% for HIV negative) increase for fluorescence microscopy (Eyangoh *et al.*, 2008). Fluorescence microscopy could potentially increase the sensitivity of smear microscopy and improve the efficiency of facilities, since these microscopes allow a larger area of the smear to be visualized, resulting in improved sample processing time. Fluorescence microscopy has been shown to have superior diagnostic capacity compared to conventional microscopy, especially in HIV positive patient samples. Fluorescence microscopy has an average of 10% more sensitivity than conventional smear microscopy (Steingart *et al.*, 2006). LED lamps are, however, not readily available in all countries. Although these improvements will increase the usefulness of microscopy, results still need to be confirmed by other diagnostics, most commonly through culture.

1.3.3 Culture-based TB diagnostics

Culture is currently the definitive routine diagnostic and it is the gold standard for TB diagnosis as well as DST (Kalantri *et al.*, 2005). This method relies on culturing clinical samples on solid or liquid media, and in the case of DST, in conjunction with various anti-TB drugs. Solid media is based on egg or agar such as Löwenstein-Jensen, Ogawa, Middlebrook (Saito, 1998). The slow replication rate of *M. tb* limits the speed of culture-based detection and drug susceptibility testing with results available in only about 6-8 weeks (Dinnes *et al.*, 2007). During this extended period of time the patient has the potential to contribute to the spread of the epidemic until an effective drug regimen is administered to the patient. Culture results are not always reliable since not all clinical samples can be cultured. Additionally, patients may have multiple strains of TB which may have varying drug resistance profiles and may represent varying amounts of the total *M. tb* bacterial population in the patient. Drug treatment provides a purifying selection against drug suseptable strains and provides grounds for positive selection for drug resistant strains. However, it is also possible that a drug sensitive strain may be the predominant strain amidst drug resistant strains which could lead to false diagnosis of drug susceptibility if the ratio is less than 1%. Sensitivity is also reduced by the initial decontamination step during which many *M. tb* bacilli are killed. Culture based TB diagnosis requires a specialized biohazard level 3 area and skilled workers, which is unsuitable for resource limited TB centers.

There have been various attempts to improve culture, as exemplified by the BACTEC, MGIT (Mycobacteria Growth Indicator Tube) and CONTAS systems. The BACTEC method makes use of media containing radio-labled carbon-14 and works on the principle that mycobacterial growth is proportional to radio-labeled CO_2 released, which can then be detected (Roberts *et al*., 1983). This means that mycobacterial growth can be detected before colonies are observed, shortening the detection time. Despite the improvement in time to detection, the assay still requires a lengthy 5-15 days for a positive result (Rusch-Gerdes *et al*., 1999). Furthermore the BACTEC system cannot perform speciation of mycobacteria and cannot culture all isolates, thus certain isolates will still have to be cultured on solid media (Roberts *et al*., 1983). The BACTEC system is also labor intensive and expensive, making it unsuitable for use as a routine TB diagnostic (El Sayed & Goda, 2007).

The mycobacteria growth indicator tube (MGIT) makes use of liquid culture in Middlebrook 7H9 broth as well as a fluorescence quenching-based oxygen sensor. A UV source is used to take fluorescence readings which are proportional to mycobacterial growth (El Sayed & Goda, 2007). The assay has similar sensitivity and specificity as the BACTEC culture method and the same pitfalls. The MGIT system requires 3 to 15 days for a positive result, is incapable of speciation and drug susceptibility testing capabilities are limited (El Sayed & Goda, 2007, Rusch-Gerdes *et al*., 1999). Based on this, attempts have been made to develop simplified culture techniques.

The nitrate reductase assay (NRA) is a culture based rapid drug resistance diagnostic for *M. tb*. Media containing potassium nitrate (such as Löwenstein-Jensen) turns pink as *M. tb* reduces potassium nitrate to nitrite and this signal in conjunction with the presence of rifampicin or isoniazid in the media is indicative of drug resistance. The assay has a sensitivity and specificity of 97% and 100%, respectively, for rifampicin resistance testing and 96% and 99% for isoniazid resistance testing. The assay requires 6.5 days for liquid media growth detection and 7-12 days for solid media. Based on these results, the WHO has also endorsed the method for rapid drug resistance testing (Vittor *et al*., 2011).

TK MEDIA® is a colorimetric detection system for TB detection where the media also changes color in response to mycobacterial growth (Baylan *et al.*, 2004). The media changes into different colors for either *M. tb* growth, no growth or contaminated growth. Mycobacterial growth is detectable within 2 weeks, whereas conventional testing with Lowenstein-Jensen (LJ) medium requires a minimum of 4 weeks to obtain results (Baylan *et al.*, 2004). TK MEDIA® offers the advantage of a simplified visual readout more suitable for low resource settings and the ability to distinguish between *M. tb* growth and contamination (Pai *et al.*, 2006b). However, contaminant gram-positive bacteria can give color changes similar to mycobacteria and would require further testing to confirm (Baylan *et al.*, 2004). Additionally, the media is pH sensitive and samples that have not been sufficiently neutralized thus affect the time of media color change (Baylan *et al.*, 2004). However, the media has been shown to have similar sensitivity and specificity to LJ culture (Baylan *et al.*, 2004). TK MEDIA ® currently only offers drug resistance testing to four drugs (isoniazid, rifampicin, streptomycin and ethambutol) and does not support speciation (Baylan *et al.*, 2004). TK MEDIA® offers advantages over other culture methods but its shortcomings described above, combined with a 2 week waiting period, makes it unsuitable for wide-spread use.

Microscopic Observation Direct Susceptibility (MODS) assay is a culture-based TB diagnostic developed to reduce the time to detection of culture. Micro-wells are filled with liquid media and inoculated with the bacterial sample, with some wells also containing rifampicin and isoniazid. Bacterial growth leads to colony formation which is detected using an inverted microscope. Growth in the wells which contain drugs are indicative of drug resistance. MODS have a growth detection time of 7 days and simultaneously tests for rifampicin and isoniazid resistance (in comparison to ~13 days for LJ and additional culture time requirement for drug resistance detection). The assay is relatively rapid for a culture-based diagnostic and is also accurate and cheap. Two studies have evaluated the performance of the test on HIV positive patients and the sensitivity was 87-98% with a specificity of 93-94%. The test also had a diagnostic time of 7-8 days in comparison to 11 days for liquid culture and 21 days for solid media (Arias *et al.*, 2007, Ha *et al.*, 2010). The assay has a reduced biohazard risk, since the cultures are sealed in a transparent bag. Additionally, the assay is less prone to contamination than conventional culture (Ha *et al.*, 2010). Due to these favorable aspects, the WHO

has recently endorsed MODS as TB diagnostic (World Health Organisation, 2010). Although the assay has a favorable performance in comparison to conventional culture, there are a few drawbacks. Colonies of non-tuberculous mycobacteria (NTM) could be difficult to distinguish *M. tb* from other mycobacteria and thus skilled operators are required (Parsons *et al*., 2011). The assay is also more complex than smear microscopy and requires biosafety culture facilities which are not feasible for low resource settings (Parsons *et al*., 2011).

The thin layer agar method is another method of microscopic observation which is performed on solid media. *M. tb* is grown on plates containing a thin layer of agar, some also containing rifampicin or isoniazid for DST, followed by microscopic observation for growth. Standard microscopes are suitable for microscopic observation using the thin layer agar method. The assay, when performed on smear positive samples, has a reported diagnostic time of 10.6 days with a sensitivity of 97.3% (Martin *et al*., 2009a). The thin layer agar method has also been tested on smear negative samples, which had a reported median time to growth detection of 14 days with a sensitivity of 74% (Martin *et al*., 2009b). The assay has the same advantages as other solid media based assays, but with a slightly lower sensitivity and slower diagnostic time. However, further studies are needed to validate the diagnostic assay. The long waiting period for growth detection and requirement for biohazard facilities and trained staff, results in this method not being suitable as a routine diagnostic.

1.3.4 Nucleic acid amplification assays

Nucleic acid amplification tests (NAAT) are polymerase chain reaction (PCR) based methods and includes in-house and commercial assays as well as nucleic acid probe amplification assays. NAAT diagnostics have become a frequent diagnostic tool for TB, mainly due its rapid and sensitive results. NAAT based TB diagnosis are based on TB-specific DNA amplification, which targets genes such as IS*6110*, *MBP64*, *rpoB* and *hsp65*, and can be done directly on clinical samples such as sputum (Cheng *et al*., 2004, Noordhoek *et al*., 2004, Pai *et al*., 2006b). NAATs are also very suitable for high throughput, especially if automation is possible. A systematic review of 106 NAATs (commercial and in house assays, on respiratory samples) have shown that the overall sensitivity and specificity is 86% and 97%, respectively, in the pooled analysis (Dinnes *et al*., 2007). Smear positive samples had a

sensitivity of 97% and smear negative samples had a sensitivity of 73%. The study also showed low sensitivity for extrapulmonary samples. The high smear positive sensitivity, low smear negative and extrapulmonary sensitivity has also been reported in other studies (Cheng *et al.*, 2004, Nahid *et al.*, 2006, Pai *et al.*, 2006b). The high specificity of NAAT allows for a preliminary early diagnosis to help set up appropriate drug regimens while waiting for culture confirmation results (Cheng *et al.*, 2004, Greco *et al.*, 2006). The high sensitivity on smear positive samples allows for ruling out of TB, however, this is not the case of smear negative samples due to the lower and varying sensitivity (Greco *et al.*, 2006, Pai *et al.*, 2006b). Thus PCR can also not replace culture and can only be used to help interpret the results (Nahid *et al.*, 2006). PCR also cannot distinguish between dead and live mycobacteria and is susceptible to amplicon contamination, both of which could lead to false positives. Further drawbacks of PCR are that it is expensive (especially the commercial kits), and requires skilled staff and infrastructure, which is not suitable for use in the field (Dinnes *et al.*, 2007). PCR-based TB detection currently only acts in conjunction with other less rapid tests. There is thus a need to improve PCR methods for TB detection and the Xpert®MTB/RIF and LAMP TB diagnostic tests are two examples of recent improvements.

The Xpert®MTB/RIF is an automated cartridge-based NAAT system which concentrates and purifies sputum, followed by PCR amplification of *M. tb* genomic and rifampicin-resistance genes. The assay requires relatively minimal training, has a detection limit of only 5 genome copies or 131 cfu/ml, and generates results in less than 2h (Van Rie *et al.*, 2010). The performance of the Xpert® MTB/RIF assay was tested in a multi-country study consisting of 1730 patients (Boehme *et al.*, 2010). The assay has a high specificity of 98% but with a variable sensitivity of 98.2% for smear-positive TB and 72.5% for culture positive smear-negative TB (Boehme *et al.*, 2010). The assay also has the drawback of requiring a constant electrical power supply, biosafety facilities, annual calibration and trained personnel (Van Rie *et al.*, 2010).

Loop-mediated isothermal amplification (LAMP) is a simplified nucleic acid amplification method that makes use of uninterrupted isothermal DNA amplification, followed by visual fluorescence detection, and has recently been applied to *Mycobacterium* detection (Iwamoto *et al.*, 2003). LAMP makes use of

Bst DNA polymerase, enabling auto-cycling strand displacement and production of large amounts of product DNA at a constant temperature (Iwamoto *et al.*, 2003). The use of *Bst* DNA polymerase also confers a relatively high specificity of primers at 67°C (Boehme *et al.*, 2007). Isothermal DNA amplification saves time in comparison to normal PCR, since there are ramping steps while cycling through temperatures. The primers used are species-specific for the *gyrB* gene and consists of four primers that recognize six regions of the *gyrB* gene, giving the assay high specificity (Boehme *et al.*, 2007, Iwamoto *et al.*, 2003). DNA is extracted from sputum samples and the DNA and LAMP reagents are mixed in one tube followed by amplification and detection (Boehme *et al.*, 2007). In the latest version of the assay (developed by Eiken Chemical Company together with the Foundation of Innovative New Diagnostics (FIND)) the reaction mixture contains calcein and manganese ion, and this allows fluorescence detection only in the case of DNA amplification (Boehme *et al.*, 2007). Calcein is a fluorescent chelating reagent which is quenched by manganese ion. However, pyrophosphate is produced by the LAMP reaction which binds to manganese ion to restore the fluorescent properties of calcein in the case of a positive LAMP reaction. Fluorescence (from calcein) is detected by the naked eye using a UV-source and combined with the turbidity caused by manganese pyrophosphate, creates an easy to detect positive result. The assay's capability for detection of *M. tb* has been shown to outperform microscopy in both smear positive and smear negative (culture positive) samples (Boehme *et al.*, 2007). The assay also takes about the same amount of time as smear microscopy to perform, with negligible reader to reader bias (Boehme *et al.*, 2007). The assay has the advantages of needing little laboratory equipment (UV source and heating block), being relatively rapid (about 60 min), and requiring a low level of technical skills. However the assay shares various downfalls with other PCR-based TB diagnostics. For example the sensitivity is low (48.8%) in smear negative, culture positive samples. Non-specific amplification also occurs if the LAMP reaction is not stopped (by incubating for 2 min at 80°C) within 70 min after the end of amplification, and the assay currently lacks diverse speciation capability (only *M. tb, M. avium* and *M. intracellulare* cannot be differentiated). The assay also does not have an internal amplification control, making the results questionable for routine TB diagnostics. Another drawback of the assay is the lack of the ability for drug susceptibility testing in its present format, so that it would still require subsequent culturing of clinical samples. Thus the LAMP

assay for TB clearly offers advantages over microscopy, but cannot replace current diagnostics and only acts as an adjunct to culture.

Nucleic acid probe assays are based on detection of DNA through hybridization to a probe. The assays generally make use of initial culture and cell lysis, followed by hybridization to probes of specific design for strain speciation and in some cases drug resistance detection. The AccuProbe assay is a nucleic acid probe speciation test that can detect *M. tb* complex, *M. avium* complex, *M. intracellulare*, *M. kansasii* and *M. gordonae* from lysed culture in about 1-2 hours (Dinnes *et al*., 2007). However, a separate test must be performed for each organism. An improvement on this assay is the Line probe assays which are PCR-based reverse hybridization assays. Two prominent line probe assays for TB are in use, the INNO-LiPA Rif for use on solid culture isolates and the Genotype MTBDR (and Genotype MTBDR*plus*) assay for use on solid culture, liquid culture and smear positive samples. Line probe assays are based on PCR amplification of bacterial DNA, followed by detection through hybridization to a probe-containing strip. Regions of the bacterial DNA are amplified, which allow detection of the *M. tb* complex, as well as drug resistance. The INNO-LiPA Rif assay amplifies the *rpoB* gene (conferring rifampicin resistance) and the Genotype MTBDR*plus* amplifies both the r*poB* gene as well as the isoniazid resistance genes by detecting mutations in the *katG* gene and inhA promoter. A recent study which focused on the application of the line probe assay for DST showed a sensitivity of 99% (for both rifampicin and isoniazid) and a specificity of 99% with a turnaround time of 1-2 days (Barnard *et al*., 2008). The line probe assays share the downfalls of conventional PCR such as the requirements for a DNA extraction room and separate PCR room, the sensitivity of the assay for amplicon contamination (causing false positives), the requirement for skilled operators and expensive equipment and the need for initial culture (except in the case of smear positive samples for the Genotype MTBDR*plus*). It is thus necessary to culture smear negative samples, leading to diagnostic delay. Despite the high accuracies, the Accuprobe, INNO-LiPA and MTBDR*plus* tests have costs, equipment and staff requirements that limit their diagnostic use.

1.3.5 Antigen-based assays

There are various TB diagnostics that make use of antigen detection as well as sensitization to antigens. These tests are used in conjunction with other TB diagnostics for a definitive diagnosis, since they can only diagnose latent TB (except for the skin patch test) and are incapable of DST. The tests can be subdivided into skin- and blood-based tests. The skin-based tests are the Tuberculin Skin Test (TST) and skin patch TB tests. The blood tests are the QuantiFERON™-TB Gold test (QFT-GIT; Cellestis Ltd, Carnegie, Australia), QuantiFERON-TB Gold In-Tube test (QFT-GIT; Cellestis Ltd, Carnegie, Australia) and T-SPOT.TB test (Oxford Immunotec, Abingdon, UK). The main difference between the skin and blood tests (other than antigen compositions) are in the way the antigens are administered and measured. In the skin patch test the antigens are placed on top of the skin, eliciting an immune response on the skin. In the TST method the antigens are injected below the epidermal layer, causing an inflammatory reaction, where after the resulting indurated area is measured (Andersen & Heron, 1993). In the blood tests, the T-cells are stimulated *in vitro* to produce Interferon-γ which is measured by ELISA in conjunction with a plate reader (Pai *et al*., 2006a).

The tuberculin skin test (TST) is one of the oldest TB diagnostics, yet is still frequently used (Andersen *et al*., 2000, Richeldi, 2006). It has low sensitivity and specificity and this limits its use as a diagnostic (Andersen & Heron, 1993, Richeldi, 2006). The antigens used in the test is obtained from a purified-protein derivative (PPD) of a culture filtrate of heat-killed tubercle bacilli (Richeldi, 2006). PPD contains over 200 antigens that are not only present in *M. tb,* but also many other species of mycobacteria, including the vaccine strain *M. bovis* BCG (Bacille Calmette ét Guerrin) (Andersen *et al*., 2000, Richeldi, 2006). This gives the test a low specificity. Patients in contact with mycobacteria other than *M. tb* and patients with prior BCG vaccination could falsely test positive for latent *M. tb* infection (Richeldi, 2006). HIV infected and other immune-compromised patients have a substantially reduced immunologic response to PPD and thus a lower sensitivity to TST (Vittor *et al*., 2011). The test also has a boosting effect whereby the patient becomes increasingly sensitized to the various antigens in the PPD each time the test is taken, creating false positives (Richeldi, 2006). Following PPD injection into the patient's skin, an inflammatory response is measured after 3 days (Andersen *et al*., 2000). The test is easy to administer and suitable for field use, however experienced workers are needed to

accurately interpret the results. Furthermore, patients have to come back to the diagnostic center for the result to be read and this leads to patient fall out. Compounding the problem, the reading of the induration is subject to reader bias. These factors make the test unreliable and inefficient.

An improvement on TST is the Sequella TB skin patch test, a trans-dermal patch that elicits a distinctive skin response in patients with active TB. The test is currently being developed as a commercial diagnostic. The test makes use of mycobacterial antigen MPB64, an antigen secreted by *M. tb*, *M. bovis* and certain strains of BCG during active growth (Andersen *et al*., 2000, Nakamura *et al*., 2001). The patch has been shown to distinguish between active and latent TB and cured TB (Nakamura *et al*., 1998, Nakamura *et al*., 2001, Pai *et al*., 2006a). The skin patch test has previously been shown to have high sensitivity and specificity and the results are available within 3-4 days (Nakamura *et al*., 1998, Nakamura *et al*., 2001, Pai *et al*., 2006a). The test also has the advantage that it does not require skilled staff or expensive infrastructure. However, the test is not without problems. Concerns of false diagnosis of TB in patients with *M. bovis* infections or BCG vaccinated individuals have been raised, which would require further testing to confirm results. The diagnostic also requires a repeat visit, leading to patient fallout, and it is unknown what the performance of the test will be in HIV patients. As with the TST, tests that rely on healthy immune responses to antigen challenge are of concern in HIV infected and other immune-compromised patients. Performance is also of concern in children, patients with a dark skin color and drug treated patients (Nakamura *et al*., 2001, Pai *et al*., 2006a). The TST and skin patch tests are minimally invasive tests, yet also have disadvantages. TB diagnostics based on assays of blood samples offer alternative diagnostic methodologies.

Blood tests rely on measurement/quantification of interferon-γ production by T-cells in response to *M. tb* antigens (Dinnes *et al*., 2007, Richeldi, 2006). Patients with an *M. tb* infection are sensitized to the antigens used in the blood tests (Dinnes *et al*., 2007). When their blood samples are exposed to these antigens, interferon-γ is produced by the lymphocytes (Dinnes *et al*., 2007). Early blood tests made use of PPD and consequently also had trouble distinguishing between *M. tb* and atypical mycobacteria (yet could distinguish between *M. tb* and *M. avium*) and gave false positives due to BCG vaccination

(Dinnes *et al*., 2007, Pai *et al*., 2006a). The QuantiFERON™-TB Gold, QuantiFERON™-TB Gold In-Tube test (QFT-GIT) test and the T-Spot TB test, however, are blood tests that make use of specific antigens from region of difference 1 (RD1) in the *M. tb* genome (Dinnes *et al*., 2007, Richeldi, 2006). The antigens used are the 6kDa early secretory antigenic target (ESAT-6) and the 10kDa culture filtrate protein (CFP-10) as well as TB7.7 (Dinnes *et al*., 2007, Pai *et al*., 2006a, Richeldi, 2006). These antigens are not present in non-tuberculous mycobacteria, except for *M. kansasii, M. szulgai*, and *M. marinum* (which could lead to cross reactions in the case where patients are infected with one these strains). Although the interferon-γ release assays are based on the same antigens they have a few differences in the way that they detect their antigens. The The QuantiFERON™-TB Gold test uses whole blood with an unknown amount of leukocytes and measures the amount of interferon-γ in the supernatant of the blood sample (Richeldi, 2006). In contrast, the T-Spot TB test quantifies the amount of interferon-γ producing T-cells and reads the amount of individual T-cells producing interferon-γ (Richeldi, 2006). These tests require 1-2 days for results (Pai *et al*., 2006a). The blood tests are not subject to reader bias, as is the case with the TST test, since they make use of automated equipment (Dinnes *et al*., 2007). There is also no need for a return visit and there is no "boosting" effect where the patient is sensitized by repeat tests as is the case with the skin tests (Dinnes *et al*., 2007, Richeldi, 2006).

The blood tests are thus a valuable asset for rapid screening of TB patients and for advising on which patients to further screen for TB with conventional tests. A meta-analysis by Pai and co-workers determined that the pooled sensitivity was 78% for QuantiFERON-TB Gold, 70% for QFT-GIT, and 90% for T-Spot TB (Pai *et al*., 2008). However there is no gold standard for latent TB detection, making comparison studies indefinite (Pai *et al*., 2006a). The drawbacks of the blood tests are that they cannot confirm active TB and only indicates a likelihood of TB infection (Pai *et al*., 2006a). Furthermore, samples must be processed within 12-16 hours and the test and interpretation of results require skilled staff, (Dinnes *et al*., 2007, Pai *et al*., 2006a, Richeldi, 2006, Vittor *et al*., 2011). The tests also uses infrastructure not ideal for use in the field or low resource settings with limited staff (Pai *et al*., 2006a). Furthermore, there is still a chance for tuberculosis mycobacterial cross reactivity or cross-reactivity due to BCG vaccination, as a large number of tuberculosis mycobacterial species

contain homologs of ESAT-6 and CFP-10. Interferon-γ release assays are expensive to process and require a 24h waiting period to generate results. These attributes make IGRAs less than ideal as TB diagnostics. As an alternative to detecting antigens by means of blood or skin tests, the Capilia TB assay (TAUNS Laboratories, Inc.) is performed directly on culture isolates. The assay is a lateral-flow immunochromatographic assay which, as with the skin patch test, detects the MPB64 antigen (Muyoyeta *et al.*, 2010). Following initial MGIT culture (how many days) a small amount of the culture is added to the sample wells which contain a strip. The culture is incubated with the strip for 15 min after which readout from the strip is taken. In one study the Capilia assay had a sensitivity and specificity of 99.6% and 99.5% respectively compared to a DNA strip test (Muyoyeta *et al.*, 2010). A further study found that the assay had a sensitivity and specificity of 98.4% and 97.9% respectively with results available in 20 min (Muchwa *et al.*, 2012). Despite the rapid time for results generation, the assay relies on initial culture which is time consuming and requires specialized biohazard facilities which is not suitable for field settings. The assay has also been shown to be liable to false negatives for *M. tb* complex samples as well as false positives for non-tuberculious mycobacterial isolates due to mutations in the mpb64 gene (Muchwa *et al.*, 2012, Muyoyeta *et al.*, 2010).

1.3.6 Mycobacteriophage-based TB diagnostics

1.3.6.1 Mycobacteriophage replication assays

Mycobacteriophages are *Mycobacterium*-specific phages, the first of which was identified in 1947 (Gardner & Weiser, 1947). Since then, over 250 mycobacteriophages have been isolated (Hatfull & Jacobs, 1994, McNerney, 1999). Mycobacteriophages isolated to date have double stranded DNA and are of the siphoviridae or myoviridae morphotype (Hatfull *et al.*, 2008). Initially, mycobacteriophages were considered for use in taxonomic studies and phage typing (see Section 1.5.3.1) (Redmond & Ward, 1966). David and coworkers developed a bacteriophage replication assay for mycobacteria which makes use of sensor cells to overcome the slow replication rate of *M. tb* (David *et al.*, 1980, Jones & David, 1971), In their assay, the *M. tb* sample is incubated with mycobacteriophage D29, treated with an antiviral agent and then plated onto a lawn of *M. smegmatis.* Progeny phages are detected by plaque formation which is possible since D29 can infect both *M. tb* and *M. smegmatis*. The role of the antiviral agent is to kill off excess phages that did not take part in the initial infection, to

avoid false positives due to mycobacteriophages remaining in the culture media. Inactivation of excess mycobacteriophage is possible by treatment by acid, sodium hydroxide as well as ferrous (iron II) salts (ferrous iron is less harsh to the cells harboring the mycobacteriophage and thus preferable) (McNerney *et al.*, 1998). Bacteriophage replication assays have been tested in various studies with varying performance outcomes as shown in Table 1. Bacteriophage replication assays offer the advantage of detecting smear negative culture positive samples and indicates successful drug treatment since killed cells are not detected despite positive smear results (Prakash *et al.*, 2009). However, the detection limit of bacteriophage replication assays has been estimated to require at least 100-300 bacilli/ml for a positive result (Albay *et al.*, 2003). This is 10 fold higher than for culture.

Table 1. Bacteriophage replication assay studies highlighting varying levels of sensitivity and specificity.

Sensitivity (%)	Specificity (%)	Samples	Setting	Reference
75.2	99	1618	South Africa	(Albert *et al.*, 2002)
81.6	97.7	514	Pakistan	(Muzaffar *et al.*, 2002)
87.5	96.9	192	Turkey	(Albay *et al.*, 2003)
58.3	99.1	2008	Spain	(Alcaide *et al.*, 2003)
63.6	82.6	38	Egypt	(Marei *et al.*, 2003)
44.1	92.4	496	Zambia	(McNerney *et al.*, 2004)
93	96	370	India	(Hemvani *et al.*, 2012)

Mycobacteriophages gained popularity when shown capable of screening antibacterial agents and discerning between drug resistant isolates and drug sensitive isolates to streptomycin (Tokunaga & Sellers, 1965), kanamycin (Nakamura *et al.*, 1967), rifampicin (Jones & David, 1971), clofazimine, colistin, dapsone and (to a lesser extent) isoniazid (David *et al.*, 1980). This is possible since the above listed anti-tuberculosis drugs block phage replication in susceptible strains and allow replication in drug resistant strains, allowing grounds for detecting drug resistance. Screening for ethambutol and isoniazid resistance, however, require several days incubation of the sample with the drugs, since these drugs do not block phage replication directly and they are only active in certain cell growth stages (David *et al.*, 1980, Wilson *et al.*, 1997).

Several studies have investigated the use of bacteriophage replication assays for *M. tb* drug resistance screening. A review which included 11 studies of bacteriophage replication-based DST showed a 100% detection rate of rifampicin resistance for 10 of the 11 studies when compared to culture (McNerney & Traore, 2005). Shortly thereafter, a bacteriophage replication assay for drug resistance screening was performed in South Africa, which included 133 samples and had a sensitivity of 100% and specificity of 98.8% (Albert *et al.*, 2004). A meta-analysis on bacteriophage replication based DST included 14 studies which showed that bacteriophage replication assays have a high sensitivity and specificity. The majority (58%) of the studies had more than 95% sensitivity and specificity when used to test culture isolates (Pai *et al.*, 2005). However, concerns over false positives, potential for contamination and indeterminate results were raised, since they hinder use of bacteriophage based DST assays.

A variation on the bacteriophage replication assay is the use of a micro-well format for drug resistance testing (Gali *et al.*, 2006, McNerney *et al.*, 2000). The assay makes use of initial incubation of *M. tb* samples with anti-TB drugs in a micro-well format. This requires 24h incubation for rifampicin and 72h for isoniazid. The sample is then mixed with mycobacteriophage D29 and incubated. Ferrous ammonium sulfate ($FeNH_3SO_4$) is then added to inactivate excess mycobacteriophage. Aliquots are then placed onto a lawn of *M. smegmatis* and the plates are observed for plaques. Plaques would thus indicate successful mycobacteriophage replication and thus the presence of viable mycobacteria in the sample. In the case of plates containing drugs, plaque presence would also indicate drug resistance in the mycobacterial sample. A study on 70 isolates showed that the sensitivity and specificity of the assay was 86.1% and 92.6% for isoniazid resistance and 100% sensitivity and specificity for rifampicin resistance detection (Gali *et al.*, 2006). The format of the assay has the advantage of decreased potential operator exposure to *M. tb* due to the small volumes handled, and is more suitable for handling large amounts of samples. McNerney and co-workers also modified the micro well assay end point measurement to make use of a colorimetric redox reaction (McNerney *et al.*, 2007). Excess mycobacteriophages are inactivated by sulfuric acid, followed by a neutralization step using sodium hydroxide. The samples are then transferred to a microplate together with *M. smegmatis* and incubated overnight. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide) is then added to each well and incubated for a further 16h. A colour change from yellow to purple indicates successful *M. smegmatis* growth, which is indicative of a drug resistant *M. tb* isolate. A purple sample similarly indicates a susceptible strain, since phage replication was not possible. The MTT microplate assay had a sensitivity and specificity of 90.9% and 98.8% when tested on 96 clinical samples with sequencing used as gold standard (McNerney *et al*., 2007). The microplate assays share the problems of low specificity for low level drug resistance detection with other bacteriophage replication assays and also requires extensive time for DST.

A follow-up meta-analysis investigated bacteriophage replication DST assays which consisted of 31 studies (Minion & Pai, 2010). The analysis determined that the commercial assays had an overall sensitivity and specificity of 96% and 95%, respectively, in-house assays had a sensitivity and specificity of 99% and 98%. The contamination rates were 0-36% (higher in direct sputum samples), which leads to indeterminate results. The assays have since been updated to include an antibiotic which reduces indeterminate results by 68%, without reducing the sensitivity and specificity (Minion & Pai, 2010). With the current performance of bacteriophage replication assays it is clear that they cannot replace culture methods, due to inadequate sensitivity, specificity and minimal variety of DST. Bacteriophage replication assays do however offer a rapid alternative to microscopy for low resource settings where microscopy is less feasible (Albert *et al*., 2002, Kalantri *et al*., 2005, Minion & Pai, 2010, Traore *et al*., 2007). The bacteriophage replication assay has the advantage of only detecting viable cells, in contrast to PCR, which makes it less prone to false positive case detection. However false positive drug resistance is problematic. The assay requires an extensive 2 days for diagnosis, 2-4 days for DST and also requires specialized culture facilities and skilled operators, which are less feasible for field use.

1.3.6.2 Reporter Mycobacteriophages

1.3.6.2.1 Luciferase Reporter Phage assays

Luciferases are enzymes that catalyze light producing reactions in the presence of ATP, O_2 and the substrate luciferin (Meighen, 1991). Jacobs and co-workers initially developed a luciferase bearing mycobacteriophage for the detection of *mycobacterium tuberculosis* (Jacobs *et al*., 1993). This was

performed by cloning the *M. bovis* BCG *hsp60* promoter together with the firefly luciferase gene (*Fflux*) into the lytic mycobacteriophage Tm4. The recombinant mycobacteriophage was able to express luciferase in *M. smegmatis* and *M. tb*. The luciferase reporter phage (LRP) assay makes use of 7-8 days culture of *M. tb* samples followed by 48h incubation of the samples with anti-TB drugs. The cultured samples are then infected with the LRP for 1-5h and the substrate (luciferin) is added. A luminometer is used for detection of light production, yielding a detection limit of 10^4 bacteria/ml (Jacobs *et al*., 1993). Several studies have subsequently investigated and improved luciferase reporter mycobacteriophages for use as TB diagnostics and for DST. The first attempt to improve upon the LRP assay made use of a recombinant mycobacteriophage L5, which utilized the same promoter and reporter gene as the initial LRP (Sarkis *et al*., 1995). The L5 based LRP assay showed an improved sensitivity for *M. smegmatis* detection, however was unable to infect *M. tb* (Sarkis *et al*., 1995). Similarly, a recombinant mycobacteriophage D29 was constructed and applied to detection of *M. bovis BCG* (also utilizing the same promoter and reporter gene as the initial LRP) (Pearson *et al*., 1996). Despite the D29 based LRP having an increase in relative light units of one log above the initial Tm4-based LRP assay, the assay still had a poor sensitivity (Pearson *et al*., 1996). One of the problems with the initial Tm4-based LRP assay was the detection limit, which could be improved by increasing control of when the LRP replicates and subsequently leads to cell lysis. This would leads to a build-up of reporter enzyme before cell lysis and thus an increased detection limit. To achieve the goal of gaining control over the timing of cellular lysis, mutations were introduced to the mycobacteriophage Tm4-based LRP which led to the production of a temperature sensitive and conditionally replicating LRP (Carriere *et al*., 1997). The conditionally replicating LRP had an improved detection limit of 120 bacteria/ml, however this detection limit was achieved using a 12h LRP infection and was performed on BCG. For DST the assay required 24h incubation of the bacterial sample with the anti-TB drugs followed by a 2h LRP infection step. The improved detection limit was attributed to a decreased rate of bacterial lysis, leading to prolonged build-up of luciferase and increased levels of light production (Carriere *et al*., 1997). Another problem with the initial Tm4-based LRP assay was the wide host range, which include several species of mycobacteria. In order to adjust the host range a selective growth inhibitor was investigated (Riska *et al*., 1997). Riska and co-workers tested the use of a growth inhibitor to differentiate between luciferase production due to the presence of non-

tuberculous mycobacteria in the sample and that of *M. tb* following infection with the luciferase mycobacteriophage (Riska *et al*., 1997). *p*-nitro-α-acetylamino-β-hydroxy propiophenone (NAP) has an inhibitory effect to the *M. tb* complex (but not to nontuberculous mycobacteria). NAP was incorporated into the luciferase reporter assay to achieve an assay capable of discerning between *M. tb* and non-tuberculous mycobacteria within 24h. The assay also investigated the use of a Polaroid film box (named the "Bronx box") as an alternative light detection method to the expensive luminometer (Riska *et al*., 1999). The LRP assay was performed in a microwell plate format and light production was measured by both a luminometer and the Polaroid film ("Bronx box") method. Despite the cost reduction that the Polaroid film method offers, it still requires use of a biohazard unit to perform the assay which limits its use. Limited reports of testing of the LRPs on clinical samples have been published. A study on 71 Mexican sputum samples showed that the LRP assay had a poor sensitivity of 76% on culture positive samples and a specificity of 93% (Banaiee *et al*., 2001). The low sensitivity was attributed to a high contamination rate. The same study also investigated the use of NAP to distinguish between *M. tb* complex and *M. tb* strains, of which 94% were correctly identified. Drug susceptibility required a median time of 2-4 days and was in agreement with BACTEC 460 results in 98.5% of the cases tested. A further modification of the LRP assay was the use of the LRP phAE142 (Bardarov *et al*., 2003). Mycobacteriophage phAE142 was constructed to utilize the P_{left} promoter from mycobacteriophage L5 to drive *Fflux* expression. Propagation of phAE142 required a special strain of *M. smegmatis* which expresses a repressor protein for the P_{left} promoter in order to avoid mutations in the *Fflux* gene (Bardarov *et al*., 2003). The phAE142 based LRP assay was tested in several studies, which showed a high sensitivity and specificity (Banaiee *et al*., 2003, Bardarov *et al*., 2003, Hazbon *et al*., 2003). The assay was performed on cultured samples, required a median of 3 days for DST and had a detection limit (for accurate DST) of $0.5X10^5$ to $1X10^5$ cfu/ml (Banaiee *et al*., 2003, Bardarov *et al*., 2003). Dusthackeer and co-workers evaluated the potential to detect both dormant and active *M. tb* bacilli by testing various mycobacterial promoters that are potentially active during dormancy in LRP assays (Dusthackeer *et al*., 2008). The promoters of *hsp60,* isocitrate lyase (*icl*) and alpha crystalline (*acr*) genes from *M. tb* were cloned to drive *Fflux* expression in both mycobacteriophage Tm4 and Che12. The authors reported success in detecting dormant and active *M. tb* in clinical samples using mycobacteriophage Tm4 based constructs and could detect as low as 10^5 cfu/ml in clinical samples

(Dusthackeer *et al.*, 2008). The ability of Tm4 to infect dormant cells has been attributed to motifs in the Tm4 structure (Mt3 motif in the tape measure protein) allowing infection of stationary phase cells (Piuri & Hatfull, 2006).

Luciferase reporter phages have several drawbacks that limit their use. The means of detecting light production is problematic since the LRP assays require luminometers which are expensive and require skilled operators and biohazard facilities, which is non-viable for use in the field studies (McNerney & Traore, 2005). The "Bronx box" which uses a Polaroid film offers a lower cost alternative, but has the same problems as the luminometer based assays. LRP assays tested to date have good sensitivity and specificity when performed on cultured samples, but little is known of their performance directly on sputum samples (Pai *et al.*, 2006b). The LRP assays require a high cfu/ml for DST, which necessitates initial culture in addition to the minimum of 2-3 days for the LRP assay (McNerney & Traore, 2005). Despite the problems with the LRP assays, they are currently the fastest option for phenotypic drug susceptibility assays (Banaiee *et al.*, 2003).

1.3.6.2.2 Fluorescent mycobacteriophages

Piuri and co-workers created recombinant Tm4 mycobacteriophages that expresses fluorescent proteins for the detection of *M. tb* (Piuri *et al.*, 2009). A modified green fluorescence protein (*EGFP*) as well as *ZsYellow* fluorescence protein was cloned into mycobacteriophage Tm4, together with the *M. bovis* BCG *Hsp60* promoter. The diagnostic was performed by incubating the fluorescent mycobacteriophages for 16-30h together with cultured samples. The samples were then fixed with 2% paraformaldehyde and washed with buffer, before finally evaluating them using fluorescence microscopy. DST was similarly performed, however it required that the samples be incubated in the presence of antibiotics and recombinant mycobacteriophage, whereafter fluorescence indicated drug resistance. The assay could detect drug resistance to several antibiotics, including kanamycin, isoniazid (requires additional 24h incubation), rifampicin, streptomycin and ofloxacin (ciprofloxacin and sparfloxacin according to preliminary results). The sensitivity was 94% for isoniazid, rifampicin and streptomycin and the specificity was 90%, 93% and 95%, respectively, for the listed drugs using the proportion method as reference (Rondon *et al.*, 2011). The time to detection was 2 days post culture

for rifampicin, streptomycin and ofloxacin and 3 days post culture for isoniazid resistance detection (Rondon *et al*., 2011). The assay has not yet been adapted for clinical material and has insufficient sensitivity and specificity to warrant large scale application (Rondon *et al*., 2011).

One concern for use of mycobacteriophages as TB diagnostic is the possibility of host phage-resistance. Common mechanisms for phage-resistance is through restriction, CRISPRs (clustered regularly interspaced short palindromic repeats) and alteration of the cell surface or cell membrane (Labrie *et al.,* 2010). However, little is known of the mechanisms involved for resistance to mycobacteriophages by mycobacteria. It has been shown that CRISPRs are present in *M. tb*, however with little sequence homology to the current collection of mycobacteriophage genomes (Hatfull 2010). It has however been shown that overexpression of the *M. smegmatis* wild-type *mrp* gene leads to resistance to mycobacteriophage D29 and that *M. smegmatis* lipid extracts have been shown to be able to inhibit the binding of certain mycobacteriophages (Hatfull 2010). One possible means to circumvent the possibility of mycobacteriophage resistance would be to implement the use of a mycobacteriophage cocktail strategy in which various mycobacteriophages with different cell receptor regognition and infection mechanisms are employed.

Reporter mycobacteriophages could potentially act as a point of care diagnostic suitable for use in low resource settings. However, this would require that the biohazard risk be minimal and that detection of the reporter signal be possible using low-cost equipment. As reporter mycobacteriophage based assays are capable of DST, it is also possible that these diagnostics could provide low-cost DST tests. If these problems could be addressed, it would be possible for reporter mycobacteriophages to act at the forefront of TB diagnostics in the form of a cheap diagnostic with wide spread use. Implementation of such diagnostics would render other diagnostics redundant with the exeption of cases where extensive DST testing is required or where the diagnostic speed similar to the Xpert®MTB/RIF is required. As a means of quality assurance, it would also be nessisary to reduce or remove the possibility of false positives which would be possible through selective culture, use of of *M. tb* specific mycobacteriophages or use of *M. tb* specific promoters.

1.4 TB diagnostics conclusion

The need for rapid, simple, cheap and effective TB diagnostics remains largely unmet. Each of the discussed TB diagnostics has limitations that cause them to inefficient for wide-spread TB detection and an optimal diagnostic thus remains elusive. Recent advances in TB diagnostics have broadened the options; however they are in their infancy and require further refinement. The downfalls of the current routine TB diagnostics are highlighted in Table 2.

Table 2. Drawbacks of current TB diagnostics

Diagnostic	Drawbacks
Culture	Expensive, time consuming, requires biohazard facilities, not suitable for low resource settings
Chest radiography	Inaccurate, not suitable for field use.
Microscopy	Limited throughput, requires experienced staff, limited sensitivity and specificity, cannot perform DST or speciation
NAAT	Expensive, requires skilled staff, requires equipment unsuitable for low resource settings.
Antigen based assays	Not an accurate indicator for active TB, cannot perform DST or speciation
Bacteriophage replication assays	Requires a biohazard facility, prone to contamination, time consuming
Luciferase reporter phage assays	Time consuming, requires initial culture, increase biohazard risk due to substrate addition, requires expensive luminometer, not suitable for field use
Fluoromycobacteriophages	Requires expensive fluorescence microscope, utilizes suboptimal promoter, no-means of signal or bacterial concentration

Each of these issues could be addressed to produce a superior diagnostic tool. In order to fully comprehend the current drawbacks as well as to grant insight as to potential improvements, it is prudent to perform a comprehensive literature review on all phage based diagnostics as a whole. The following is a review of bacteriophage based diagnostics with specific application to pathogen detection.

1.5 Phage-based detection of bacterial pathogens

1.5.1 Introduction

Pathogenic bacteria pose a global health threat and cause extensive morbidity and mortality each year. Salmonella infection, for example, was estimated at 1.4 million cases annually in the USA with numbers potentially much higher in third world countries (Voetsch *et al*., 2004). Increased spread of drug resistance among pathogenic bacteria and multiple drug resistance, best exemplified by the drug resistant *M. tb* epidemic, is a cause for concern. Transmission of drug resistance has also been observed among various pathogens such as the gram negative *Citrobacter freundii*, *Klebsiella pneumoniae*, *Acinetobacter, Proteus mirabilis*, *Pseudomonas aeruginosa* and *Escherichia coli* as well as the gram positive *Staphylococcus aureus* and *Enterococci (Centers for Disease Control and Prevention (CDC), 2010, de Costa & Mavalankar, 2010, Krishna, 2010, Walsh & Fanning, 2008).* A recent example of the alarming rate at which drug resistance spreads is the spread of NDM-1 (New Dehli Metallo-β-lactamase) between clinical isolates of the Gram-negative *Enterobacteriaceae* from India to Europe, USA and to Japan (Mazzariol *et al*., 2012, Yong *et al*., 2009). The NDM-1 enzyme is particularly problematic since it is resistant to almost all β-lactams and easily transfers resistance between bacterial species (Poirel *et al*., 2010). The rise in drug resistance and dwindling drug treatment options emphasize the dire need for rapid and effective diagnostics to contain the spread of bacterial pathogens. The requirements for these novel diagnostics may fluctuate between individual applications for pathogen detection. For example, a diagnostic for *Bacillus anthracis* requires utmost priority on speed, since infection may prove fatal within hours. In contrast pathogen detection in food and water requires that the sensitivity be the highest priority. Generally though, diagnostics need to strike a balance between their speed, sensitivity, specificity, ease of use and cost. Although culture has been a wide-spread method to detect bacterial pathogens, as well as determine their drug resistance, the method is inefficient. Culture is limited to viable and culturable cells which is problematic for sensitive detection of pathogens since viable but not culturable (VBNC) cells, such as pathogenic *E. coli,* is common in water (Liu *et al*., 2010). Furthermore, culture of certain pathogens requires specialized biohazard facilities, such as for *M. tb* and *Bacillus anthracis,* which is not applicable for field use. Molecular methods provide a rapid alternative to culture yet are expensive and are also not applicable for wide-spread use, due to cost and the need for skilled operators.

Bacteriophages offer key features that could provide simple solutions to the diagnostic needs for bacterial pathogens.

1.5.2 Bacteriophage background

Bacteriophages are naturally occurring viral particles that infect and replicate in their bacterial hosts. Bacteriophages are the most abundant organisms on earth above all other species and it has been estimated that 10^{25} phages commence a new infection cycle every second, (Pedulla *et al*., 2003). Bacteria and bacteriophages are thus under immense selective pressure to continuously evolve accordingly in order to survive in each other's presence. The vast pool of bacteriophage biomass offers a wealth of tools to understand and combat bacteria. Of particular importance are the potential tools that may arise from bacteriophages of pathogenic bacteria. Bacteriophages offer several unique properties to be exploited to create superior diagnostics that could fulfill the criteria required to effectively detect pathogenic bacteria and curb the spread of epidemics. Bacteriophages are generally host-specific, capable of infecting only a specific species or even strain, with some exceptions such as *Listeria* phage A511 (which can infect an entire genus) (Loessner *et al*., 1995).

The life cycle of bacteriophages are visually illustrated in Figure 1. The life cycle starts with the phage tail fibers and attachment proteins recognizing specific receptors on the surface of the host cell, followed by binding (adsorption) of the bacteriophage to its host. The receptors include outer membrane proteins such as cell-surface proteins, polysaccarydes, lipopolysaccharides, flagella, sex pili and lipoproteins (Neufeld *et al*., 2003, Neufeld *et al*., 2005). Adsorption to the host is followed by either insertion of the entire phage particle, or more commonly, the phage DNA into the host. Bacteriophage infection and replication is however influenced by factors such as bacterial growth stage, co-factor concentrations (such as Ca^{+} and Mg^{+}) as well as temperature (temperature sensitive phages can only replicate at specific temperatures). Under favorable conditions that permit infection, bacteriophage genes are subsequently expressed by the host's own cellular machinery. Several bacteriophages even express genes that code for their own tRNA to order to compensate for codon usage biases and the genetic differences between host and bacteriophage (Bailly-Bechet *et al*., 2007).

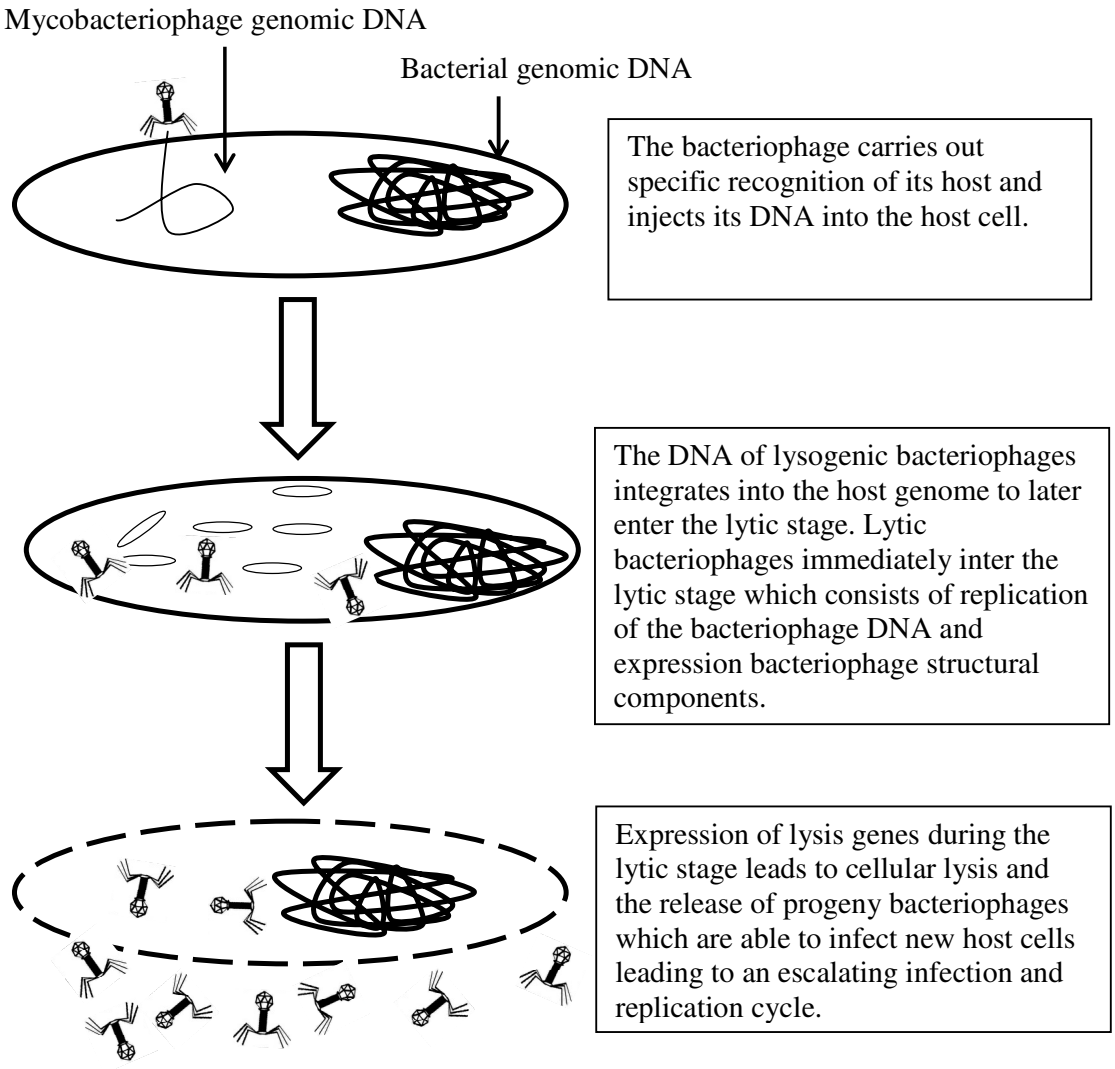

Figure 1: Bacteriophage reproductive cycle.

The amount of phages that infects or adsorbs to a single bacterium is referred to as the multiplicity of infection (MOI). If the MOI is greater than the threshold of adsorption, a phenomenon called “lysis from without” (LO) occurs. LO leads to cellular lysis without the production of progeny phage. In the case of a lower MOI, infection leads to successful expression of bacteriophage genes. Some of the first genes to be expressed in several bacteriophages are those responsible for superinfection immunity. Superinfection immunity refers to the ability of certain bacteriophages to block infection and replication of additional bacteriophages that enter the bacterial cell. This is possible by expression of repressor proteins. For example mycobacteriophage L5 expresses a 183-amino-acid product that confers superinfection immunity against additional L5 mycobacteriophages (Donnelly-Wu *et al.*, 1993). Following bacterial infection, bacteriophages have differing life cycle potentials. Certain bacteriophages are lytic in nature, incapable of entering the lysogenic phase, whereas other bacteriophages are temperate, capable of both lytic and lysogenic phases. Lysogenic bacteriophages can integrate their genomes into the host genomic DNA to create what is called a lysogen. This lysogen can stay dormant and be copied along with the bacterial genomic DNA in cellular division or be activated, usually from bacterial stress responses, and enter the lytic phase. Lysogens can also alter the bacterial phenotype and even increase pathogenicity (Eklund *et al.*, 1971, Figueroa-Bossi *et al.*, 2001, O'Brien *et al.*, 1984). During lytic growth the phage genome replicates and its lytic genes are expressed to also produce its head and tail proteins. The phage DNA is packaged into the phage heads, followed by attachment of the phage tails. In addition to the phage capsid genes, holin and lysin genes are also expressed during the lytic cycle. Holins and lysins control the timing of cellular lysis. Holins alter or degrade cytoplasmic membranes, granting lysins access to the peptidoglycan leading to cell lysis (Tanji *et al.*, 2004). Holins and lysins are thus under immense genetic selection pressure to achieve the optimal progeny phage production in order to assure an optimal burst size. The burst size varies widely between individual bacteria, mycobacteriophage D29, for example, has been observed to have an average burst size of 98 phage particles and is affected by both culture conditions and growth stage (Sellers *et al.*, 1962). The progeny released following infection of one bacterial host cells leads to an enormous phage amplification culminating in a potentially exponential escalation of bacteriophage progeny in the presence of viable bacterial hosts. In summary the unique features that bacteriophages present are their extreme host specificity, and sensitivity, and their ability

to rapidly replicate in their host (producing escalating progeny numbers). The following section provides a summary of how these unique properties of bacteriophages have been exploited to create bacteriophage-based diagnostics for several bacterial pathogens.

1.5.3 Bacteriophage-based diagnostics

1.5.3.1 Plaque-based assays

Initially, bacteriophages were only utilized to determine bacterial taxonomy and phage typing. Phage typing is a method of bacterial characterization by susceptibility testing to various bacteriophages, which enables the bacterial genus and species to be determined (Redmond & Ward, 1966). The method is based on detection of plaques on bacterial lawns and has been applied to several bacteria, of which a few are listed in Table 3.

Table 3. Examples of phage typing assays

Bacterial species	Reference
Bacillus anthracis	Abshire,T.G. 2005
Bacillus cereus	Ahmed,R. 1995
Brucella spp	Corbel,M.J. 1987
Campylobacter spp	Khakhria,R. 1992
Clostridium spp	Sell,T.L. 1983
Escherichia coli	Grif *et al.*, 1998, Nicolle *et al.*, 1952, Khakhria,R. 1990
Enterococci spp	Caprioli,T. 1975
Listeria spp	Loessner,M.J. 1990, Gasanov *et al.*, 2005
Salmonella spp	Scholtens, 1962, Hickman-Brenner,F.W. 1991, Castro,D. 1992, Kuhn,J. 2002, Majtanova & Majtan, 2006,
Shigella spp	Slopek,S. 1969
Staphylococci spp	Sekaninova,G. 1998
Proteus spp	Sekaninova,G. 1998
Vibrio cholerae	Chakrabarti,A.K. 2000
Yersinia spp	Baker,P.M. 1982

The main caveat of using phage typing assays as a diagnostic, is that they rely on the host bacterial replication rate and lawn formation rate, which although fast for *E. coli* or *B. anthracis*, is slower for other bacteria such as *Mycobacteria (Abshire et al., 2005, McNerney & Traore, 2005)*. The assays also have the potential for contamination and have a specificity which is subject to the infection range of the bacteriophages (Kalantri *et al*., 2005, Pai *et al*., 2005). Plaque formation is however not the only diagnostic avenue that bacteriophages offer. Bacteriophages have several unique features which can be exploited to create novel diagnostics, one of which is the highly specific binding affinity for their host.

1.5.3.2 Detection of host lysis

The specificity with which bacteriophages infect their hosts grants specificity as to which cells subsequently undergo cell lysis. Detection of the release of host intracellular components due to bacteriophage cell lysis is the basis for several diagnostics (Blasco *et al*., 1998, Neufeld *et al*., 2003). Bacterial cell lysis releases several cellular components that can be detected or measured, such as adenosine triphosphate (ATP), adenylate kinase (AK) and β-D-galactosidase (Blasco *et al*., 1998, Neufeld *et al*., 2003). Specific detection of host lysis can be performed in mixed bacterial populations, since bacteriophages are strain specific (Blasco *et al*., 1998, Neufeld *et al*., 2003, Yemini *et al*., 2007). Detection of host lysis and release of ATP has previously been performed in industry by the addition of luciferase and luciferin (Blasco *et al*., 1998). However this method has a low detection limit of about 10^3 to 10^4 cells (Blasco *et al*., 1998). An improvement on the method is to detect adenylate kinase (AK) released by host bacteria following bacteriophage cell lysis. AK is an essential enzyme in most bacterial cells that catalyzes the equilibrium reaction:

ATP + AMP ←→ 2ADP

Addition of ADP drives the reaction to produce ATP and this can be used to fuel light production in bioluminescence reactions, creating a sensitive detection assay for AK. Blasco *et al* investigated the use of bacteriophage for specific bacterial lysis in combination with the AK detection assay (Blasco *et al*., 1998). The assay, when applied to *E. coli* detection, could detect fewer than 10^4 cfu/ml in less than 1h and 2h for *Salmonella newport* detection. This was later improved to 10^3 cfu/ml for *E. coli* as well as

Salmonella, with a detection time of less than 2h, by optimizing the MOI, bacterial growth stage and duration of infection (Wu *et al*., 2001).

β-D-galactosidase is another common cellular constituent which is released following host lysis by bacteriophages. Neufeld *et al* developed an electrochemical assay that detects the release of β-D-galactosidase amperometrically (Neufeld *et al*., 2003). β-D-galactosidase is released by *E. coli* K-12 MG1655 as it is undergoes cell lysis by bacteriophage lambda vir gene. The target sample is then filtered to separate the lysed cellular components from intact cells which potentially also contain β-D-galactosidase. The current resulting from the activity of β-D-galactosidase, is measured electrochemically in real-time as it hydrolyzes p-aminophenyl-β-D-galactopyranoside to produce p-aminophenol (which is oxidized at the carbon anode). Use of filtration of the target sample (and pre-incubation) allowed the assay to detect as low as 1 cfu per 100 ml in 6-8h. The assay has the advantage of detecting enzymatically active cells which are not necessarily culturable, in contrast to phage replication assays which can only detect viable cells. The assay requires a pre-incubation step for concentrations of bacteria lower than $2.5X10^3$ cfu/ml, for example detecting 100 cfu/ml requires 3-4h pre-incubation. The electrochemical assay was later also adapted for detection of *Bacullus cereus* and *M. smegmatis* (Yemini *et al*., 2007). The assay consisted of filtering of the target samples, infection of the target bacteria with bacteriophages, incubation with the substrate until lysis was complete, filtration of the sample followed by electrochemical analysis. The bacteriophage B1-7064 and substrate *para*-amino-phenyl-α-D-glucopyranoside was used for *B. cereus*. Mycobacteriophage D29 and substrate *para*-amino-phenyl-β-D-glucopyranoside was used for *M. smegmatis.* The modified assay could detect 10 cfu/ml in 8h. Assays based on host cell lysis detection have the inherent risk of background signal from non-target bacteria that possess similar reporter signals. One means to circumvent the background risk is through methods that focus on the specificity that phage binding provide without utilizing reporter signals from host lysis.

1.5.3.3 Specific binding and adherence to host

Currently, specific bacterial antigen detection is based on antibodies, due to the high specificity that they offer. Bacteriophages bind to their bacterial hosts with similar high specificity, however they have the added advantages of having a reliable specific binding and a significantly cheaper large scale production cost. This has led to the investigation of the diagnostic uses of the phage particle in a bacterial detection assay as well as assays that make use of phage binding proteins.

1.5.3.3.1 Bacteriophage biosorbents

Immuno-PCR is based on antibody capture of a specific antigen followed by PCR amplification. In general, the method relies on covering a surface with host/antigen specific antibodies followed by wash steps to remove unbound antibodies. The sample to be probed is then added which potentially contains the target organism followed by wash steps to remove excess material which is unbound to the antibodies. The sample is then PCR amplified to detect target DNA. A variation on the method is to use bacteriophage as antigen capture particle, or rather biosorbent. In principle the method consists of immobilization of bacteriophage onto a surface, removal of excess bacteriophage, addition of the sample potentially containing the target host bacteria, followed by wash steps and various detection methods. An example of this method was the use of Sapphire phage (Amersham Inernational) for detection of *Salmonella* (Bennett *et al*., 1997). The Sapphire phage was immobilized (by overnight incubation) onto microplates with various surface properties as well as onto a dipstick format. The bacterial detection assay for the plate format involved adding the crude bacterial sample to the plate wells followed by an incubation step, a wash step, a PCR of the lysate and finally visualization by gel-electrophoresis. In the dipstick format, detection was based on addition of a fluorescent dye followed by fluorescent microscopy. The authors reported poor performance due to inefficient phage immobilization, stating that their method allowed both head and tail to immobilize onto the solid surface due to passive absorption (Bennett *et al*., 1997). The authors also stated that active immobilization of the phage head, exposing the phage binding proteins, would be necessary for optimal bacterial capture and that this would require knowledge as to the structure of the phage surface chemistry. As such, an improvement on this method would be through the use of bacteriophage binding proteins instead of the entire phage particle.

Investigation of one such binding protein followed the discovery that the C-terminal region of a binding protein from γ-phage specifically binds to the cell wall of *Bacillus anthracis* (Fujinami *et al.*, 2007). This particular binding protein is called Phage-Lysin-Gamma (PlyG) and is a lysin protein. The region of PlyG conferring the binding activity was cloned to contain a Glutathione S-transferase (GST) tag and the recombinant protein was designated PlyGB-GST. The bacterial detection assay consisted of blotting bacterial suspensions onto a nitrocellulose membrane, blocking the membrane and exposing it to the PlyG-GST fusion proteins. Horseradish peroxidise-conjugated mouse anti-GST and a tetramethylbenzidine membrane peroxidise substrate was added and the resultant signal was then visualized using an imaging system. This assay is more rapid (about 3 hours) and is more sensitive than the plaque-based detection methods using γ-phage (Fujinami *et al.*, 2007). The specificity was similar to γ-phage methods with a detection limit of 10^3 cfu/ml (Fujinami *et al.*, 2007). The authors also stated that the method is cheaper and simpler to perform compared to PCR and immunological assays. The authors speculated that the assay could be improved by labeling the recombinant PlyG protein with stable quantum dot nanocrystals (Fujinami *et al.*, 2007). To this goal, a recombinant biotin-tagged PlyG together with streptavidin-conjugated quantum Dot nanocrystals was investigated for use as an improved diagnostic for *Bacillus anthracis* (Sainathrao *et al.*, 2009). The assay consisted of incubating biotin-tagged PlyG with bacterial samples, followed by addition of streptavidin-conjugated quantum dot nanocrystals. Fluorescence was measured by fluorescence microscopy or fluorometry using a micro-plate reader. The assay was very rapid and showed high sensitivity. It was able to detect single cells, yet the technique requires fluorescence microscopy or fluorometry (Sainathrao *et al.*, 2009). In contrast to using bacteriophage binding proteins, use of bacteriophages in their entirety have also been investigated as cell-markers for bacterial detection.

1.5.3.3.2 Labeled phage

The specificity with which bacteriophages bind to their hosts has led to the investigation of using stained and labeled bacteriophages for bacterial detection. Horseradish peroxidase (HRP), a common enzymatic reporter for various assays such as in ELISA (enzyme-linked immunosorbent assay) has been utilized as enzymatic label for bacteriophages for *E. coli* detection (Willford *et al*., 2011). The Shiga toxin producing *E. coli* specific bacteriophages CBA120, AR1 and bacteriophage 56 were selected for chemical labeling using a commercial HRP labeling kit. The detection assay consisted of taking a swab which is used to inoculate selective media containing immunomagnetic beads. The immunomagnetic beads, which are specific for shiga toxin producing bacteria, are then incubated for 8h together with the swab. The beads are then washed to remove excess bacteriophage and are incubated with the HRP labeled bacteriophages followed by another wash step to remove excess bacteriophage. At this stage one of two substrates are added, a colorimetric substrate or a luminescent substrate. The colorimetric substrate leads to a colour change to yellow and the luminescence substrate resulted in light production which was measured with a luminometer. The assay`s sensitivity without enrichment was between $6.8x10^4$ cfu/ml to $3.85x10^5$ cfu/ml which decreased to 1 cfu/ml with 8h enrichment (Willford *et al*., 2011). A similar method combined immuno-separation of *E. coli* O157 with detection of fluorescently labeled bacteriophage binding to bacteria (Goodridge *et al*., 1999a). The method consisted of immuno-separation of the target bacteria by means of magnetic beads (Dynal Inc., Lake Success, N.Y.) a wash step to remove excess bacteria followed by incubation with labeled bacteriophage. The bacteriophage used in this study was bacteriophage LG1, which had been stained with the nucleic acid dye YOYO-1 (Molecular probes, Inc., Eugene, Oreg.). The beads were subsequently magnetically separated and washed to remove excess labeled bacteriophage followed by flow cytometry. The assay was able to detect 10^4 cfu/ml in 8h (Goodridge *et al*., 1999a). The assay was also adapted to detect *E. coli* O157 in food samples such as ground beef and raw milk (Goodridge *et al*., 1999b). The assay was able to detect 2.2 cfu/g in 7h in ground beef and could detect between 10 and 100 cfu/ml in raw milk in 12h. In contrast to utilizing stained bacteriophages, a means to reduce production steps (and cost) is to utilize recombinant bacteriophages that express fluorescent proteins which are fused to structural proteins. An example of this was the use of a recombinant T-even type PP01 bacteriophage for detection of enterhemorrhagic *E. coli* strain

O157:H7 (Oda *et al*., 2004). The recombinant PP01 bacteriophage was engineered by homologous recombination to contain a green fluorescent protein (GFP) tag to a structural protein – the small outer capsid protein (Oda *et al*., 2004). The research team found N-terminal fusions of GFP to the small outer capsid protein to have optimal fluorescence intensity post host infection, indicating functionality of the fluorescent fusion protein. Furthermore, the GFP-small outer capsid protein fusion increased phage binding affinity to their host but decreased stability in acidic and alkaline solution in comparison to wild type phage. The detection assay consisted of incubation of the bacterial sample with the recombinant bacteriophage for 10 min and a wash step to remove excess bacteriophage, after which labeled bacteria could be detected by fluorescence microscopy. The assay showed increasing fluorescence intensity due to bacteriophage replication after 1h incubation of *E. coli* O157:H7 with the recombinant phage and reached maximum fluorescence at 3h (Oda *et al*., 2004). The assay could detect culturable cells as well as viable but non-culturable (VBNC) cells by modifying the MOI and incubation time. A low MOI and incubation time of greater than 1h was used to detect culturable cells using a fluorescent microscope, since this allowed for expression of the reporter gene. VBNC cells were detected by using a higher MOI to detect the adsorbed fluorescent phages on bacterial host cell membrane using a fluorescent microscope. In lieu of the lytic nature of PP01, which could potentially decrease sensitivity in microscopy based bacterial detection, a recombinant T4 bacteriophage incapable of lysis was investigated for use for *E. coli* detection (Tanji *et al*., 2004). The bacteriophage T4 was engineered to have a mutation in its lysozyme gene (named *Gpe*). The recombinant T4 was also created to have a GFP fusion to the small outer capsid protein. The recombinant bacteriophage, named T4e-/GFP maintained infectivity and host fertility without the ability to lyse the host. The T4e-/GFP assay consisted of incubation of the recombinant bacteriophage with the bacterial sample, a wash step to remove excess bacteriophage, followed by fluorescence microscopy. The assay required a detection time of 10-30 min for detecting both viable *E. coli* as well as viable but non-culturable *E. coli* and could discern between the two cellular states in less than an hour (Tanji *et al*., 2004). The assay was also applied to the detection of *E. coli* in sewage influent, which showed that the recombinant bacteriophage T4e-/GFP could not infect all strains of environmental *E. coli* indicating the need for a bacteriophage with a wider host range (Namura *et al*., 2008). This lead to the investigation of a variety of bacteriophages that infect environmental *E. coli*, of which two were found to be superior

in their host range (Namura *et al.*, 2008). The two bacteriophages, IP008 and IP052, were modified to replace their lytic genes with a GFP gene in addition to a GFP fusion to their small outer capsid genes (Namura *et al.*, 2008). The combined host range of the recombinant bacteriophages allowed the assay to detect 35 out of 70 strains of *E. coli* in a few hours using fluorescence microscopy (Namura *et al.*, 2008). It has been suggested, however, that the use of GFP is problematic since it has a low signal to noise ratio due to background and cellular auto-fluorescence, as well as stability and photo-bleaching problems (Edgar *et al.*, 2006). The advantages of streptavidin-tagged quantum dot nanocrystals in conjunction with biotin-tagged bacteriophages was investigated as a departure from stained and fluorescent protein tagged bacteriophage methods (Edgar *et al.*, 2006). A recombinant E. coli phage T7 was constructed for detection of *E. coli* by fusing a biotinylation peptide tag to the T7 major capid protein (named gp10a). The assay consisted of incubation of the recombinant bacteriophage with the bacterial sample together with streptavidin-coated quantum dots. Following infection of viable bacterial cells, the recombinant bacteriophage T7 becomes biotinylated by the native *E. coli* biotinylation enzymes. The biotinylated bacteriophages subsequently bind to the streptavidin-coated quantum dots, which can then be visualized by fluorescence microscopy. The researchers found that visual detection of target bacteria was possible within 1h and that the detection of as low as 10 cfu/ml of sample was possible. Individual cells, labeled phage as well as individual quantum dots could be visualized (Edgar *et al.*, 2006). The lytic nature of bacteriophage T7 has, however, been suggested to hamper the sensitive detection of single cells (Yim *et al.*, 2009). To circumvent this problem the use of a non-lytic or conditionally replicating bacteriophage is necessary. To achieve this goal, bacteriophage lambda gt11 was chosen since it contains mutations which make it temperature sensitive. Bacteriophage lambda gt11 was engineered to contain a biotin-binding peptide fusion to the bacteriophage major coat protein gpD (Yim *et al.*, 2009). The biotin-tagged bacteriophages were harvested following temperature induced replication. The progeny bacteriophages are then biotinylated by the *E. coli* host biotinylation enzymes, after which the bacteriophages could be purified. The purified and biotinylated bacteriophage lambda gt11 was then added, together with streptavidin coated quantum dots, to the bacterial sample and incubated at room temperature. Since lambda gt11 is temperature sensitive, the bacteria do not undergo lysis at room temperature, intact cells could be visualized using fluorescence microscopy or flow cytometry. Although the fluorescent properties of quantum dot nanocrystals are

potentially superior to common fluorophores, the assay could be simplified, and potentially be made more versatile, by making use of phage-based expression of a reporter signal, which does not necessitate addition of substrate or dyes for detection. Although single cell detection is possible, the main drawback of the assay is that it requires addition of substrate (streptavidin-coated quantum dots) and the fluorescence signal is thus not self-driven as in the expression of reporter proteins. Additionally, the use of flow cytometry and fluorescence microscopy does not lend itself readily for field use. This warrants the investigation of not only fluorescent proteins as reporter signal, but the investigation of all potential reporter proteins or signals.

1.5.3.4 Reporter gene expression

Reporter bacteriophages are specific as to which bacterial hosts they infect which also grants specificity as to which bacteria express the reporter gene(s). Considering reporter bacteriophages that do not make use of incorporation of the reporter signal into structural components, as discussed in the above Sections, certain benefits become apparent. These reporter bacteriophages have the inherent advantage of only infecting viable cells and thus do not give false positives for drug-killed cells as with PCR (Dinnes *et al*., 2007). Furthermore, the reporter signal can also be expressed in dormant bacteria (Dusthackeer *et al*., 2008). To date, several bacteriophage reporter assays have been developed which will be explored in this section.

1.5.3.4.1 Ice Nuclease

Supercooled water can remain liquid below 0℃, but can rapidly undergo a chain reaction of freezing when ice nuclei, or a nucleating agent is introduced to it (Franks, 2003). Various organisms have the ability to cause nucleation of ice in supercooled water, such as *Pseudomonas, Erwinia* and *Xanthomona* (Corotto *et al*., 1986). The first recombinant bacteriophage to make use of ice nucleation as reporter signal utilized the *inaW* gene from *Pseudomonas fluorescens* (Wolber & Green, 1990). The *inaW* gene was transferred to bacteriophage P22 for specific detection of *Salmonella.* Expression of the *inaW* gene causes ice nucleation at temperatures below -9.3℃ which allows the cells to freeze. Detection of ice formation is aided by making use of a fluorescent freezing-indicator dye (Wolber & Green, 1990). When tested on *Salmonella,* the assay could detect less than 10 cfu/ml in mixed

bacterial populations indicating that an enrichment step is not required (Wolber & Green, 1990). The assay was later shown to be able to detect as low as 2 cfu/ml (Wolber, 1993). A commercial assay based on the ice nuclease reporter bacteriophage P22 was also developed called the Bacterial Ice Nucleation Diagnostic (BIND) assay which could detect less than 3 cfu/ml in 3h (Irwin *et al.*, 2000). Assays based on ice nucleation have the advantage that background bacteria are unlikely to contain similar gens to *inaW* which makes the assay specific. Sadly, no further ice nuclease reporter bacteriophage assays have since been developed, perhaps due to the need of accurate temperature control devices which would be less than ideal for field use as well as due to favorable performance of other reporter proteins.

1.5.3.4.2 Enzymatic reporters

The utilization of enzymes as reporter genes has the advantage that a small amount of enzyme can produce a relatively large amount of reporter signal. To date, several studies have investigated the use of enzymes as reporter genes for reporter bacteriophages. One study investigated the use of alkaline phosphatase as reporter enzyme for detection of *E. coli* TG-1 (Neufeld *et al.*, 2005). The researchers utilized helper bacteriophage M13KO7 to deliver a commercial plasmid which leads to expression of alkaline phosphatase. Although not a true reporter bacteriophage (since it lacks a bacteriophage genome), the assay is an example of how bacteriophages can deliver enzymatic reporter genes to their hosts and lead to expression in target bacteria. The diagnostic assay is based on infecting *E. coli* with the helper bacteriophage which then leads to the expression of alkaline phosphatase (AP). AP reacts with *p*-aminophenyl phosphate to produce *p*-aminophenol in an oxidation reaction which is measured using an electrochemical cell. The assay could detect 1 cfu/ml in 2-3h (Neufeld *et al.*, 2005). The phagemid assay is, however, not a true reporter bacteriophage since the phasmid cannot replicate and thus the only beneficial bacteriophage feature that it offers is through specificity of host infection.

The prokaryotic LacZ gene is a more widely used reporter gene and the activity of its product enzyme can be monitored using various fluorogenic and chromogenic substrates. The LacZ gene comes from the lac operon and encodes β-galactosidase, which catalyzes the hydrolysis of β-galactosides such as

lactose into monosaccharides. When used as a reporter gene, its product is forced to hydrolyse easily detectable substrates such as the chromogenic substrate X-gal (5-bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside). During X-gal hydrolysis, subunits are formed that have a deep blue colour generating a signal easily detectable to the naked eye. The enzyme, in its active form, is a tetramer of four identical monomers creating a protein of around 465kDa with a conformation that makes fusion proteins possible on the N-termini (Juers *et al.*, 2000). Since several bacteria express β-galactosidase, it is important to make sure no background expression leads to false positives when using the LacZ gene as reporter gene. One means to increase specificity is to make use of immuno-magnetic separation in order to first specifically capture the target organism before substrate is added. Unpublished results have reported on the development of a recombinant β-galactosidase expressing bacteriophage T4 for *E. coli* detection (Goodridge & Griffiths, 2002). The assay consisted of adding the recombinant bacteriophage together with a chemiluminescent substrate (GalactoStar) to the bacterial sample. The recombinant T4 bacteriophage was reported to have a detection limit as low as 10^2 cfu/ml and as low as 1 cfu/ml using an 8h incubation (Goodridge & Griffiths, 2002). Although the use of enzymes allow a very low sensitivity, the assays that make use of them are liable to contamination. These assays require the addition of substrate and the potential that background bacteria contain similar enzymes are high which would lead to false positives.

1.5.3.4.3 Bioluminescence

Bioluminescence is the emission of light in living organisms from enzyme-catalyzed reactions. Luciferase is an example of an enzyme that catalysis a yellow-green light-producing reaction in the presence of both ATP and a suitable substrate such as luciferin. The prokaryotic *lux* and eukaryotic *luc* bioluminescence genes have been extensively investigated and utilized as reporter genes. Several luciferases have been isolated and cloned which provide tools for bacterial detection. Luciferase genes have also been cloned into bacteriophages to offer specific light production in target bacteria. An example of this was the construction of an auto-inducer expressing bacteriophage for the detection of viable *E. coli* cells (Ripp *et al.*, 2006). The assay is based on the *luxCDABE* operon of *Vibrio fischeri*. The *luxAB* component synthesizes luciferase and is under control of the regulatory genes *luxI* and *luxR*. *luxI* codes for a regulatory protein called an autoinducer which interacts with *luxR* which in

turn stimulates transcription of *luxCDABE* and *luxI.* As the concentration of autoinducer rises so too does transcription of luciferase, luxI and *luxR* binding which creates an auto-amplified loop generating increasing levels of bioluminescence. The assay makes use of a recombinant bacteriophage lambda which expresses the regulatory *luxI* gene following infection of *E. coli* (Ripp *et al.*, 2006). The assay consists of adding recombinant bacteriophage lambda to the bacterial sample together with additional bacteria which contain the *luxCDABE* and *luxR* genes (but not the *luxI* gene). The expression of *luxI* following the specific cell infection and host lysis by bacteriophage lambda creates an auto-amplified bioluminescent signal. The assay was capable of detecting 10^8 cfu/ml within 1.5h and could detect 1 cfu/ml with a pre-incubation step in 10h. This method was also applied to the detection of *E. coli O157:H7* in food samples using a 96-microwell format (Ripp *et al.*, 2008). The microwell assay could detect 1 cfu/ml in 6 to 6.5h following a pre-incubation step of 6h and could detect 10^2cfu/ml in 5h without pre-incubation. The *luxI* expressing bacteriophage lambda assay was also tested on *E. coli* K12 variant XL1-Blue (Birmele *et al.*, 2008). The lux expressing strain used in this assay was *E. coli* OHHLux which expresses luciferase in the presence of LuxI and which is resistant to bacteriophage lambda. The modified assay could detect 1cfu/ml in 24h in spite of bacterial starvation or the presence of lactonase producing bacteria indicating that the assay is resilient to contamination.

In contrast to utilizing only regulatory proteins to drive lux expression in trans, the entire lux operon has also been transferred to create a reporter bacteriophage that does not need helper bacteria for signal production. In fact, the very first luciferase reporter bacteriophage was constructed to use the entire *lux* operon from *Vibrio fischeri* which was cloned into bacteriophage lambda Charon 30 (Ulitzur & Kuhn, 1987). To do so the *lux* operon was first cloned into a plasmid and subsequently cloned into the lambda Charon 30 chromosome. The recombinant bacteriophage was capable of detecting 10-100 cfu/ml of *E. coli* in 1h (Ulitzur & Kuhn, 1987). Since then several luciferase bacteriophages have been constructed to express luciferase in their natural hosts as shown in Table 4.

Table 4. Luciferase reporter bacteriophage assays

Bacterial species	Reference
Bacilus anthracis	Schofield,D.A. 2009
E. coli	Ulitzer Kuhn 1987
E. coli	Duzhii zavilgelskii 1994
E. coli O157:H7	Waddel & poppe 2000
Enterobacteriaceae	Kodikara 1991
Listeria monocytogenes	Loessner 1996
Listeria monocytogenes	Loesner 1997
M.bovis BCG	Pearson,R.E. 1996
M.bovis BCG	Carriere,C. 1997
Mycobacterium avium Subsp *Paratuberculosis*	Sasahara,K.C. 2004
Mycobacterium smegmatis	Sarkis,G.J. 1995
Mycobacterium tuberculosis	Jacobs,W.R.,Jr 1993
Mycobacterium tuberculosis	Riska,P.F. 1999
Mycobacterium tuberculosis	Banaiee 2003
Mycobacterium tuberculosis	Riska,P.F. 1997
Mycobacterium tuberculosis	Bardarov,S.,Jr 2003
Mycobacterium tuberculosis	Dusthackeer,A. 2008
Salmonella	Steward 1989
Salmonella	Kuhn 2002
Salmonella spp	Chen Griffiths 1996
Salmonella typhimurium	Turpin et al 1993
Staphylococcus aureus	Pagotto 1996

A luciferease reporter bacteriophage for *Listeria monocytogenes* detection was developed by utilizing the *Vibrio harveyi luxAB* genes (Loessner *et al.*, 1996). The *luxAB* genes were cloned into bacteriophage A511 downstream of the major capsid protein by means of homologous recombination. The *luxAB* based assay enabled bioluminescence detection in 2h with a detection limit of $5X10^2$ to 10^3 cfu/ml in 2h and 1 cfu/ml in 24h by means of enrichment. The researchers later also adapted the technique for microwell use as well as for enumeration of bacteria in food samples (Loessner *et al.*, 1997). Another means in which luciferase reporter bacteriophages have been constructed was by means of transposon mutagenesis (Waddell & Poppe, 2000). A 3.6kb insertion containing the *luxA* and *luxB* genes was transferred to the chromosome of bacteriophage ΦV10 by means of transposon mutagenesis which was investigated for the detection of *E. coli* O157:H7. The reporter bacteriophage

assay was dependent on n-decanal substrate addition for bioluminescence and could detect E. coli cells in approximately 1h. A luciferase reporter bacteriophage was developed for the detection of *Mycobacterium avium* spp *paratuberculosis* based on mycobacteriophage Tm4 (Sasahara *et al*., 2004). A recombinant mycobacteriophage Tm4 was constructed to express the firefly luciferase (*fflux*) gene under control of the BCG hsp60 promoter post host infection. In the assay, the substrate D-lucifersin was added and incubated with the bacterial sample together with the recombinant mycobacteriophage after which luminescence was measured using a luminometer. Detection of luciferase activity was possible after 4h with a maximum peak after 48h when tested on 10^6 cfu/ml culture. The assay had optimal performance when results were measured 24-48h after infection of more than 1000 cells. Luciferase assays have also yielded the necessary diagnostic speed needed for detection of certain bacterial pathogens such as *Bacillus anthracis* (Schofield & Westwater, 2009). The *Vibrio harveyi luxA and luxB* genes were cloned into bacteriophage W β. Following infection of the sample containing target bacteria and addition of the substrate, n-decanal, the assay could detect 10^3 cfu/ml in 1h. The researchers also transferred the same genes, by means of homologous recombination to the plaque-diagnostic phage A2211 for the detection of *Yersinia pestis* (Schofield *et al*., 2009). The luciferase genes were cloned downstream of the A1, A2 and A3 promoters. The phage diagnostic was applied to diagnosis of attenuated *Yersinia pestis* cultivated isolates and positive bioluminescence was detected using a bioluminescence multiplate reader. Bioluminescent signals were detectable 12-15 min after phage and substrate (n-decanal) was added to the sample in a dose dependent manner. The assay could detect 100 cfu/ml in 1h. Luciferase assays have the common problem of requiring substrate addition. This can only be circumvented by cloning the entire lux operon, however, the listed assays that make use of the entire operon have a lower sensitivity than those that make use of substrate addition. Luciferase assays also require expensive luminescence detection equipment such as a luminometer, in addition to biohazard facilities and skilled staff which is not suited for low resource settings.

1.5.3.4.4 Fluorescent protein expression

Fluorescent proteins have been used extensively in molecular biology to study cellular mechanisms such as cell division, localization of proteins, quantification of protein expression levels, timing of expression etc. Fluorescent proteins have also served as a means of bacterial detection, often through fusion proteins to bacteriophage structural protein. A GFP expressing bacteriophage was developed for the specific detection of *E. coli* XL1-blue by cloning GFP into bacteriophage lambda TriplEx (Clonetech Laboratories Japan, Ltd) (Funatsu *et al*., 2002). The assay made use of fluorescence microscopy to detect fluorescing cells which required 4-6h hours for flourophore expression and maturation. The authors concluded that the optimal phage concentration (for maximal host infection) should be 10^9 pfu/ml in this system. The system showed strain specificity, by infecting *E. coli* but not *M. smegmatis* in mixed bacterial cultures. Similarly, reporter bacteriophages have been developed for the detection of mycobacteria which is discussed in Section 1.3.6.2. The benefit of using fluorescent proteins as reporter genes in reporter bacteriophages is that only viable hosts become infected and subsequently express the fluorescent protein (Funatsu *et al*., 2002). A further advantage of fluorescent proteins is that the fluorescent signal remains detectable for weeks after the diagnostic assay is performed in contrast to bioluminescence which has a time-dependent reporter signal (Piuri *et al*., 2009).

1.6 Conclusions

Assays that are based on luciferase production and fluorescent protein detection have been shown to be relatively simple and have the best performance in being rapid, with a high sensitivity and specificity. However, the means of detection are the main drawbacks. The luciferase assay makes use of a costly luminometer and the fluorescence-based assays make use of costly fluorescence microscopy, both of which require skilled staff. Furthermore, the luciferase assays involves working with live *M. tb* bacilli, which requires specialized biohazard facilities. Refinement of the reporter mycobacteriophage assays seem to be the best option for development of a usable TB diagnostic.

1.7 Problem statement

Several reporter bacteriophage assays exist that utilize various combinations of bacteriophages, promoters, reporter genes and means of bacterial concentration and signal concentration methods. Many of these assays are of relevance to diagnostics, however, for detection of *M. tb* several specific requirements need to be met. Specifically, a TB diagnostic is needed that can reach even into resource-poor settings. This requires rapid, simple to perform and relatively cheap assays, that perform at high sensitivity and specificity and no not require highly skilled staff or expensive infrastructure.

1.8 Hypothesis

We hypothesize that a rapid, simple, specific and low cost TB diagnostic could be developed utilizing mycobacteriophages which express affinity-tagged reporter proteins using strong promoters specifically active when infecting *M. tb.*

1.9 Aim

The overall aim of this study is to develop a reporter mycobacteriophage that could be utilized in future as a rapid, specific and low cost TB diagnostic.

1.10 Objectives

1) To isolate suitable mycobacterial promoters to drive expression of reporter genes.
2) To construct and evaluate affinity-tagged reporter constructs
3) To construct and evaluate reporter mycobacteriophages

1.11 Study design

1.11.1 Promoters

M. tb promoters will be isolated and compared to promoter activity of the *Hsp60* promoter by means of β-galactosidase assays. The most suitable promoter will be selected and evaluated as a promoter to drive expression of various reporter constructs.

1.11.2 Reporter constructs

Reporter constructs will be created using the promoter identified in 1.11.1, as well as the *Hsp60, pSmyc, p19K* and *pACE* promoter. mRFP1, DsRed and LacZ will be used as reporter genes, and histidine and biotin affinity tags will be used as tools for signal concentration. The expression vectors pSD21, p19Kpro, pDMN1 and pACE will be investigated in various combinations to find optimal expression. The expression and functionality of these constructs will be tested in the laboratory strain *M. smegmatis.*

1.11.3 Mycobacteriophages

The most suitable reporter constructs from 1.11.2 will be cloned into suitable mycobacteriophages. These reporter mycobacteriophages will be evaluated in *M. tuberculosis.*

1.12 Rationale

1.12.1 Promoters

The *Hsp60* promoter is a problematic and suboptimal promoter. Despite over 130 mycobacterial promoters being characterised over the years, the Hsp60 promoter still remains one of the most popular promoters to drive mycobacterial gene expression (Gomez & Smith, 2007). The Hsp60 promoter has the befit of promoter activity in *M. smegmatis, M. bovis BCG* and *M. tb* (Al Zarouni & Dale, 2002). The Hsp60 promoter has long been considered a strong mycobacterial promoter, however recent studies have cast doubt over its stability and usefulness (Al Zarouni & Dale, 2002). It has been shown to cause unstable constructs and that constructs containing the Hsp60 promoter, which drives expression of a foreign gene, cause frequent deletions within the promoter itself (Haeseleer,F. 1994, Kumar,D. 1998, Al Zarouni,M. 2002, Al Zarouni & Dale, 2002). The authors of this

study hypothesised that the deletions are due to excessively strong promoter activity leading to cell death, creating a selection pressure for constructs with mutated, deleted or non-functional promoters (Al Zarouni & Dale, 2002). It is thus necessary to make use of a more consistent and reliable mycobacterial promoter when constructing reporter mycobacteriophages.

We propose to use promoter screening methods to compare novel and existing mycobacterial promoters. There are various mycobacterial promoter screening methods available and they commonly make use of reporter genes to indicate promoter activity, for example the β-galactosidase gene, chloramphenicol acetyltransferase (CAT) reporter gene and GFP expression (Mulder *et al.*, 1997). During promoter screening the candidate promoter region is cloned into the promoter probe vector containing the reporter gene and this enables a quantitative comparison of different promoter strengths (Mulder *et al.*, 1997). Various promoter isolation attempts have used cloning of random mycobacterial genomic DNA (Triccas *et al.*, 2001). However, screening of targeted regions can increase the likelihood of discovering promoter elements and also grant insight into the regulatory mechanisms of the region. Previous promoter isolation studies in our laboratory focused on the promoters situated in the *M. tb* ESAT-6 gene clusters and utilized β-galactosidase assays (Botha, 2006, Gey van Pittius, 2002). It has been shown that these regions contain promoter elements of varying strength (Botha, 2006, Maciag *et al.*, 2009). The ESAT-6 gene clusters contain various promoters that might offer mycobacterial promoters more suitable than the Hsp60 promoter.

1.12.2 Reporter genes

There are several choices for a reporter gene; however we propose the use of a fluorescent protein or enzymatic reporter to be superior. GFP, originally isolated from the jellyfish *Aequorea victoria*, is currently one of the most well-known fluorescent proteins and has widespread application as a cellular marker and reporter gene (Tsien, 1998). It has a fluorescence absorbance / excitation peak at 395nm and another minor peak at 475nm, the corresponding emission peak is at 508nm (Phillips, 1997). GFP has relatively strong resistance to photo-bleaching; however the protein has shown some limitations and drawbacks. The short wavelength of GFP creates problems with light scattering and background due to cellular auto-fluorescence (Tsien, 1998). GFP fluorescence is also sensitive to pH (Tsien,

1998). Since the initial discovery and cloning of GFP there has been a considerable increase in the amount of available fluorescent proteins for use as reporter protein (Chudakov *et al*., 2010). Mutants of GFP have been made to emit blue, cyan, and yellow light (Rodrigues *et al*., 2001). Recently, a red fluorescent protein called DsRed has been isolated, which offers vast improvements as reporter protein (Baird *et al*., 2000).

DsRed is a 28-kDa polypeptide made up of four monomers that form a tetramer which has bright red fluorescence (Yarbrough *et al*., 2001). This protein was recently isolated from a corallimorpharian of the *Discosoma* genus (Yarbrough *et al*., 2001). DsRed fluorescence has a longer wavelength than GFP with an excitation maxima at 558nm and emission maxima at 583nm, which minimizes problems with light scattering and cellular autofluorecence (Rodrigues *et al*., 2001, Yarbrough *et al*., 2001). Unlike GFP, DsRed has a negligible pH dependence of absorbance or fluorescence through a wide pH range of pH 5 to pH 12 (Baird *et al*., 2000). It has also been found that DsRed is quite resistant to photobleaching at exposure intensities of typical spectrofluorometers and microscopes (Baird *et al*., 2000). DsRed does, however, have a few drawbacks as genetic marker. It is an obligate tetramer as proven by analytical ultracentrifugation. Thus, it is possible that interaction between proteins to which DsRed is fused could interfere with normal DsRed tetramerization. DsRed also requires over 24h of maturation for optimal emission (Yarbrough *et al*., 2001). After 24h the red fluorescence is at 50% of its maximal peak and requires more than 48h to reach more than 90% peak (Baird *et al*., 2000). This could pose a problem if used in a rapid TB detection assay since it could slow down the time to detection. The DsRed crystal structure shows that it is a dimer of dimers (Yarbrough *et al*., 2001). Figure 1 shows a diagram of the interaction between the dimers.

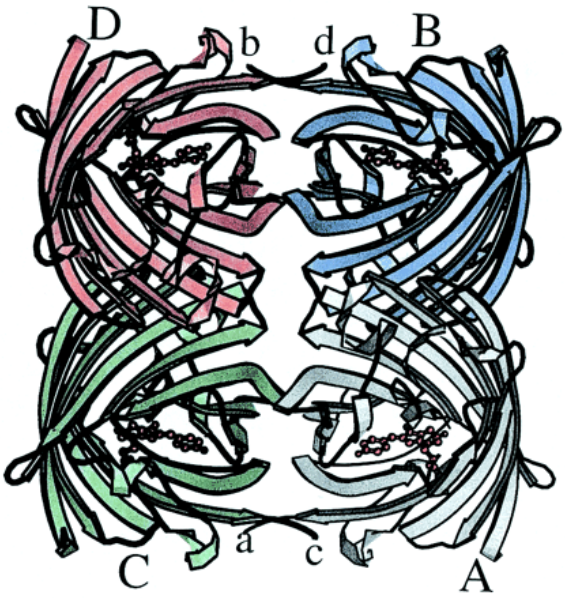

Figure 1. Ribbon diagram of DsRed tetramer (figure taken from Yarbrough *et al.*2001). Uppercase A-D indicate monomers and lowercase a-d indicate carboxy termini.

In its tetramer form the surfaces of the A and C chain as well as the carboxy termini makes contact. The carboxy termini of tetrameric DsRed are different from those in GFP. In GFP they are free whereas in DsRed the a- and c-carboxy terminus are interacting (Yarbrough *et al.*, 2001). The chromophore-containing region is situated in the interacting region of the A and C monomers, thus suggesting that proper chromophore function requires the protein to be in its tetrameric form. It has been suggested that the Leu-225 at the carboxy termini will likely interact with a protomer inherent to the tetramer and thus potentially create non-functional caboxy-terminal protein fusions (Yarbrough *et al.*, 2001). However, various amino-terminal fusions have been made successfully (Baird *et al.*, 2000, Yarbrough *et al.*, 2001). A monomeric version of the DsRed gene, called mRFP1, has been developed in order to circumvent some of the problems with DsRed.

mRFP1 was generated by inserting amino acids in key regions of the DsRed subunit to create a stable monomeric red fluorescent protein (Campbell *et al.*, 2002). This monomer has an excitation peak of 584nm and an extinction peak of 607nm, which are about 25nm red-shifted from DsRed, and which confer less cellular autofluorescence than DsRed (Campbell *et al.*, 2002). Since it is a monomer, it

does not require such an extensive time period to fully mature and is estimated at 10 times faster to maturation than DsRed (Campbell *et al.*, 2002). The monomeric state makes the protein much more suitable for protein fusions (Campbell *et al.*, 2002). mRFP1 has a similar brightness to DsRed despite a lower extinction coefficient, quantum yield, and photostability than DsRed (Campbell *et al.*, 2002). These benefits of mRFP1 over DsRed deems the protein more suitable for a rapid TB detection assay.

In a previous study which compared the usefulness of GFP and DsRed as a reporter, it was demonstrated that luciferase can be detected more rapidly than DsRed and GFP due to the slow maturation times of the two fluorescent proteins (Hakkila *et al.*, 2002). The study also stated that luciferase-based cellular detection is deemed more sensitive, since there is no luminescent background signal, whereas cellular auto-fluorescence requires more fluorescent protein for detection (Hakkila *et al.*, 2002). Although both these statements are true for rapidly growing hosts like *E. coli*, it is not really relevant in the case of *M. tb* detection. In the case of *M. tb* detection, high concentrations of host bacteria are required for luciferase detection (at least 10^5 cfu/ml according to the Tm4-based luciferase mycobacteriophage assay discussed in Section 1.3.6.2.1). Since this concentration of bacteria is not common in sputum samples, a culture step is required, which extends the diagnostic to 2-3 days (see Section 1.3.6.2.1). This necessitates a specialized biohazard facility, which limits the usefulness of luciferase based assays. Additionally, luciferase assays make use of a lumometer and sample reading during a specific timeframe, which would not be ideal for use in the field. In contrast, a fluorescent protein remains detectable up to months after expression and fixing of *M. tb* by means of a fixative such as paraformaldehyde (Piuri *et al.*, 2009). DsRed and mRFP1 both offer superior wavelengths compared to GFP, and confer far less cellular auto-fluorescence (Campbell *et al.*, 2002). These properties make DsRed and mRFP1 suitable reporters for *M. tb* detection. We thus propose the use of the fluorescent proteins DsRed and mRFP1 for use as reporter signal in a mycobacteriophage based diagnostic.

Enzymes such as β-galctosidase have previously been shown to grant high sensitivity for bacterial detection (Neufeld *et al.*, 2003). The main problem with its use was due to the high prevalence of similar enzymes in other bacteria which could lead to false positives (Yemini *et al.*, 2007). We propose

to circumvent this problem by means of a purification step which is possible by creating an affinity tag fusion to the β-galctosidase gene.

1.12.3 Affinity tags

The final area for improvement on the current reporter mycobacteriophage assays is with regards to amplification/condensation of the reporter signal, which we propose to do through the use of affinity tags. Affinity tags make isolation of almost any protein possible, as long as a soluble fusion protein is possible and the fusion tag is exposed to the external protein milieu. Use of affinity tags on reporter proteins allows for separation and purification of the reporter as well as condensing the reporter signal. This effectively increases the reporter signal strength and can lead to a higher sensitivity. Furthermore, this decreases the potential background signal from contaminant bacteria. There are various affinity tags available, a popular choice is histidine tags (typically made up of 6 histidines placed at either the N- or C-terminus of the protein (Arnau *et al*., 2006). Histidine tags readily bind to immobilized transition metals like Ni^{+2}, yet can also be eluted to derive a purified protein eluate (Kimple & Sondek, 2002). Biotin tags are another good option since they are capable of more efficient protein capture and greater yield due to its extremely strong interaction with streptavidin (Arnau *et al*., 2006). Since the bond is so strong, a denaturing elution step, often using heat, is involved (Kimple & Sondek, 2002). Thus, diagnostics that make use of biotin tags should use detection methods that do not require elution to observe a reporter protein signal lest the reporter protein also be denatured.

1.12.4 Conclusion

In conclusion, the improved reporter mycobacteriophage we intend to construct will make use of a strong and stable mycobacterial promoter and a reporter protein with simple detection abilities such as fluorescent proteins or a colorimetric reaction, as well as a signal amplification method such as an affinity tag.

Chapter 2 Materials and Methods

2.1 Bacterial strains and culture conditions

2.1.1 Bacterial strains

Escherichia coli (E. coli) strain XL1-Blue (Stratagene), *Mycobacterium smegmatis (M. smegmatis)* strain *mc²155* (ATCC 700084) and *Mycobacterium tuberculosis* (*M. tb*) strain H37Rv (ATCC 35734) was used in this study.

2.1.2 Bacterial culture

2.1.2.1 *E. coli* liquid cultures

E. coli liquid cultures were grown overnight in 10 ml Lysogeny Broth (LB), (Appendix A.2.6), at 37°C in a shaker at 200 rpm with the appropriate antibiotics (Appendix A.1). Freezer stocks were made as described in Section 2.1.2.5.

2.1.2.2 *E. coli* solid media culture

Cells were plated out on LB agar plates (Appendix A.2.7) containing the appropriate antibiotics (Appendix A.1). Once the plate(s) dried enough to not allow running drops of liquid, the plate(s) was (were) inverted and incubated overnight at 37°C.

2.1.2.3 *M. smegmatis* liquid cultures

M. smegmatis liquid cultures were grown for 2 days in 11 ml 7H9 media (Appendix A.2.1) at 37°C on a shaker at 200 rpm with the appropriate antibiotics (Appendix A.1). ZN staining (Section 2.1.2.6) was done on *M. smegmatis* cultures to determine if contamination was present. Freezer stocks were made of pure cultures (Section 2.1.2.5).

2.1.2.4 *M. smegmatis* solid media culture

Cells were plated on Middlebrook 7H11 plates (Appendix A.2.3) containing the appropriate antibiotics (Appendix A.1). The plates were sealed with parafilm, inverted and incubated overnight (or up to two days depending on the colony size) at 37°C.

2.1.2.5 Freezer stocks

Freezer stocks of bacterial liquid cultures were made by transferring 150 µl sterile glycerol and 750 µl of the cell culture to a fresh 2 ml Eppendorf tube. The freezer stock was then mixed using a vortex and stored at -80°C.

2.1.2.6 Ziehl-Neelsen (ZN) staining

ZN stains were performed to confirm pure cultures of *M. smegmatis.* An aliquot of 150 µl of mycobacterial culture was spread onto a microscope slide and allowed to air dry. The slide was intermittently heated by flaming 3-4 times to heat-fix the cells. The slide was flooded with Carbol Fuchsin (Appendix A.3.8) and heated intermittently, carefully as to not boil the liquid, for 2-5 min until steam formation was visible. The slide was then washed with H_2O and decolorized with 3% acid alcohol (Appendix A.3.1) and then washed with H_2O. Thereafter the slide was counterstained with methylene blue (Appendix A.3.19) for 1-2 min and washed with H_2O. After drying, the slide was examined under a microscope using oil-immersion and 1000 times magnification. Red/pink cells indicated mycobacterial cells and blue cells indicated non-acid fast cells (indicative of contamination).

2.1.2.7 Electrocompetent *E. coli*

Electrocompetent *E. coli* was made for electroporation of vectors. A starter culture was made by inoculating 1 µl of stock *E. coli* competent cells into 50 ml LB media (Appendix A.2.6) containing 500 µl tetracycline (Appendix A.1.4). After overnight growth at 37°C with shaking at 200 rpm, 2 ml of the starter culture was inoculated into fresh LB media containing tetracycline to selectively culture the tetracycline resistant XL1-Blue strain of *E. coli* (section 2.1.1). This was grown until the optical density (OD) at 600nm, measured in a LKB Biochrom Ultrospec 4051, was between 0.7 and 0.8, at which point the cells were at the exponential growth stage. The cultures were spun down for 10 min at 4°C in a Sorvall RC-5B refrigerated centrifuge (Du Point Instruments) at 4000 rpm, using a GSA rotor. The cell culture pellets were washed with 10% glycerol (Appendix A.3.15) in an amount equal to the original culture volume. The spin and wash step were repeated two more times and after the last step the cells were pooled into a 50 ml centrifuge tube. The cells were spun down in an Eppendorf Centrifuge 5810R at 4000 rpm for 10 min and resuspended in 10% glycerol using 2 ml per liter of

initial culture. Cells were aliquoted (100 µl) into Eppendorf tubes, flash frozen using liquid nitrogen and stored at -80°C.

2.1.2.8 Electrocompetent *M. smegmatis*

Electrocompetent *M. smegmatis* was made for electroporation of vectors. A starter culture was made by inoculating 1 µl of *M. smegmatis* competent cells into 100 ml Middlebrook 7H9 (Appendix A.2.1) and grown to an OD measurement at 600nm, in a LKB Biochrom Ultrospec 4051, of 0.5. The cells were then placed on ice for 1h and centrifuged at 3000g for 5 min at 4°C in a Sorvall RC-5B refrigerated centrifuge (Du Point Instruments). The cell pellet was washed twice in 30 ml ice cold 10% glycerol (Appendix A.3.15) after each centrifugation. The cell pellet was then resuspended in 4 ml 10% glycerol and 200 µl cells were aliquoted into into Eppendorf tubes, after which it was stored at -80°C.

2.2 PCR

2.2.1 PCR Primers

A list of all primers used in this study is shown in Table 5. The descriptions of these primers can be found in subsequent Sections. Primers were designed using Primer Premiere software to obtain the theoretically optimal primer pairs where possible. Primers were diluted to 50pmol/µl using nuclease free H_2O.

Table 5. List of primers and oligonucleotides

Primer name	Sequence (5`→3`)	Tm (℃)	Purpose
LambdaCosF	ACGCGTGAGCTCCCCGGTGGTGTTATCTGGC	70	Construction of pDMNLCosmRFP, Section 2.6.4.4
LambdaCosR	TCTAGAGGTACCTTAATTAATTGTTGACTTCCATTGTTCATTCC	61	Construction of pDMNLCosmRFP, Section 2.6.4.4
D29repF	CATATGTGTGCGTTCGCACGCACAGGCCCGG	70	Construction of pYub412mRFPNdeIBglII, Section 2.6.4.3
D29repR	AGATCTCTAGGCGCCGGTGGAG	62	Construction of pYub412mRFPNdeIBglII, Section 2.6.4.3
DsRed-Hisr	TCTAGACTAGTGGTGGTGGTGGTGGTGCAGGAACAGGTGGTG	70	Construction of pSD21Hsp60DsRedCHis, Section 2.6.3.3
ESAT6-f	GGATCCTTGTTGTCGTTGGCGGTCA	78	Construction of pSD21Hsp60DsRedCHis, Section 2.6.3.3
Hsp60mRFP1f2	TAAGGTACCCATGGCCTCCTCCGAGGACGTCATCA	67	Construction of pSD21Hsp60mRFPNHis, Section 2.6.3.7
Hsp60mRFP1r2	GCAGTCGACTCAAGCTTCGAATTCTTAGGCGCCG	67	Construction of pSD21Hsp60mRFPNHis, Section 2.6.3.7
HSP60PROMHISR3	GGTACCGCGTGGTGGTGGTGGTGGTGCCACTGCTGCTCCAT	75	Construction of pSD21Hsp60DsRedNHis, Section 2.6.3.1, Construction of pJem15Hsp60NHis, Section 2.6.3.11, Construction of P19KproHsp60DsRedNHis, Section 2.6.3.2
Int1LacZF	AGATCTTGCTCAGCATATTCGTGGGC	61	Construction of pJem15Int1NHis, Section 2.6.3.12
Int1LacZR	AGATCTGTGGTGGTGGTGGTGGTGCTCCATTGTATACCGTCTCAC	70	Construction of pJem15Int1NHis, Section 2.6.3.12
INT1RFPr	GGTACCUCGTGGTGGTGGTGGTGGTGCTCCATTGTATACCGTCTCAC	71	Construction of pSD21Int1DsRedNHis, Section 2.6.3.5
mRFPreporter1-f	CGATCGTGTGCGTTCGCACGCACAGGCCCGG	739	Construction of pYub412mRFPPvuI, Section 2.6.4.1
mRFPreporter1-r	CGATCGCTAGGCGCCGGTGGAG	66	Construction of pYub412mRFPPvuI, Section 2.6.4.1
p19kmrfpf2	CTAAAGCTTGACCACAACGGTTTCCCTCT	62	Construction of p19KProp19KmRFPNHis, Section 2.6.3.8
P19kmrfpr3	ATCGATTTAGGCGCCGGTGGAGTGG	64	Construction of p19KProp19KmRFPNHis, Section 2.6.3.8

RepNdeFixF	AAGCTTGGATGCATCATCATCATC ATCATGCGATGGCCTCCTCCGAG GACG	71	Construction of pYub412mRFPNdeIBglII, Section 2.6.4.3
Rep4EcoRVR	GATATCCTAGGCGCCGGTGGAG	61	Construction of pYubmRFP, Section 2.6.4.5
Rep4SalIF	GTCGACTGTGCGTTCGCACGCAC AGGCCCGG	73	Construction of pYubmRFP, Section 2.6.4.5
RepTm4SapF	GCTCTTCCTGTGCGTTCGCACGC ACAGGCCCGG	73	Construction of pYub412mRFPSapI, Section 2.6.4.2
RepTm4SapR	GGAAGAGCCTAGGCGCCGGTGG AG	67	Construction of pYub412mRFPSapI, Section 2.6.4.2
RCMRFPF	AAGCTTGGCATCATCATCATCATC ATATGGCCTCCTCCGAGGACG	67	Construction of pDMN1mRFPNHis, Section 2.6.3.10
RCMRFPR	GTTAACCTAGGCGCCGGTGGAG	62	Construction of pYub412mRFPNdeIBglII, Section 2.6.4.3
Rv1779-80f	GGATCCTGCTCAGCATATTCGTG GGC	73	Construction of pSD21Int1DsRedNHis, Section 2.6.3.5, Construction of pSD21Int1DsRedNHis, Section 2.6.3.5, Promoter screening
Rv1779-80r	GGTACCTGTATACCGTCTCACGTC AC	63	Promoter screening
Rv1781-82f	GGATCCTCGGGCAAGTTCGACCA G	72	Promoter screening
Rv1781-82r	GGTACCGCCACGCTAGATCACCT	66	Promoter screening
Rv1783-84f	GGATCCCAGCGGGTTGCCCACAT T	75	Promoter screening
Rv1783-84r	GGTACCATGGTGTCGGTCCGGCT A	71	Promoter screening
RV178384f2	CCTCGGTTCGTGAGTTTGC	64	Promoter screening
Rv1786-87f	GGATCCGAAATGGCGCTCATCCT	70	Promoter screening
Rv1786-87r	GGTACCCAACCAGTCCTCCCTCT C	66	Promoter screening
Rv1787-88f	GGATCCCACAGTGTGGTTGCCCG	72	Promoter screening
Rv1787-88r	GGTACCCATGCGATCTCCTGCTTA	66	Promoter screening
Rv1789-90f	GGATCCATGGCTCGCCCACCCTT C	75	Promoter screening
Rv1789-90r	GGTACCGTCCATTCCGAACCCTTT	68	Promoter screening

Rv1790-91f	GGATCCGTCTCCGTCGGCGGGATA	75	Promoter screening
Rv1790-91r	GGTACCGACATGCTGTCTTCCTCT	64	Promoter screening
Rv1791-92f	GGATCCGAGGCGGCCAACGCAG	76	Promoter screening
Rv1791-92r	GGTACCCATGTTGCCTGTCTCCTTA	65	Promoter screening
Rv1793-94f	GGATCCACAGCGCCGTCGGCTCCA	79	Promoter screening
Rv1793-94r	GGTACCGATCCATCGCTACCTCAGC	69	Promoter screening
Rv1794-95f	GGATCCAGTGGAAAACACACAGCA	66	Promoter screening
Rv1794-95r	GGTACCCATCGTCCTGTACCCCCT	68	Promoter screening
SapF2	GCTCTTCCGCTTTGTGCGTTCGCACGCACAGGCCCGG	74	Construction of pYub412mRFPSapI, Section 2.6.4.2
SapR2	AAGCGGAAGAGCCTAGGCGCCGGTGGAG	70	Construction of pYub412mRFPSapI, Section 2.6.4.2
Sp6	ATTTAGGTGACACTATAG	48	Sequencing primer for pGem-T-Easy
T7	TAATACGACTCACTATAGGG	56	Sequencing primer for pGem-T-Easy

2.2.2 PCR conditions

PCR amplification was done in an Eppendorf Mastercycler Gradient Cycler using the ROCHE Faststart PCR kit using the following PCR program and reaction mixture shown in Table 6 and 7.

Table 6. PCR reaction mix composition

Component	Amount
H_2O	57.2 µl
10X PCR buffer	10 µl
GC mix	20 µl
dNTPs	8 µl
Forward primer	2 µl
Reverse primer	2 µl
Faststart Taq (Roche)	0.8 µl
DNA	1 µl***

Table 7. PCR cycling conditions

Temperature	Time	
95℃	4 min	
95℃	1 min 30sec	38 cycles
*X℃	1 min	
72℃	**Y min	
72℃	10 min	
4℃	∞	

*Where X is equal to the lowest thermal melting point (Tm) between the two primers minus two degrees (primer Tm as indicated in Table 5).

**Where Y is the amount of minutes allotted for product extension (1 min extension time per 1kb of product length was used, for example 2kb would require 2 min extension).

2.2.3 PCR colony screening

After each electroporation into competent cells (*E. coli*, Section 2.3.13.1 and *M. smegmatis*, Section 2.3.13.2), a PCR colony screening was done to confirm the presence of the plasmid containing the desired insert. Single colonies were picked with a pipette tip, mixed by pipeting up and down in the PCR reaction mixture and the pipette tip was then placed into 200 µl LB media (Appendix A.2.6) until the PCR results were known. Colonies which were PCR positive for the insert (following gel electrophoresis (Section 2.3.1) were then grown in liquid media (Section 2.1.2.1 for *E. coli* colonies and Section 2.1.2.3 for *M. smegmatis* colonies). The PCR method used was the same as described in

Section 2.2.2, however only 1/10th of the reaction mix was used and a shorter PCR program was used as shown in Table 8 and 9:

Table 8. PCR reaction mix composition

Component	Amount
H_2O	5.72 µl
10X PCR buffer	1 µl
GC mix	2 µl
dNTPs	0.8 µl
Forward primer	0.2 µl
Reverse primer	0.2 µl
Faststart Taq (Roche)	0.08 µl
DNA	1 µl
Total	10 µl

Table 9. PCR Program composition

Temperature	Time	
95℃	4 min	
95℃	45sec	30 cycles
*X℃	45sec	
72℃	**Y min	
72℃	2 min	
4℃	∞	

*Where X is equal to the lowest thermal melting point (Tm) between the two primers minus two degrees (primer Tm as indicated in Table 5).

**Where Y is the amount of minutes allotted for product extension (1 min extension time per 1kb of product length was used, eg. 2kb would require 2 min extension)

2.3 DNA Manipulations

2.3.1 Gel Electrophoresis

Agarose gels were made and run as follows: In the case of small DNA fragments, in general a 0.7% to 1% agarose gel was made by dissolving 0.7 to 1g D1-LE agarose (merk) in 100 ml 1X Sodium Borate buffer (SB, Appendix A3.39) using a microwave. Five microliters of Ethidium Bromide (Appendix A.3.14) was added to the gel before casting the gel. In the case of larger DNA fragments (or when higher resolution was required) a lower agarose concentration (down to 0.5%) as well as TAE buffer (Appendix A.3.44) was used instead of SB buffer. DNA samples were loaded with 6X loading buffer (Fermentas) and run with either roche DNA Molecular Weight Marker X, Fermentas “Generuler DNA Ladder Mix”, “Generuler 100bp DNA ladder plus” or Generuler 1kb DNA ladder. Gel electrophoresis was generally done at 6.5V/cm for large DNA molecules. Gel electrophoresis for PCR colony

screening was generally performed using 11-22V/cm. Gels were visualized and using a UV transilluminater and documented with a digital camera.

2.3.2 Field Inversion Gel Electrophoresis

Field Inversion Gel Electrophoresis (FIGE) was applied for the resolution of very large DNA fragments such as mycobacteriophage genomic DNA. For optimal resolution, Fermentas LE GQ Agarose was used at a concentration of 0.4% using TAE buffer (Appendix A.3.44) and was cast and run at 4°C. In the case where subsequent DNA recovery from the gel was required, Cambridge Seakem low melting point agarose was used. Low melting point gels were to 0.5% agarose by adding 0.5g Cambridge Seakem low melting point agarose to 100 ml TAE. The agarose was fully dissolved by heating in a microwave and cast at 4°C. Samples were mixed with loading buffer as described in Section 2.3.1 and the FIGE gel was run on a Hoefer Scientific Instruments (HSI) PC750 Pulse Controller using the following settings: 60V (3.15V/cm), 18h run length, 50 msec. pulse, forward:reverse pulse ratio of 2.5:1 at 4°C. Following FIGE, the gel was stained with ethidium bromide (Appendix A.3.14), by placing the gel in a container with 500 ml TAE and 40 µl ethidium bromide under (very) gentle shaking for 20 min (Staining is performed after electrophoresis since ethidium bromide hinders the migration of very large DNA particles). Photos of gels were taken using a transilluminator and digital camera.

2.3.3 DNA purification

DNA purification of ligations, digests and of gel slices (following electrophoresis) was done using the Promega Wizard® SV Gel and PCR Clean-Up System according to the standard protocol. In the case of purification from gels: DNA fragments of interest were excised from agarose gels using a scalpel, 10 µl membrane binding solution (supplied with the Promega kit) was added per mg of gel slice. The gel slice and membrane binding solution mixture was heated at 65°C until dissolved. In the case of ligations and digests: An equal amount of membrane binding solution was added to the volume of the sample. The DNA/membrane binding mixture was then placed into a spin column (supplied with the Promega kit) and incubated for 1 min. The spin column was attached to a vacuum adapter to pull the liquid through the spin column. 650 µl membrane wash solution was added and also sucked through the spin column. This was followed by adding 500 µl membrane wash solution and a repeat of the

vacuum step. The spin column was transferred to a collection tube and centrifuged at maximum speed in an Eppendorf centrifuge 5415D for 5 min. The spin column was placed into a new Eppendorf tube, 20-35 µl H_2O (heated to 65°C) was added to the membrane and incubated for 2 min at room temperature. The spin column was then centrifuged for 1 min at maximum speed to elute the purified DNA into the Eppendorf tube. The DNA concentration was determined (Section 2.3.7) and the DNA was stored at -20°C until further use.

2.3.4 DNA purification from agarose by phenol extraction

A phenol extraction method was used for recovery of very large DNA fragments from low melting point agarose gels following gel electrophoresis and FIGE (Section 2.3.2). Gel slices were placed into 2 ml Eppendorf tubes (or larger tubes in the case of larger gel slices) and weighed. For each mg of gel slice, 5 ml of TE (Appendix A.3.43) was added. The tube was incubated at 65°C until the gel slice was fully melted. The tube was allowed to partially cool and an equal amount of equilibrated phenol (Appendix A.3.28) was added. The tube was then centrifuged at 4000g in an Eppendorf centrifuge 5415D for 15 min at room temperature. The aqueous phase was drawn off and placed into a new tube containing an equal amount of phenol followed by another centrifugation step. This was repeated until the aqueous phase was clear. The final aqueous phase was transferred into a new Eppendorf tube containing an equal volume of phenol:chloroform (Appendix A.3.29), the tube was then centrifuged for 15 min at room temperature. The aqueous phase was then placed into a tube containing an equal volume of chloroform and centrifuged for 15 min at room temperature. The aqueous phase was then placed into a new Eppendorf tube and 3M Sodium acetate (Appendix A.3.38) was added to give a concentration of 0.3M. Two and a half volumes of 95% ethanol (Appendix A.3.13) was added and the tube was then placed at -80°C for 5-10 min. The tube was centrifuged at maximum speed for 15 min. The tube content was carefully decanted so as not to disturb the DNA pellet, 500 µl 70% ethanol (Appendix A.3.12) was added and centrifuged for 15 min. The ethanol was removed by careful pipetting and the pellet was allowed to air dry overnight at room temperature. The pellet was resuspended in a volume of 10-50 µl nuclease free water (warmed to 65°C), the DNA concentration was determined (Section 2.3.7) and stored at -20°C.

2.3.5 Plasmid DNA isolation

Plasmids were isolated using the Promega Wizard Miniprep Kit. Cell cultures were centrifuged in an Eppendorf Centrifuge 5810R at 3000 rpm for 10 min. The cell pellets were resuspended in 300 µl resuspension solution (supplied with the kit) and transferred to a 2 ml Eppendorf tube. Three-hundred and fifty microliters of cell lysis buffer (supplied with the kit) was added and the tubes were inverted 3 times. Ten microliters of alkaline phosphatase (supplied with the kit) was added, the tube was inverted 3 times and incubated for 3 min. A total of 400 µl neutralization solution (supplied with the kit) was added and the tubes were inverted 3 times. The tubes were centrifuged in an Eppendorf centrifuge 5415D for 10 min at max speed (132 000 rpm). The supernatant was transferred to a spin column (supplied with the kit) and vacuum was applied to suck the supernatant through the membrane. An amount of 600 µl membrane wash solution (supplied with the kit) was added and sucked through, followed by 500 µl membrane wash solution. The spin column was then centrifuged in an Eppendorf centrifuge 5415D for 5 min at 13 200 rpm. The spin column was placed into a new 1.5 ml Eppendorf tube and 20-50 µl H_2O, heated to 65°C, was added to the membrane and incubated for 2 min. The tube containing the spin column was then centrifuged for 30sec, the spin column was discarded, the DNA concentration was determined (Section 2.3.7) and the DNA was then stored at -20°C until needed.

2.3.6 Cosmid DNA isolation

Cosmid DNA isolation was done using the nucleobond® AX 100 cosmid extraction kit. A 100 ml culture was grown overnight as described in Section 2.1.2.1, the cells were pelleted by centrifuging for 10 min at 4000 rpm in an Eppendorf Centrifuge 5810R at 4°C. The cell pellets were resuspended in 8 ml Buffer S1 with added RNAse (supplied with the kit). An amount of 8 ml Buffer S2 (supplied with kit) was added, the suspension was inverted 6-8 times, and incubated for 5 min. Thereafter 8 ml Buffer S3 (supplied with the kit) was added, the tube was inverted 6-8 times and incubated on ice for 5 min. The lysate was then loaded onto a H_2O moistened filter and the flow through was collected. The flow-through was loaded onto a cartridge (equilibrated with 2.5 ml “buffer N2” (supplied with kit)). The cartridge was then washed 3 times with 4 ml “buffer N3” (supplied with kit). The DNA was eluted by adding 5 ml buffer N5 (pre-warmed to 50°C, supplied with the kit). An equal volume of isopropanol

was added to the purified cosmid and the tube was centrifuged at 16000g for 30 min, 4℃ in an Eppendorf centrifuge 5415D. The DNA was then washed with 70% Ethanol (Appendix A.3.12, 4℃) and centrifuged 10 min at 16000g in an Eppendorf centrifuge 5415D at room temperature. The pellet was then air dried and dissolved in DNAse free H_2O. The DNA concentration was determined using a Nanodrop (see Section 2.3.7) and the DNA was stored at -20℃.

2.3.7 DNA concentration determination

DNA sample concentrations were measured with a Nanodrop ND-1000 spectrophotometer at 260nm absorbance using H_2O (Promega, nuclease free) as blank.

2.3.8 Sequencing

DNA samples for PCR products, plasmids and cosmids were sent for sequencing at the sequencing laboratory of the central analytical facility at the University of Stellenbosch (www.sun.ac.za/saf). PCR products were sent undiluted (approximately 50ng/µl), plasmid sequencing samples were diluted to 100ng/µl, cosmid DNA was not diluted and was used at concentrations of higher than 600ng (ethanol precipitation was used to concentrate the DNA if needed) and primer concentrations were diluted to 1.1pmol/µl.

2.3.9 Restriction enzyme digests

Restriction enzyme (RE) digests were incubated for 16 hours at 37°C (except in the case of *BclI* which was incubated at 55°C). The RE digest mixture composition shown in Table 10 was used.

Table 10. Restriction enzyme mixture composition

Component	Amount
DNA	xµl (1-10 µg)
RE	2 µl
10X RE buffer	3 µl
H_20	25-xµl*
Total	30 µl

*Where X is the amount of DNA required for the given digest.

Samples that required an additional digest were purified by a DNA clean up step (Section 2.3.3) followed by elution with 20.5 µl H_2O. Two microliters RE and 2.5 µl 10X RE buffer was added and incubated as above. Once the digests were complete the samples were mixed with 6X loading buffer (Fermentas) and run on a gel as described in Section 2.3.1.

2.3.10 Partial restriction enzyme digests

A 100 µl reaction mixture was made up containing the DNA to be digested and 1X restriction enzyme buffer. The reaction mixture was divided up into five tubes, with tube 1 containing 30 µl, tube 2-4 containing 20 µl each and tube 5 containing 10 µl. All tubes were kept on ice. The first tube received 3U/µg of RE which was mixed. From the first tube, 10 µl was transferred to tube 2 and mixed. Subsequently, 10 µl from tube 2 onwards was transferred up to tube 5 using a different pipette tip each time. The tubes were incubated at 37°C for 15 min and analysed by gel electrophoresis (Section 2.3.1).

2.3.11 Dephosphorylation

Vector RE digests were followed by dephosphorylation prior to ligations to decrease background due to self-ligation of vectors. Eight microliters of DNA sample was mixed with 1 µl of Shrimp Alkaline Phosphotase (SAP, ROCHE) and 1 µl 10X SAP buffer (Roche) and incubated at 37℃ for 10 min. This was followed by a deactivation step of 15 min at 65℃.

2.3.12 Ligations

A vector:insert ratio of 1:3 was used for ligations. The amount of insert required was calculated with the following equation shown below and the reaction composition shown in Table 11. Ligation reactions were incubated at 4℃, overnight followed by a 15 min deactivation of the ligase enzyme by incubating at 65℃

$$\frac{\text{Vector (100ng)} * \text{(size of insert (in bp))}}{\text{(Size Vector (in bp))}} \times \frac{3}{1} = \text{ng of insert needed}$$

Table 11. Ligation reaction composition

Component	Amount
Vector	yµl (100ng)
Insert	xµl (varies according to formula as indicated above)
T4 DNA ligase	1 µl
10X Ligase buffer	1 µl
H_20	8 - x - y
Total	10 µl

2.3.13 Bacterial Transformation

2.3.13.1 Transformation of competent *E. coli*

Transformation was performed by electroporation using a Bio-Rad Gene Pulser. Forty-five microliters of competent *E. coli* (Section 2.1.2.7) was mixed with 2 µl ligation product (Section 2.3.12), or 15ng vector DNA in the case of vector amplification, in a Bio-Rad Gene Pulser Cuvette using a Bio-Rad electroporater at 2.5kV, 200Ω, 125 µF. Cells were resuspended in 600 µl super optimal broth with "catabolite repressor" (SOC), (Appendix A.2.10) and incubated 1h at 37℃ with shaking at 200 rpm. Seventy-five microliters of the cells were then plated out on LB plates (Appendix A.2.7) containing the appropriate antibiotics (Appendix A.1) according to each vector's antibiotic resistance (Appendix B).

2.3.13.2 Transformation of competent *M. smegmatis*

Frozen *M. smegmatis* competent cells (Section 2.1.2.8) were thawed on ice before use. The cells were then centrifuged in an Eppendorf centrifuge 5415D at 1.7g for 2 min. The cells were resuspended in ice cold 10% glycerol (Appendix A.3.15). The centrifuge and resuspension steps were repeated. Two micrograms of DNA was mixed with 200 µl competent *M. smegmatis* and placed in a Bio-Rad Gene Pulser Cuvette. Electroporation was done using a Bio-Rad electroporator at 2.5kV, 1000Ω, 125 µF. Time constants between 10 and 20 were deemed acceptable, otherwise a further DNA cleanup was performed (Section 2.3.3) and an additional glycerol wash step was performed. After electroporation, the cells were incubated in 1 ml Middlebrook 7H9 (Appendix A.2.1) with OADC (Appendix A.2.8) for 3h at 37℃ with shaking at 200 rpm. Seventy-five microliters of the cells were plated onto 7H11 (Appendix A.2.3) plates with the appropriate antibiotics (Appendix A.1) according to the vector's antibiotic resistance (Appendix B).

2.3.14 Lambda packaging of cosmid DNA

2.3.14.1 Packaging reaction

The Epicentre MaxPlax Lambda Packaging extract was used to efficiently transform large cosmids into *E. coli*. A MaxPlax Lambda Packaging extract was thawed on ice and 25 µl of the tube contents was mixed with 10 µl substrate DNA (about 0.2 µg) in a 1.5 ml Eppendorf tube. The tube was incubated for 90 min at 30℃. Twenty-five microliters additional packaging reagent was added to the tube and

incubated a further 90 min at 30°C. Five-hundred microliters phage dilution buffer (Appendix A.3.27) and 25 µl chloroform was then added to the tube and briefly vortexed. The lambda packaged cosmid was then titered using the *E. coli* host strain LE392MP (host strain was prepared as described in Section 2.3.14.2 and titered as described in Section 2.3.14.3).

2.3.14.2 Preparation of host strain bacteria

LB agar (Appendix A.2.7) was made up and cooled to about 40°C. An amount of 48.5 ml was transferred into a sterile 50 ml tube and 1 ml 1% Maltose (Appendix A.3.18) was added (giving a 0.2% w/v concentration) and 500 µl 1M $MgSO_4$ (Appendix A.3.22, giving a 10mM concentration) was added, the tube contents were mixed and plated out. *E. coli* bacterial strain LE392MP (Stratagene) was streaked out onto the LB agar plates (containing 10mM $MgSO_4$ and 0.2% Maltose) and incubated at 37°C overnight. Single colonies were picked and grown in 10 ml LB media (containing 200 µl 1% maltose and 100 µl 10mM $MgSO_4$ at 37°C with shaking till the culture reached an OD (measured at 600nm in a LKB Biochrom Ultrospec 4051) of 0.8. The cells were then stored at 4°C until use.

2.3.14.3 Titering of the packaging reaction

A serial dilution range from 10^{-2} to 10^{-9} was made of the packaged phage in phage dilution buffer (Appendix A.3.27). The initial dilution was made by adding 10 µl of the packaged phage to 990 µl phage dilution buffer to create a 10^{-2} dilution. A dilution range from 10^{-3} to 10^{-9} was then made by serially taking 100 µl of the previous dilution and adding it to the next Eppendorf tube containing 900 µl dilution buffer (with thorough mixing in between dilutions and using a new tip for each titer). One-hundred microliters of each dilution was placed into a new Eppendorf tube and incubated with 100 µl of the *E .coli* host strain (Section 2.3.14.2) for 15 min at 37°C. The content of the tube was then plated out on LB agar plates (Appendix A.2.7) containing the appropriate antibiotics (Appendix A.1) depending on the cosmid used for packaging.

2.4 Mycobacteriophage Isolation, purification and DNA extraction

2.4.1 Mycobacteriophage isolation from soil

Soil samples were collected in universal tubes (Sterilin), by filling the tubes halfway with moist soil. MP buffer (Appendix A.3.23) was added to the samples to the point where the buffer covered the top of the sample (about 8-10 ml). Fifty microliters concentrated *M. smegmatis* was added to the samples and incubated overnight at 37°C in an incubator with mixing every 30 min for the first 4h. After the soil settled at the bottom of the samples, 2 ml of the liquid was transferred to a 2 ml Eppendorf centrifuge tube and centrifuged at 2000g for 5 min in an Eppendorf centrifuge 5415D. The supernatant was filtered through a Millipore MILLEX®-GV polyvinylidene difluoride (PVDF) 0.22 µm filter unit. One-hundred microliters of the filtered phage solution was incubated with 250 µl *M. smegmatis* for 30 min at 37°C. Between 7-10 ml of preheated (about 45°C) top agar (Appendix A.2.4) was mixed with each sample and immediately plated onto a 7H10 agar plate (Appendix A.2.2). The plate was incubated at 37°C for 24-48h and plaque presence was indicative of mycobacteriophages with *M. smegmatis* as part of their host range in the soil sample. Plaques were picked using a pipette tip and placed into 50 µl MP. A serial dilution was made of the phage isolate ranging from 10^{-2} to a 10^{-6} dilution. This was done by placing 10 µl from the 50 µl initial mycobacteriophage dilution into 990 µl MP buffer to yield a 10^{-2} dilution. 100 µl of the 10^{-2} dilution was then placed into a fresh Eppendorf tube containing 900 µl MP buffer to yield a 10^{-3} dilution. 100 µl of the 10^{-3} dilution was then added to a new tube containing 900 µl MP buffer to yield a 10^{-4} dilution, the process of serial dilution was similarly continued until a dilution of 10^{-6}. The various dilutions were mixed with 250 µl competent *M. smegmatis* and incubated for 30 min at 37°C. The samples were then mixed with 10 ml preheated top agar and plated onto 7H10 agar plates and incubated 24-48h at 37°C. To ensure the plaques contain only one type of mycobacteriophage, a single plaque was picked among one of the plates with the best plaque separation, placed into 50 µl MP buffer and the above-mentioned titration and plating was repeated about 4-5 times. After the final plating step, the plate with the most confluent plaques, creating the best “web-like” pattern was selected, 10 ml MP buffer was added and the plate was incubated at 4°C overnight. The MP buffer was removed by pipetting and subsequently filtered through a Millipore MILLEX®-GV polyvinylidene difluoride (PVDF) 0.22 µm filter unit. The filtrate was collected and a final dilution range was created as mentioned above. The optimal dilution was then selected which created

the best “web-like pattern” (most confluent plaque pattern). This phage dilution was used for preparation of large scale phage stocks as described in (Section 2.4.2).

2.4.2 Preparation of large-scale mycobacteriophage stocks

A large mycobacteriophage lysate was made in order to ensure that a sufficient amount of mycobacteriophages were produced to enable visualization following CsCl ultracentrifugation. For this purpose 30 large plates (145mm X 20mm) with optimal plaque confluence (as determined by titering as described in Section 2.4.1) was prepared as follows. Competent *M. smegmatis* was created as described in Section 2.1.2.8 and 15 ml competent *M. smegmatis* was placed into a 50 ml conical tube. The appropriate amount (and titer) of purified mycobacteriophage (prepared as described in Section 2.4.1) for confluent plaque formation was multiplied by 30 and this amount was placed into the 50 ml tube containing the *M. smegmatis* cells. The tube was gently mixed by inversion a few times. The tube was incubated for 30 min at 37℃ with mixing every 5 min. Approximately 270 ml top agar (Appendix A.2.4) was added, together with the contents of to the 50 ml tube, to a sterile 500ml flask, gently mixed, and immediately plated onto 30 large petri dishes (about 9 ml per plate). The plates were then incubated at 37℃ for 24-48h (until complete lysis was observed on the mycobacterial lawn) without turning the plates upside down to allow droplets of moisture to aid in plaque dispersal. Between 10 and 15 ml MP buffer (Appendix A.3.23) was pipetted onto each plate and incubated at 4℃ overnight. The phage buffer was then removed by pipetting, placed into 50 ml conical tubes, centrifuged at 3000g using an Eppendorf Centrifuge 5810R for 20 min at 4℃. The supernatant was filtered through a Millipore millex®-GV polyvinylidene difluoride (PVDF) 0.22 µm filter unit. The volume of the filtrate was determined and NaCl was added to make a 1M solution. Polyethylene glycol (PEG 8000) was then added to create a 10% solution, followed by overnight stirring at 4℃. The solution was then centrifuged at 5500g in a GSA rotor for 15 min at 4℃. The supernatant was discarded and the pellet (containing the precipitated mycobacteriophages) was carefully transferred to a 50 ml TPP centrifuge tube and resuspended in 10-15 ml MP buffer. The pellet was slowly inverted overnight at 4℃ to resuspend the mycobacteriophages in the buffer. The tube was then centrifuged at 3000g in an Eppendorf Centrifuge 5810R for 30 min at 4℃ and the supernatant, containing the resuspended

mycobacteriophage, was transferred to a new conical centrifuge tube. The supernatant was then purified by CsCl ultracentrifugation as described in Section 2.4.3.

2.4.3 Ultracentrifugation of mycobacteriophage

Ultracentrifugation was used to separate intact mycobacteriophages from debris, following large scale amplification as described in Section 2.4.2. Fifteen grams of CsCl (United States Biochemical corp, Cleveland Ohio, ultrapure quality) was added per 10 ml of phage supernatant and gently mixed until fully dissolved. The supernatant was then transferred into an ultracentrifuge tube, weighed, carefully balanced by the addition of 15g/ml CsCl and sealed. The tubes were then placed into a type 50 Ti ultracentrifuge rotor. The rotor was placed into a Beckman ultracentrifuge and was run under vacuum at 35000 rpm for 24h at 4℃. The resulting phage band(s) were then carefully removed by pipetting and placed into Eppendorf tubes and then purified by dialysis as described in Section 2.4.4.

2.4.4 Mycobacteriophage dialysis

About 2 ml of the CsCl purified phage sample was pipetted and sealed into a pierce10000 MWCO pleated snakeskin dialysis tubing and dialysed in 1L MP buffer (Appendix A.3.23). The dialysis tube containing the phage sample was slowly stirred using a magnetic stirrer bar for 6h at 4℃. After the initial 6h, the buffer was replaced with 2L of MP buffer followed by a further dialysis overnight at 4℃ with stirring. The dialysed samples were transferred into 2 ml Eppendorf tubes and stored at 4℃. Dialysed mycobacteriophage samples where then used for electron microscopy (Section 2.4.5), as well as DNA extraction (Section 2.4.6).

2.4.5 Electron microscopy of phage particles

A dilution range of the mycobacteriophage samples were made using MP buffer (Appendix A.3.23) as described in Section 2.4.2 for optimal phage concentration for visualization on the electron micrograph. Phage samples were sent to the Department of Physics at the University of Cape Town (UCT) for transmission electron microscopy (TEM). Head and tail length were recorded and the TEM image was documented.

2.4.6 Phenol:Chloroform:Isoamyl alcohol DNA extraction of mycobacteriophage DNA

The dialysed mycobacteriophage sample was diluted by transferring 1 ml of the sample to a fresh Eppendorf tube containing 1 ml MP buffer (Appendix A.3.23) and by gentle pipetting. One-hundred microliters 10X proteinase K buffer (Appendix A.3.34), 50 µl proteinase K (Appendix A.3.33) and 900 µl of the diluted mycobacteriophage sample were mixed in an Eppendorf tube and incubated at 55°C overnight. Five-hundred microliters of the proteinase K digested phage sample was added to 500 µl Phenol:Chloroform:Isoamyl alcohol (PCI, Appendix A.3.30). The tubes were mixed by inverting and centrifuged at 13000 rpm in an Eppendorf 5415D centrifuge at 4°C for 15 min. The supernatant was removed and fresh PCI was added and the tubes were centrifuged again. This process was repeated until the white interface disappeared (after about 3-4 cycles of PCI and centrifugation). An amount of 1/10 of the volume of the sample of 3M Sodium Acetate (Appendix A.3.38) was added as well as 2.5 times the sample volume of 95% ethanol (Appendix A.3.13). The samples were mixed by inverting and incubated at -80°C for 15 min. The tubes were then centrifuged at 13000 rpm in an Eppendorf 5415D centrifuge for 30 min at 4°C and the supernatant was carefully decanted. The pellet was washed by adding 500 µl 70% ethanol (Appendix A.3.12), briefly vortexed, and then centrifuged at 13000 rpm for 15 min at 4°C. The liquid was decanted and the pellets were air dried at room temperature. Based on the size of the pellet, 50-200 µl TE buffer (Appendix A.3.44, heated to 65°C) was added to the pellet and incubated at 37°C for 15 min and then stored at -20°C until further use.

2.5. Protein isolation, separation and detection

2.5.1 Disruption of *M. smegmatis cells*

Cell lysates were derived by ribolysing using a Fastprep^{Tm} FP120 Bio101 Savant. Ten milliliters cell culture was centrifuged using an Eppendorf Centrifuge 5810R at 4000 rpm for 15 min. The cell pellet was resuspended in 500 µl PBS (Appendix A.3.26). The resuspended cell pellets were ribolysed in a QSP 2 ml conical screw microtube with 0.5mm glass beads (1/10th filled with glass beads) for 45sec at a speed of 6.5. This was followed by a cooling step on ice for 1 min. This was repeated 4 times in total.

2.5.2 Magna His purification

His-tagged proteins were purified using the Promega Magna His tag purification kit. Thirty microliters Magna-His Ni-particles were mixed with 200 µl cell lysate in a 1.5 ml Eppendorf tube. This was incubated for 2 min at room temperature. The tube was then placed in a magnetic stand for 30 sec and the supernatant was removed. The tube was removed from the stand and the lysate was resuspended with 150 µl Magna His binding/washing buffer. The tube was then placed in the magnetic stand again for 30 sec and the supernatant was removed. This wash step was repeated for a total of 3 washes. The last elution step used 50 µl elution buffer. The tube was placed in the stand and the eluate was removed and placed into a new tube. The eluate was kept at -20℃ until use.

2.5.3 Modulus microplate readings

Fluorescence readings of samples containing red fluorescent protein were taken using a Modulus Microplate Reader (Turner Biosystems) with the fluorescence module labelled “green” with excitation at 525nm and emission range of 580-640nm. A 5 sec integration time was used. One-hundred microliters of each sample was loaded into a 96 well fluorescence microplate together with positive and negative controls.

2.5.4 Bio-Rad Protein assay

The Bio-Rad Protein assay was used to determine protein concentrations of samples intended for β-gal assays as well as for Western blotting. A dilution range of Bovine Serum Albumin (BSA) dissolved in H_2O was used to create a standard curve. Sample readings were compared to the standard curve to calculate the protein concentrations. The BSA dilution range was made from a 2mg/ml stock as indicated below in Table 12.

Table 12. BSA dilutions for standard curve

BSA dilution (mg/ml)	2 mg/ml BSA stock (µl)	H_2O (µl)
0	0	10
0.2	1	9
0.4	2	8
0.5	2.5	7.5
0.6	3	7
0.8	4	6
1	5	5

Each standard received a volume of 10µl Kircheners medium (Appendix A.2.5). The Bio-Rad reagent was mixed with H_2O in a 1:4 ratio (1 part reagent to 4 parts H_2O). An amount of 980 µl of the mixture was added to each standard and sample as well as a blank (Kircheners and H_2O). The tubes were incubated at room temperature for 5 min and the absorbance was measured at 595nm in a LKB Biochrom Ultrospec 4051 absorbance reader.

2.5.5 β-galactosidase assay

β-galactosidase (β-gal) assays were performed using pJem15 promoter probe constructs (Section 2.6.2) to compare promoter strengths of various promoters. Following electroporation of the constructs into *M. smegmatis* (Section 2.3.13.2), single colonies were picked and grown in 10 ml 7H9 (Section 2.1.2.3) containing kanamycin (Appendix A.1.2). The cell culture was centrifuged at 4000 rpm for 15 min in an Eppendorf Centrifuge 5810R. The cell pellet was re-dissolved in Kircheners medium (Appendix A.2.5). Centrifugation and resuspention in Kircheners was repeated for a total of 4 times. One-hundred microliters of resuspended cells was inoculated into 10 ml Kircheners medium containing Kanamycin. The culture was grown until an OD reading of 1 (measured at 600nm in a LKB Biochrom Ultrospec 4051 absorbance reader). The cells were centrifuged and the cell pellet was resuspended in 500 µl PBS (A.3.26). The cells were then lysed (Section 2.5.1). The resulting cell lysate was centrifuged in an Eppendorf centrifuge 5415D at 13000 rpm for 10 min and the supernatant was transferred into a new tube. The protein concentration of the supernatant was then determined using the Bio-Rad protein assay (Section 2.5.4). Equal amounts of protein samples were made up to a volume of 30 µl in triplicate. The β-gal reaction was set up as in Table 13.

Table 13. β-galactosidase assay reaction composition

Component	Amount (µl)
100X Mg solution (Appendix A.3.20)	3
1XONPG solution (Appendix A.3.24)	66
Protein sample	30
0.1M Phosphate buffer (Appendix A.3.31)	201

The reaction mixture was shaken and incubated at 37°C for 30 min. To stop the reaction, 500 µl sodium carbonate (Appendix A.3.40) was added. The OD was measured at 420nm as well as at 550nm. To calculate the amount of units of β-galactosidase the following equation was used:

1 unit of β-galactosidase = 200 * (OD_{420}-(1.65 X OD_{550})) / mg of protein / minute

2.5.6 SDS Polyacrylamide Gel Electrophoresis (PAGE)

2.5.6.1 Sample preparation

Protein concentrations of ribolysed samples were measured using the Bio-Rad Protein assay (section 2.5.4) and samples were diluted to the same concentration. Twenty microliters of each ribolysed sample was placed into an 1.5 ml Eppendorf tube, mixed with 20 µl 2X sample reducing buffer (Appendix A.3.35) and boiled at 100°C for 5 min. The samples were then loaded onto a gel prepared as described in Section 2.5.6.2 and was run as described in Section 2.5.6.3.

2.5.6.2 Gel preparation

A Bio-Rad gel apparatus was assembled as per manufacturer's instruction (Bio-Rad). A 1% separating gel was made, reserving the top 2 cm for the 3% stacking gel. The gel consisted of the components listed in Table 14 and 15.

Table 14. Components for a 10% acrylamide separating gel

Component	Amount
Acrylamide/bis-acrylamide 30:0.8 (% w/v), (Appendix A.3.2)	3.33 ml
1.5 M Tris-HCl pH 8.8 (Appendix A3.47)	2.5 ml
10% SDS (Appendix A.3.36)	100 µl
H_20	4 ml
Tetramethylethylenediamine (temed, sigma)	10 µl
10% ammonium persulphate (Appendix A.3.3)	50 µl

Table 15. Components for a 3% acrylamide stacking gel

Component	Amount
Acrylamide/bis-acrylamide	1.3 ml
1M Tris-HCl pH 6.8 (Appendix A3.45)	1.25 ml
10% SDS (Appendix A.3.36)	100 µl
H_20	7.4 ml
Tetramethylethylenediamine	20 µl
10% ammonium persulphate (Appendix A.3.3)	50 µl

2.5.6.3 Running of samples

Fifteen microliter of each sample (as described in Section 2.5.6.1) was loaded into the gel (as described in Section 2.5.6.2) wells and run along with 5 µl "Prestained Protein Ladder" (Fermentas) protein ladder at 200V for 50 min using SDS-PAGE running buffer (Appendix A.3.37). Gels were run in duplicate, in order to have one for protein size analysis as well as one for Western blotting. One gel was stained with Coomassie Blue (Appendix A.3.9) for one hour, followed by overnight destaining with destaining solution (Appendix A.3.10). The other gel was used for Western blotting as described in Section 2.5.6.4.

2.5.6.4 Western blotting

After SDS polyacrylamide gel electrophoresis, the protein samples on the gel were transferred to a polyvinylidene difluoride (PVDF) membrane by Western blotting. The gel was placed on a pre-wet PVDF membrane. A Whatman sheet was placed on either side of the gel and membrane (one on the gel side and one on the membrane side.). A foam pad is then added to either side of the Whatman sheet. This “sandwich” was then placed into a Western blot apparatus filled with transfer buffer (Appendix A.3.42). A frozen icepack and magnetic stirrer were added and the transfer was allowed to take place at 100 V for 60 min. Following this, the membrane was stained with Ponceau Red dye (Appendix A.3.32) and the positions of visible protein ladder marker bands were marked with a pen (along the edge).

2.5.6.5 Antibody-based His-tag detection

For antibody-based detection, the membrane was blocked overnight with blocking buffer (Appendix A.3.5). The membrane was rinsed well with Tris Buffered Saline Tween (TBS-T) buffer (Appendix A.3.41) and incubated with the primary antibody, mouse anti-His (Invitrogen). The mouse anti-His antibody was diluted 1:5000 in TBS-T buffer. From this dilution 1 µl was used and placed onto the membrane. The membrane was incubated for 60 min at room temperature with gentle shaking. This was followed by a wash step by rinsing twice with TBS-T buffer followed by three 10 min wash steps in TBS-T buffer with shaking. The membrane was then incubated with the secondary antibody, goat-anti mouse (Caltag Laboratories). The secondary antibody (goat-anti-mouse conjugated to Horse Radish Peroxidase (HRPO)) was diluted 1:10 000. One microliter was diluted in 10 ml TBS-T buffer and incubated with the membrane for 60 min at room temperature with gentle shaking. This wash step was repeated followed by an incubation step with enhanced chemiluminescent (ECL) detection fluid (5 ml “solution 1” mixed with 5 ml “solution 2”) for 1 min. The membrane was then incubated along with an auto-radiographic film in a X-ray film cassette for periods of 10 sec, 30 sec, 2 min, 5 min and 20 min to vary the intensities of the visual result on the film.

2.5.6.6 HRP Western blot detection colorimetric-based His-tag detection

The KPL His Detector Western Blot Kit was used to detect certain His-tagged proteins. Following Western blotting the membrane was blocked with 10 ml of 1% BSA blocking buffer (Appendix A.3.7) in TBS-T buffer (Appendix A.3.41) for 1 hour at room temperature with gentle shaking. One microliter His-Detector Nickel-HRP conjugate (KPL) was added directly to the blocking solution and incubated with the membrane for 1 hour with gentle shaking. The membrane was washed with TBS-T buffer 3 times for 5 min each with gentle shaking. Ten millilitres TMB Membrane Substrate (KPL) was added to the membrane and allowed to react for 15 min. The membrane was rinsed for 30sec to stop the reaction and allowed to air dry. The membrane was then photocopied to keep a permanent record.

2.6 Cloning

2.6.1 Cloning of PCR products

PCR amplification products intended for cloning were first cloned into the Promega T-vector, pGem-T-Easy (Appendix B.5). Following a PCR cleanup (Section 2.3.3) and elution with 40 µl H_2O, the purified PCR product was used in a ligation reaction with pGem-T-easy as described in Table 16.

Table 16. Ligation components for ligation of PCR products to pGem-T-Easy.

Component	Amount
Purified PCR product	3 µl
2X Ligation buffer (Promega)	5 µl
pGem-T-Easy vector (Promega)	1 µl
T4 DNA Ligase (Promega)	1 µl
Total	10 µl

This ligation mixture was incubated at room temperature for 1h. Two microliters of the ligation was electroporated into *E. coli.* (Section 2.3.13.1) and plated onto LB agar plates (Appendix A.2.7) containing ampicillin (Appendix A.1.1) along with 100 µl X-gal (Appendix A.3.49) and 100 µl IPTG (Appendix A.3.16). White colonies were picked, PCR screened for the correct insert (Section 2.2.3), grown overnight in LB (Section 2.1.2.1) with ampicillin. Plasmid DNA was extracted and purified using the Promega SV-Wizard Miniprep kit (Section 2.3.5). The DNA concentration was then determined (Section 2.3.7) and the DNA was then sent for sequencing (Section 2.3.8) using the sequencing primers Sp6 and T7 (Section 2.2.1, Table 5) to confirm the sequence of the insert.

2.6.2 Cloning of promoter elements into pJem15

Ten intergenic regions were chosen from *M. tb* ESAT-6 gene cluster region 5 (Rv1782 to Rv1798). Based on promoters detected in other ESX gene clusters, this region potentially contains several strong promoters (Gey van Pittius, 2002). Two online promoter prediction software programs were used to identify ten intergenic regions selected from the *M. tb* H37RV ESAT-6 gene cluster 5. The first is the "Neural Network Promoter Prediction" (NNPP) software, (http://www.fruitfly.org/seq_tools/promoter.html) and the second is the "BPROM" software

(http://softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). A minimum promoter score of 0.2 was used for both programs. Regions larger than 35bp were chosen to be cloned into a promoter probe vector to detect promoter activity. Table 17 lists the details of the various regions chosen to screen for promoter activity and Appendix E.1 lists the sequences of the selected promoter regions that were screened.

Table 17. Details of the selected intergenic regions of the ESAT-6 gene cluster region 5 and their location in the *M. tb* H37Rv genome

Intergenic region	Size of intergenic region (bp)	Size of PCR product (bp)	Location in H37Rv genome (bp)	Primer pairs (details in Table 5 Section 2.2.2)
Int1 or (Rv177980)	420	254	2014379-2014799	Rv1779-80f and Rv1779-80r
Int2 or (Rv178182)	464	319	2017376-2017840	Rv1781-82f and Rv1781-82r
Int3 or (Rv178384)	670	529	2020464-2020734	Rv1783-84f and Rv1783-84r
Int4 or (Rv178687)	470	323	2024931-2025401	Rv1786-87f and Rv1786-87r
Int5 or (Rv178788)	279	128	2026298-2026577	Rv1787-88f and (Rv1787-88r
Int6 or (Rv178990)	654	498	2027871-2028525	Rv1789-90f and Rv1789-90r
Int7 or (Rv179091)	627	462	2029377-2030004	Rv1790-91f and Rv1790-91r
Int8 or (Rv179192)	344	187	2030103-2030447	Rv1791-92f and Rv1791-92r
Int9 or (Rv179394)	288	135	2030878-2031166	Rv1793-94f and Rv1793-94r
Int10 or (Rv179495)	472	312	2031868-2032340	Rv1794-95f and Rv1794-95r

Each promoter element was PCR amplified (Section 2.2) with the corresponding primers listed in Table 17, the products were run on a gel (Section 2.3.1). The correct size band (as listed in Table 17) was purified from the gel (Section 2.3.3) and cloned into pGem-T-Easy (Section 2.6.1). The plasmids were then digested with *Bam*HI and *Kpn*I (Section 2.3.9) and the digests were run on a gel. The correct size bands (Table 17) were purified from the gel. Vector pJem15 was digested with *Bam*HI and *Kpn*I, run on a gel, the large molecular size band was purified from the gel and dephosphorylated (Section 2.3.11). The digested intergenic region promoter elements were ligated (Section 2.3.12) to

the digested and dephosphorylated pJem15 to create the constructs named pJem15int1, pJem15int2, pJem15int3, pJem15int4, pJem15int5, pJem15int6, pJem15int7, pJem15int8, pJem15int9 and pJem15int10. The ligations were electroporated into *E. coli* (Section 2.3.13.1) and plated onto LB agar (Appendix A.2.8) containing kanamycin (Appendix A.1.2). A PCR colony screening was done (Section 2.2.3) using the relevant primer pairs from Table 17 and the colonies testing positive for the plamid with the correct size inserts (as listed in Table 17) were grown in liquid media (Section 2.1.2.1) containing kanamycin. The plasmid DNA was extracted (Section 2.3.5) and sequenced (Section 2.3.8) using the relevant primer pair from Table 17 to verify the insert sequence. The promoter strengths of the promoter constructs were then assayed using the β-galactosidase assay (Section 2.5.5) and the strongest promoter construct was selected for use in constructing reporter plasmids as described in Section 2.6.3.

2.6.3 Cloning to create reporter plasmid constructs

As a proof of concept for the mycobacteriophage reporter system, plasmid reporter constructs were made for evaluation of expression in *M. smegmatis.* Several variables play a role in the expression levels of a given reporter protein. It was thus necessary to establish the optimal reporter construct, and in so doing numerous plasmid reporter constructs were created and evaluated. The constructs made use of various promoters, reporter genes, affinity tags and expression vectors. Table 18 lists the various reporter constructs that were created. The methodology for creating each of the reporter constructs is referenced in Table 18.

Table 18. List of reporter plasmids.

Construct Name	Reporter Gene	Promoter	Affinity tag	Expression vector	Antibiotic resistance	Section
pSD21Hsp60DsRedNHis	DsRed	Hsp60	N-term His tag	pSD21	Kanamycin	2.6.3.1
p19KProHsp60DsRedNHis	DsRed	Hsp60	N-term His tag	p19KPro	Hygromycin	2.6.3.2
pSD21Hsp60DsRedCHis	DsRed	Hsp60	C-term His tag	pSD21	Kanamycin	2.6.3.3
pACEHsp60DsRedCHis	DsRed	Hsp60	C-term His tag	pACE	Hygromycin	2.6.3.4
pSD21Int1DsRedNHis	DsRed	Int1	N-term His tag	pSD21	Kanamycin	2.6.3.5
pACEInt1DsRedCHis	DsRed	Int1	N-term His tag	pACE	Hygromycin	2.6.3.6
pSD21Hsp60mRFPNHis	mRFP1	Hsp60	N-term His tag	pSD21	Kanamycin	2.6.3.7
p19KProp19kmRFPNHis	mRFP1	P19k	N-term His tag	p19KPro	Kanamycin	2.6.3.8
pSD21Int1mRFPNHis	mRFP1	Int1	N-term His tag	pSD21	Kanamycin	2.6.3.9
pDMN1SmycmRFPNHis	mRFP1	Smyc	N-term His tag	pDMN1	Kanamycin	2.6.3.10
pJem15Hsp60LacZNHis	LacZ	Hsp60	N-term His tag	pJem15	Kanamycin	2.6.3.11
pJem15Int1LacZCHis	LacZ	Int1	N-term His tag	pJem15	Kanamycin	2.6.3.12

2.6.3.1 Construction of pSD21Hsp60DsRedNHis

The Hsp60 promoter as well as the first 3 amino acids from the *M. tb* ESAT-6 gene was previously cloned into the vector pMB154Hsp60 (Appendix B.8). The promoter and ESAT-6 segment was PCR amplified (Section 2.2) with primers ESAT6-f and Hsp60PromHisr3 (Table 5, Section 2.2.1) to add RE sites *Bam*HI and *Kpn*I as well as a poly-Histidine tag (His-Tag, 6 amino acids in total) to the PCR product. The PCR products were run on a gel (Section 2.3.1) and the 361bp fragment was purified from the gel (Section 2.3.3). The purified PCR products were then cloned into pGem-T-Easy (Section 2.6.1) to create the construct named pGemHsp60NHis3. About 4 µg of each plasmid DNA was used in a digestion to remove the insert from pGem-T-Easy with RE *Bam*HI and *Kpn*I (Section 2.3.9). The digestion products were run on a gel and the 359bp fragment was purified from the gel. Vector pDsRed (Appendix B.3), was digested with *Bam*HI and *Kpn*I, run on a gel, and the 3034bp vector fragment was purified and then dephosphorylated (Section 2.3.11). The digested and dephosphorylated DsRed vector was ligated (Section 2.3.12) to the inserts from the digested pGemHsp60NHis3 vectors and the resulting construct was called pDsRedHsp60NHisDsRed3. This places the *DsRed* gene, encoding for a tetrametic red fluorescent protein, under the control of the Hsp60 promoter and also adds an N-terminus His-tag to the *DsRed* gene. The ligation was electroporated into *E. coli* (Section 2.3.13.1) and plated on LB agar (Appendix A.2.7) containing ampicilin (Appendix A.1.1). PCR colony screening was done (Section 2.2.3) using ESAT6-f and Hsp60PromHisr3, colonies testing positive for the plasmid containing the plasmid with the correct size

insert (corresponding to 361bp band) were grown in liquid media (Section 2.1.2.1) containing ampicillin. Plasmid DNA was extracted and purified (Section 2.3.5) and the vectors were then sent for sequencing (Section 2.3.8) using primers ESAT6-f and Hsp60PromHisr3. The inserts were removed from the pDsRed vector by digestion with RE *Xba*I. The digests were run on a gel and the 1065bp fragment was purified. pSD21 (Appendix B.12) was digested with *Xba*I, run on a gel and the large molecular weight band was purified and dephosphorylated. The digested pSD21 DNA was ligated to the inserts of the digested Hsp60NHisDsRed3 products and the construct was named pSD21Hsp60DsRedNHis3. The ligations were electroporated into *E. coli* followed by a PCR colony screening for the Hsp60 promoter and *DsRed* gene insert using ESAT6-f and Hsp60PromHisr3. Colonies testing positive for the plasmid with the correct size insert (corresponding to the 1065bp band) were grown up in liquid media containing kanamycin (Appendix A.1.2). The plasmid DNA was purified from the liquid cultures and the plasmid constructs were verified by sequence analysis using primer pairs ESAT6-f and Hsp60PromHisr3. Figure 2.1a and 2.1b is an illustration of the main cloning steps used to create the construct pSD21Hsp60DsRedNHis.

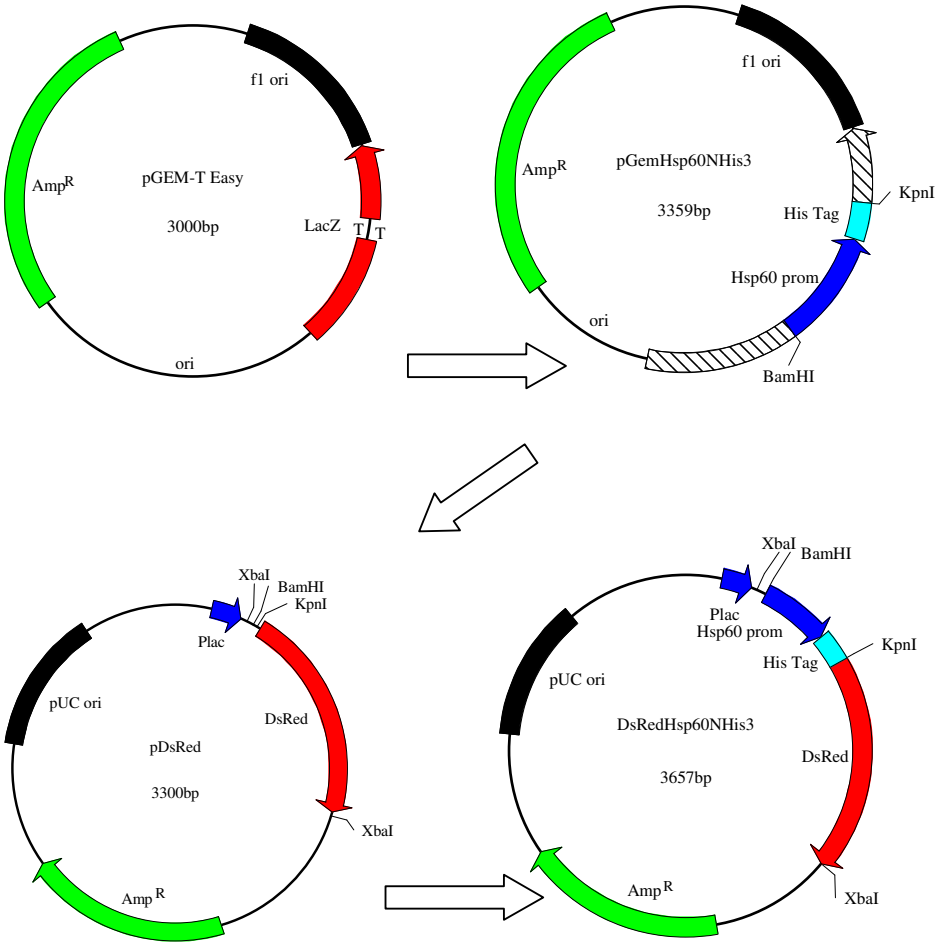

Figure 2.1a Construction of pSD21Hsp60DsRedNHis. The PCR amplification product, consisting of the Hsp60promoter and a histidine tag for N-terminal protein fusions, was cloned into pGem-T-Easy and was called pGemHsp60NHis3. pGemHsp60NHis3 was digested with *Bam*HI and *Kpn*I and the insert was ligated to the *Bam*HI and *Kpn*I digested pDsRed to give pDsRedHsp60NHis3. This places the *DsRed* gene under the control of the Hsp60 promoter and also creates an N-terminal His tag fusion to the *DsRed* gene.

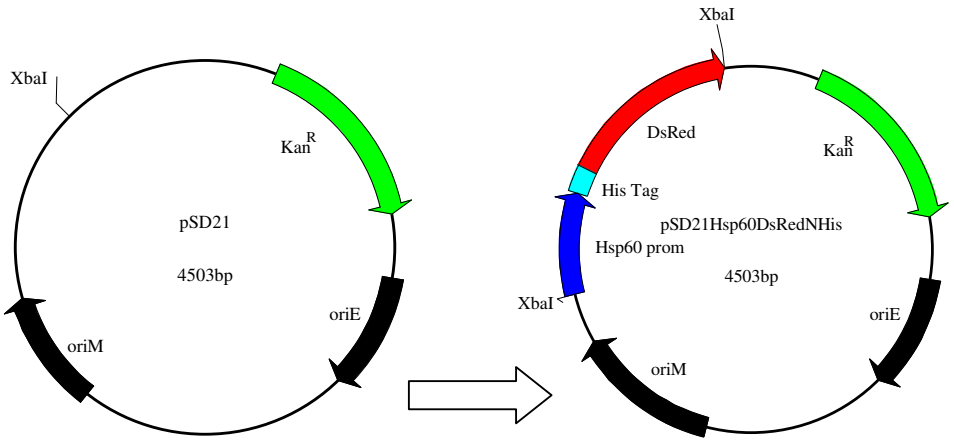

Figure 2.1b Construction of pSD21Hsp60DsRedNHis (continued). DsRedHsp60NHis3 (shown in Figure 2.1a) was digested with *Xba*I and the resulting product consisting of the Hsp60 promoter and N-terminus His tagged *DsRed* gene was cloned into the *Xba*I site of expression vector pSD21 to create pSD21Hsp60DsRedNHis.

2.6.3.2 Construction of P19KproHsp60DsRedNHis

The P19Kpro vector (Appendix B.1) was digested (Section 2.3.9) with *Xba*I and run on a gel (Section 2.3.1). The large molecular weight band was purified from the gel (Section 2.3.3) and dephosphorylated (Section 2.3.11). The *Xba*I digested P19Kpro vector was then ligated (Section 2.3.12) to the liberated insert from the *Xba*I digested pDsRedHsp60DsRedNHis (Section 2.6.3.1) to create the vector P19KproHsp60DsRedNHis. The ligation was electroporated into *E. coli* (Section 2.3.13.1). A PCR colony screening (Section 2.2.3) was performed using primers ESAT6-f and Hsp60promHisr-3 (Table 5, Section 2.2.1), to confirm the presence of plasmid containing the correct inserts. The colonies that tested positive for the plasmid with the correct insert (corresponding to the 1065bp band, consisting of the Hsp60 promoter and *DsRed* gene) were grown up in liquid media (Section 2.1.2.1) containing hygromycin (Appendix A.1.3) and the plasmid DNA was purified (Section 2.3.5). The constructs were then sequence analyzed (Section 2.3.8) using primers ESAT6-f and Hsp60promHisr-3. Figure 2.2 is a visual illustration of the main cloning steps used to create construct P19KproHsp60DsRedNHis.

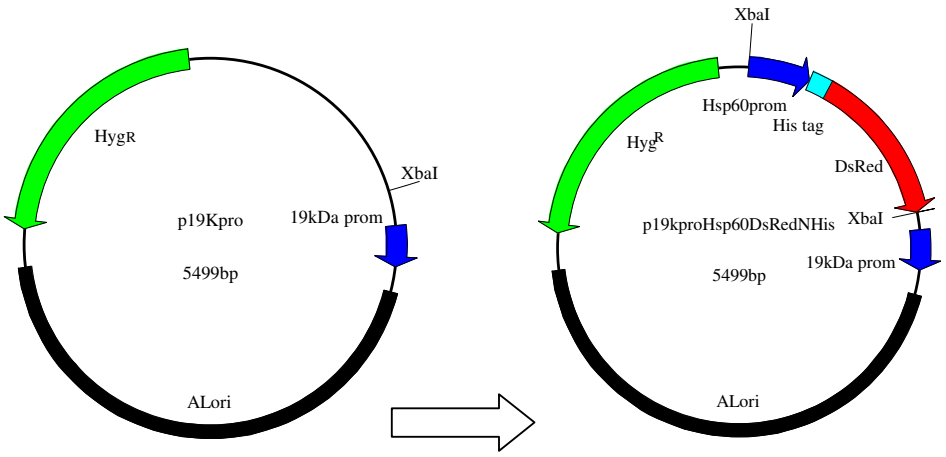

Figure 2.2 Construction of P19KproHsp60DsRedNHis. The liberated insert from the *Xba*I digested pDsRedHsp60DsRedNHis (Section 2.6.3.1) was cloned into *Xba*I digested P19Kpro to create P19KproHsp60DsRedNHis.

2.6.3.3 Construction of pSD21Hsp60DsRedCHis

The Hsp60 promoter and the *DsRed* gene was PCR amplified (Section 2.2.2) from a previously constructed vector, pHsp60DsRed (Appendix B.4), with primer pairs ESAT6-f and DsRedHisr (Table 5, Section 2.2) to add a C-terminus His-tag as well as *Bam*HI and *Xba*I RE sites. The PCR product was run on a gel (Section 2.3.1) and the 1065bp band was purified (Section 2.3.3). The PCR product was then cloned into pGem-T-Easy (Section 2.6.1) and the resulting construct was called pGemHsp60DsRedCHis. The insert was removed from pGem-T-Easy by digestion using *Bam*HI and *Xba*I (Section 2.3.9), run on a gel and the 1059bp band was purified. pSD21 (Appendix B.12) was digested with *Bam*HI and *Xba*I, run on a gel and the large molecular weight band was purified and dephosphorylated (Section 2.3.11). The digested and dephosphorylated pSD21 vector was ligated to the insert from the digested pGemHsp60DsRedCHis product and the construct was called pSD21Hsp60DsRedCHis. The ligation was electroporated into *E. coli* (Section 2.3.13.1) and plated onto LB solid media (Section 2.1.2.2) with kanamycin (Appendix A.1.2). A PCR colony screening (Section 2.2.3) was done and colonies testing positive for the plasmid with the correct insert size (corresponding to the 1065bp band) were grown in liquid culture (Section 2.1.2.1) with kanamycin. The

construct was then sequence verified (Section 2.3.8). Figure 2.3a and 2.3b is an illustration of the main cloning steps to create pSD21Hsp60DsRedCHis.

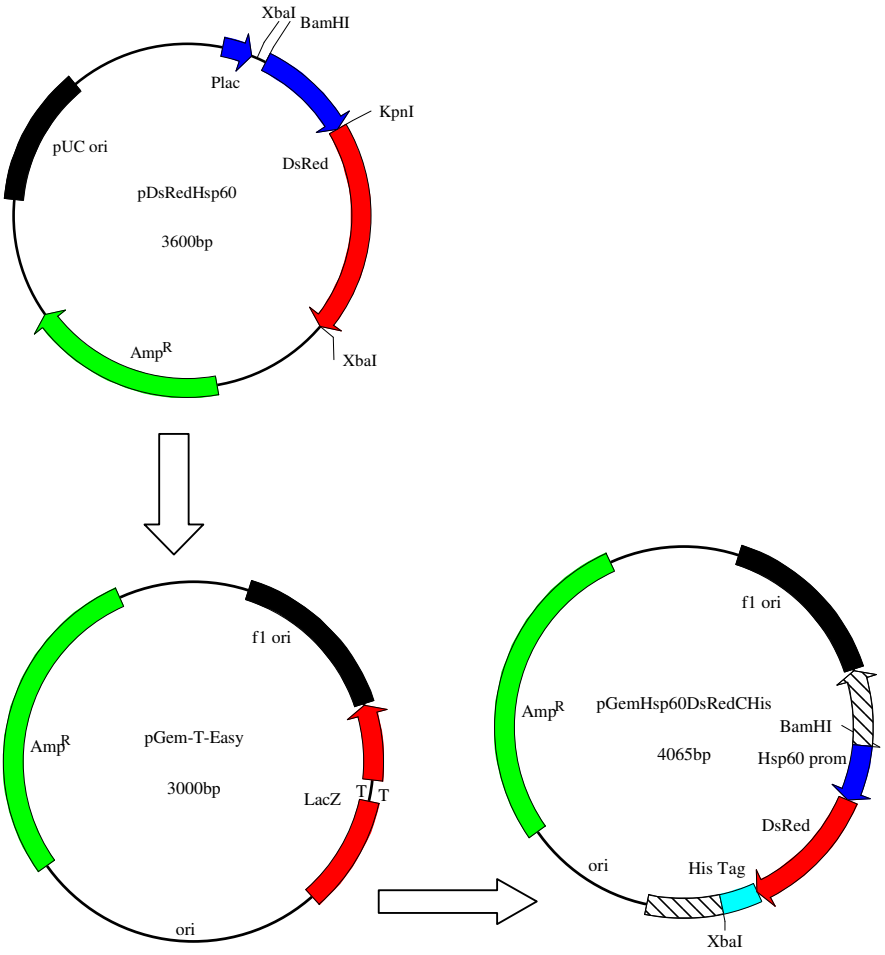

Figure 2.3a Construction of pSD21Hsp60DsRedCHis. The Hsp60 promoter and *DsRed* gene was PCR amplified from pDsRedHsp60 to add a C-term His-tag fusion to the PCR product. The PCR product was ligated to pGem-T-Easy and was called pGemHsp60DsRedCHis.

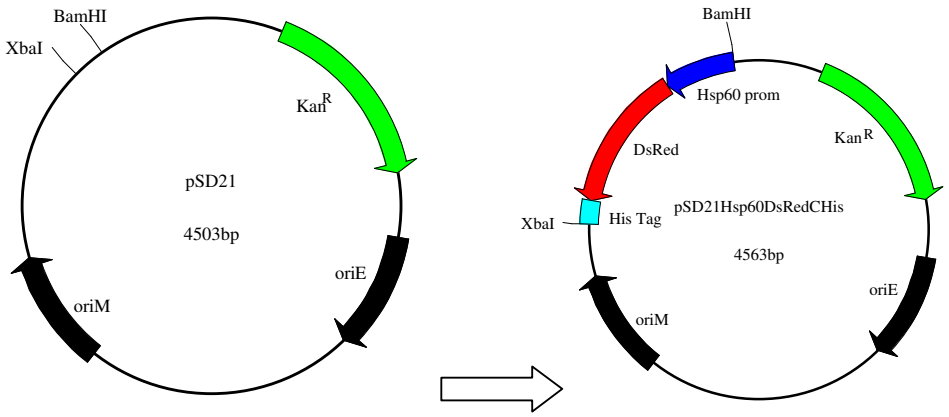

Figure 2.3b Construction of pSD21Hsp60DsRedCHis (continued). Vector pGemHsp60DsRedCHis was digested with *Bam*HI and *Xba*I and the liberated insert was ligated to *Bam*HI and *Xba*I digested pSD21 to create pSD21Hsp60DsRedCHis.

2.6.3.4 Construction of pACEHsp60DsRedCHis

The 1059bp pGemHsp60DsRedCHis *Bam*HI and *Xba*I digest product from Section 2.6.3.3 was also cloned into the expression vector pACE (Appendix B.2). The pACE vector was digested (Section 2.3.9) with *Bam*HI and *Xba*I (which removes the ACE promoter) and run on a gel (Section 2.3.1). The digested pACE vector (5315bp band) was purified from the gel (Section 2.3.3) and dephosphorylated (Section 2.3.11). The *Bam*HI and *Xba*I digested pACE vector was then ligated (Section 2.3.12) to the liberated insert from the *Bam*HI and *Xba*I digested pGemHsp60DsRedCHis (Section 2.6.3.3) to create the construct named pACEHsp60DsRedCHis. The ligation was electroporated into *E. coli* (Section 2.3.13.1) and a PCR colony screening (Section 2.2.3) was done, using primers ESAT6-f and DsRedHisr (Table 5, Section 2.2.1). The colonies testing positive for the plasmid with the correct insert size (corresponding to the 1065bp band) were grown in liquid media (Section 2.1.2.1) containing ampicillin (Appendix A.1.1) and the plasmid DNA was purified (Section 2.3.5). The construct was then sequence analyzed (Section 2.3.8) using primers ESAT6-f and DsRedHisr (Table 5, Section 2.2.1). Figure 2.4 is a visual illustration of the main cloning steps used to create pACEHsp60DsRedCHis.

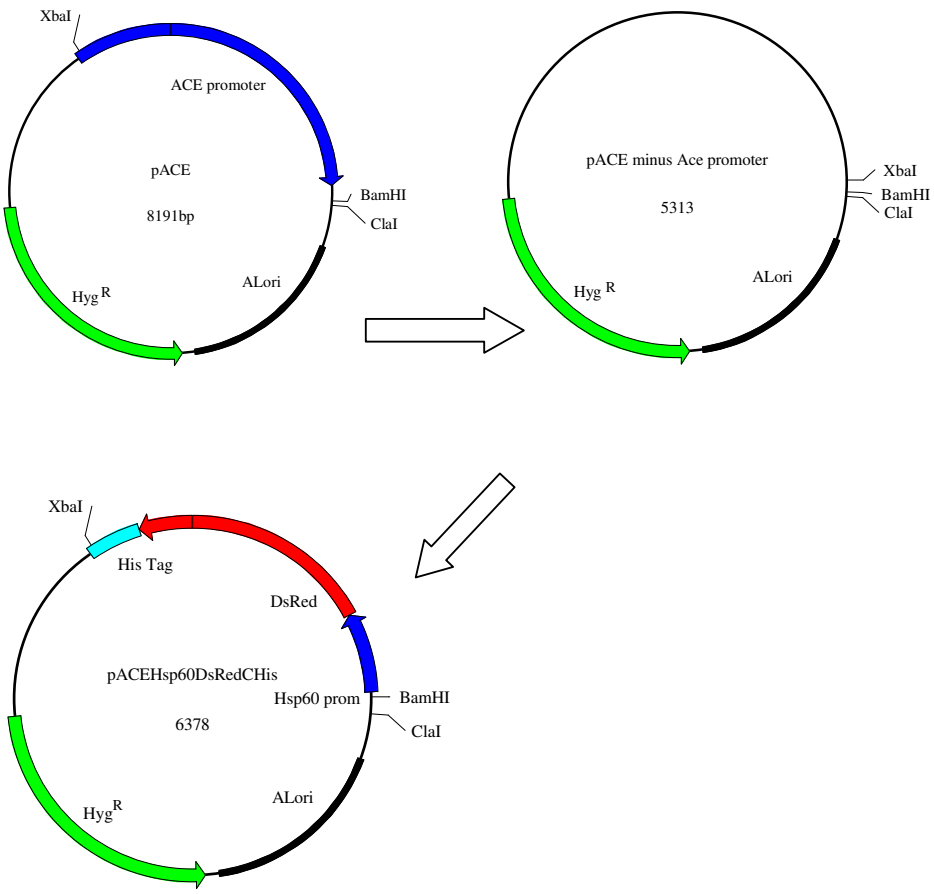

Figure 2.4 Construction of pACEHsp60DsRedCHis. The ACE promoter was removed from pACE with *Bam*HI and *Xba*I. The insert from the *Bam*HI and *Xba*I digested pGemHsp60DsRedCHis (Section 2.6.3.3) was cloned into the *Bam*HI and *Xba*I sites of pACE creating the construct pACEHsp60DsRedCHis.

2.6.3.5 Construction of pSD21Int1DsRedNHis

The intergenic region promoter element "Int1" (Section 2.6.2) was PCR amplified (Section 2.2.2) from H37Rv genomic DNA (Section 2.1.1) with the primers Rv1979-80f and Int1RFPr (Table 5, Section 2.2.1) to add *Bam*HI and *Kpn*I RE sites as well as an His-tag for N-terminal protein fusions to the PCR product. The PCR product was run on a gel (Section 2.3.1) and the 254bp band was purified (Section 2.3.3) and cloned into pGem-T-Easy (Section 2.6.1) to create the construct pGemInt1NHis. The insert was removed from pGem-T-Easy by digestion (Section 2.3.9) with *Bam*HI and *Kpn*I. The digest was

run on a gel and the 254bp band was purified. pDsRed (Appendix B.3) was digested with *Bam*HI and *Kpn*I (Section 2.3.9), the digest product was run on a gel and the large molecular weight band was purified and then dephosphorylated (Section 2.3.11). The *Bam*HI and *Kpn*I digested pDsRed vector was ligated (Section 2.3.12) to the insert from the *Bam*HI and *Kpn*I digested pGemInt1NHis to create the construct pDsRedInt1DsRedNHis. The ligation was electroporated into *E. coli* (Section 2.3.13.1) and a PCR colony screening was done (Section 2.2.3) using primers Rv1979-80f and Int1RFPr. The colonies testing positive for the plasmid with the correct insert size (corresponding to the 254bp band) were grown up in liquid media (Section 2.1.2.1) containing ampicillin (Appendix A.1.1). The plasmid DNA was purified (Section 2.3.5) and sequence verified (Section 2.3.8) with primers Rv1979f and Int1RFPr. The pDsRedInt1DsRedNHis construct was then digested with *Xba*I to remove the promoter and *DsRed* gene. The digest was run on a gel and the 993bp band was purified. pSD21 (Appendix B.12) was digested with *Xba*I, the digest was run on a gel and the large molecular weight band was purified, dephosphorylated and was ligated to the *Xba*I digested insert from pDsRedInt1DsRedNHis to create the construct pSD21Int1DsRedNHis. The ligation was electroporated into *E. coli* and a PCR colony screening was done using primers Rv1979-80f and Int1RFPr to screen for colonies with the correct insert size (corresponding to the 993bp band). The colonies with plasmids containing the correct insert size were grown in liquid media containing kanamycin (Appendix A.1.2) and the plasmid DNA was purified. The constructs were sequenced with primers Rv1979-80f and Int1RFPr. Figure 2.5a and 2.5b is a visual illustration of the main cloning steps used to create construct pSD21Int1DsRedNHis.

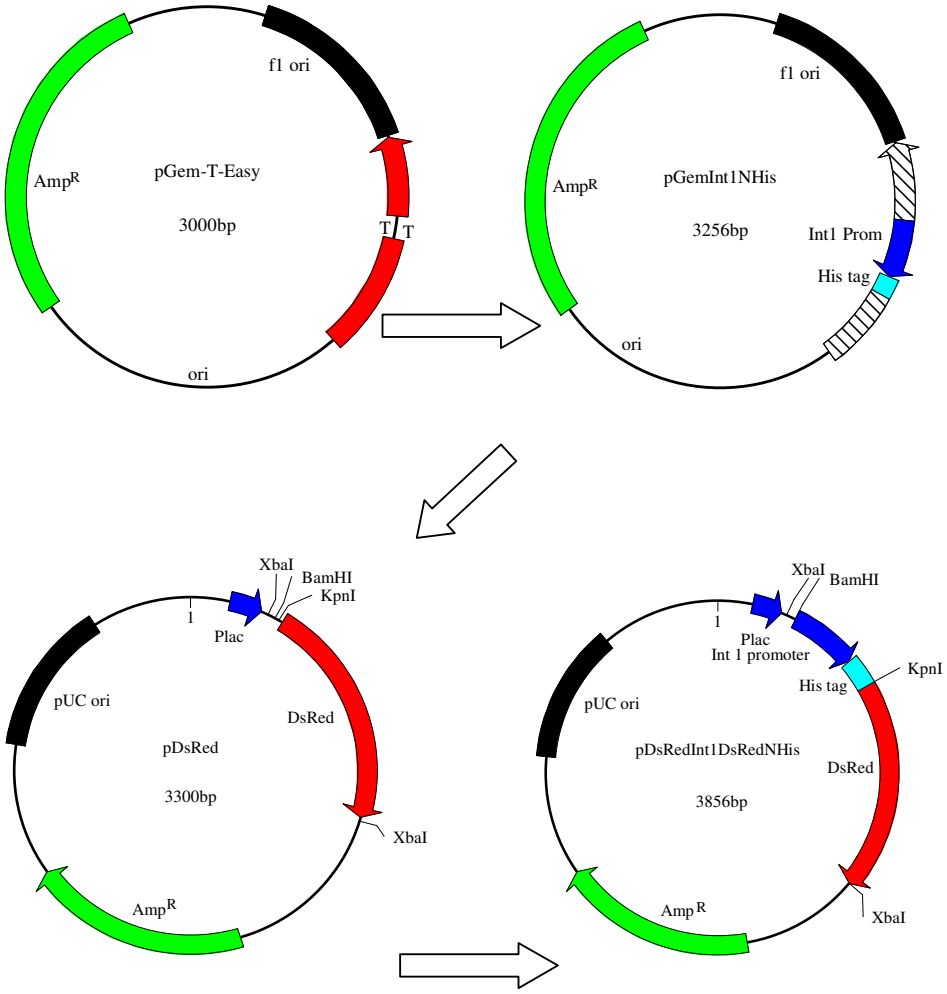

Figure 2.5a Construction of pSD21Int1DsRedNHis. The "int1" (intergenic region 1) promoter was PCR amplified to add a His-tag sequence for N-terminal protein fusions and was cloned into pGem-T-Easy. The construct was named pGemInt1NHis. The insert was removed from pGem-T-Easy using *Bam*HI and *Kpn*I and was ligated into pDsRed to create pDsRedInt1DsRedNHis.

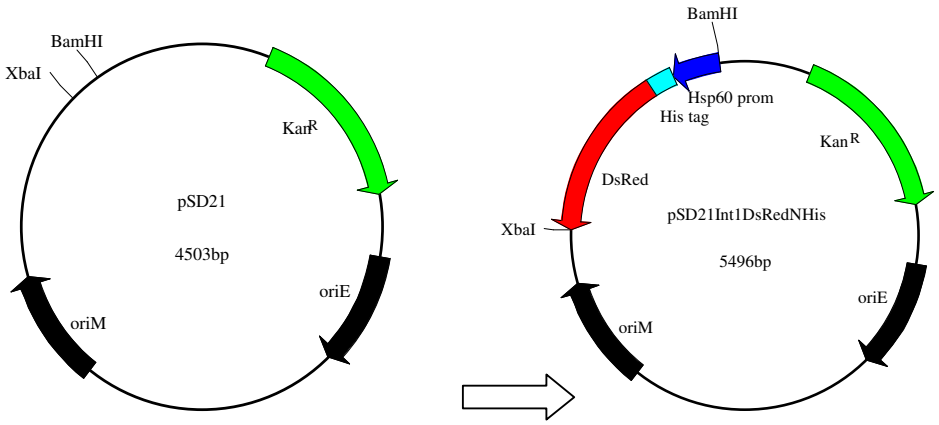

Figure 2.5b Construction of pSD21Int1DsRedNHis (continued). The promoter and *DsRed* gene was removed from pDsRed using *Xba*I and cloned into pSD21, this construct was named pSD21IntIDsRedNHis.

2.6.3.6 Construction of pACEInt1DsRedCHis

The intergenic region 1 promoter element 1 was PCR amplified (Section 2.2.2) using primers Rv1979-80f and Rv1979-80r (Table 5, Section 2.2) from H37Rv genomic DNA (Section 2.1.1). The primers add *Bam*HI and *Kpn*I RE sites to the PCR product. The PCR product was run on a gel (Section 2.3.1) and the 233bp band was purified from the gel (Section 2.3.3). The purified PCR product was ligated to pGem-T-Easy (Section 2.6.1) and this was called pGemInt1. The insert was removed from pGem-T-Easy by digestion with *Bam*HI and *Kpn*I (Section 2.3.9) and the digest was run on a gel, the 233bp band was then purified (Section 2.3.3). pDsRedHsp60DsRedCHis (Section 2.6.3.3) was digested with *Bam*HI and *Kpn*I to remove the Hsp60 promoter. The digested DNA was run on a gel and the large molecular weight band was purified and then dephosphorylated (Section 2.3.11). The digested pGemIntI fragment was then ligated (Section 2.3.12) to the *Bam*HI and *Kpn*I digested pDsRedHsp60DsRedCHis vector and was called pDsRedIntIDsRedCHis. The ligation was then electroporated into *E. coli* (Section 2.3.13.1) and plated onto LB agar plates (Appendix A.2.8) containing ampicillin (Appendix A.1.1). The colonies were PCR screened (Section 2.2.3) using primers Rv1979-80f and Rv1979-80r. Colonies testing positive for the 223bp band were then grown in liquid

media containing ampicillin and the plasmid DNA was subsequently purified (Section 2.2.5). The purified pDsRedIntIDsRedCHis DNA was then digested with *Bam*HI and *Xba*I and the 993bp band was purified. *Bam*HI and *Xba*I digested and dephosporylated pACE DNA from Section 2.6.3.4 was then ligated to the 993bp fragment from the *Bam*HI and *Xba*I digest of pDsRedIntIDsRedCHis. The ligation product was named pACEIntIDsRedCHis and electroporated into *E. coli*, a PCR colony screening was done using primer pairs Rv1979-80f and DsRedHisr. The colonies containing plasmids testing positive for the correct insert size (corresponding to the 993bp band) were grown in liquid media (Section 2.1.2.1) containing hygromycin (Appendix A.1.2). A plasmid DNA extraction was performed on the liquid cultures and the construct was analyzed by sequencing (Section 2.3.8) using primers RV1979-80f, RV1979-80r and DsRedHisr. Figure 2.6 is a visual illustration of the main cloning steps to create construct pACEInt1DsRedCHis.

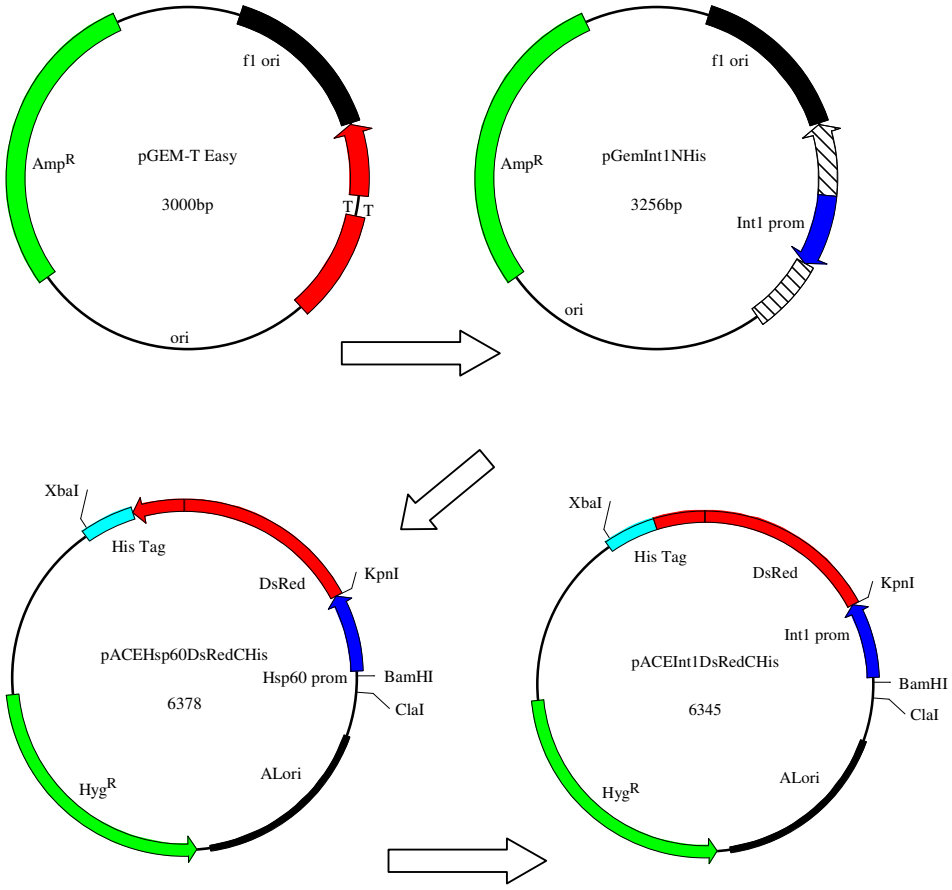

Figure 2.6 Construction of pACEInt1DsRedCHis. The initial PCR product was cloned into pGem-T-Easy and was named pGemInt1. The Hsp60 promoter was removed from pACEHsp60DsRedCHis using *Bam*HI and *Kpn*I. The int1 promoter and His tag from pGemInt1NHis were cloned into the *Bam*HI and *Kpn*I digested pACEHsp60DsRedCHis to create pACEInt1DsRedCHis.

2.6.3.7 Construction of pSD21Hsp60mRFPNHis

The *mRFP1* gene was PCR amplified (Section 2.2) from the vector pRSETB (Appendix B.11) with the primers Hsp60mRFP1f2 and Hsp60mRFP1r2 (Table 5, Section 2.2.1) which add *Kpn*I and *Sal*I RE sites to the PCR product. The PCR product was run on a gel (Section 2.3.1), the 706bp band as purified (Section 2.3.3) and was subsequently cloned into pGem-T-Easy (Section 2.6.1) to create pGem1mRFP1. The insert was removed by digesting with RE *Kpn*I and *Sal*I (Section 2.3.9). The digest was run on a gel, and the 704bp band was purified from the gel. pGemHsp60NHis2 (Section 2.6.3.1) was digested with *Kpn*I and *Sal*I, run on a gel, and the large molecular band was purified and dephosphoylated (Section 2.3.11). The purified mRFP1 insert from the *Kpn*I and *Sal*I digested pGem1mRFP2 was then ligated (Section 2.3.12) to the *Kpn*I and *Sal*I digested and dephosphorylated pGemHsp60NHis to create pGemHsp60mRFP1NHis2. The ligations were electroporated into *E. coli* (Section 2.3.13.1) and a PCR colony screening was done (Section 2.2.3) using primers Hsp60mRFP1f2 and Hsp60mRFP1r2. Colonies testing positive for the plasmid containing the correct insert size (corresponding to the 1060bp band) were grown up in liquid media (Section 2.1.2.1) containing ampicillin (Appendix A.1). A plasmid extraction was done (Section 2.3.5) and the construct was sequence verified (Section 2.3.8) using the primers Hsp60mRFP1f2 and Hsp60mRFP1r2. pSD21 (Appendix B.12) was digested with *Bam*HI and *Kpn*I, run on a gel, the large molecular band was purified and the vector was dephosphorylated (Section 2.3.11). The *Bam*HI and *Kpn*I digested insert from pGem2Hsp60mRFPNHis consisting of the Hsp60 promoter and N-term His-tagged mRFP1 gene was ligated to the *Bam*HI and *Kpn*I digested and dephosphoylated pSD21 vector to create pSD21Hsp60mRFPNHis. The ligation was electroporated into *E. coli* and a PCR colony screening was done using primers ESAT6-f and Hsp60mRFP2r. Colonies testing positive for the correct insert size were selected and grown in liquid media containing kanamycin (Appendix A.1.2). The plasmid DNA was purified from the liquid culture (Section 2.3.5) and the construct was sequence verified (Section 2.3.8) using primers ESAT6-f, Hsp60mRFP2f and Hsp60mRFP2r. Figure 2.7a and 2.7b is a visual illustration of the main cloning steps involved to create the construct pSD21Hsp60mRFPNHis.

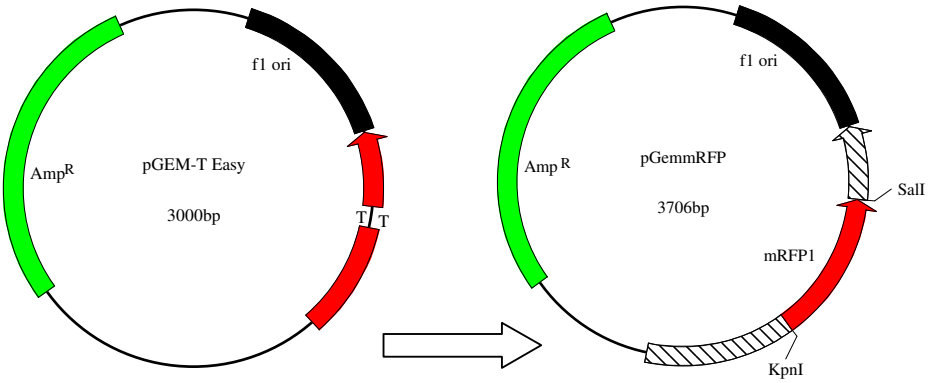

Figure 2.7a Construction of pSD21Hsp60mRFPNHis. The mRFP1 gene was PCR amplified and cloned into pGem-T-Easy to create pGemmRFP.

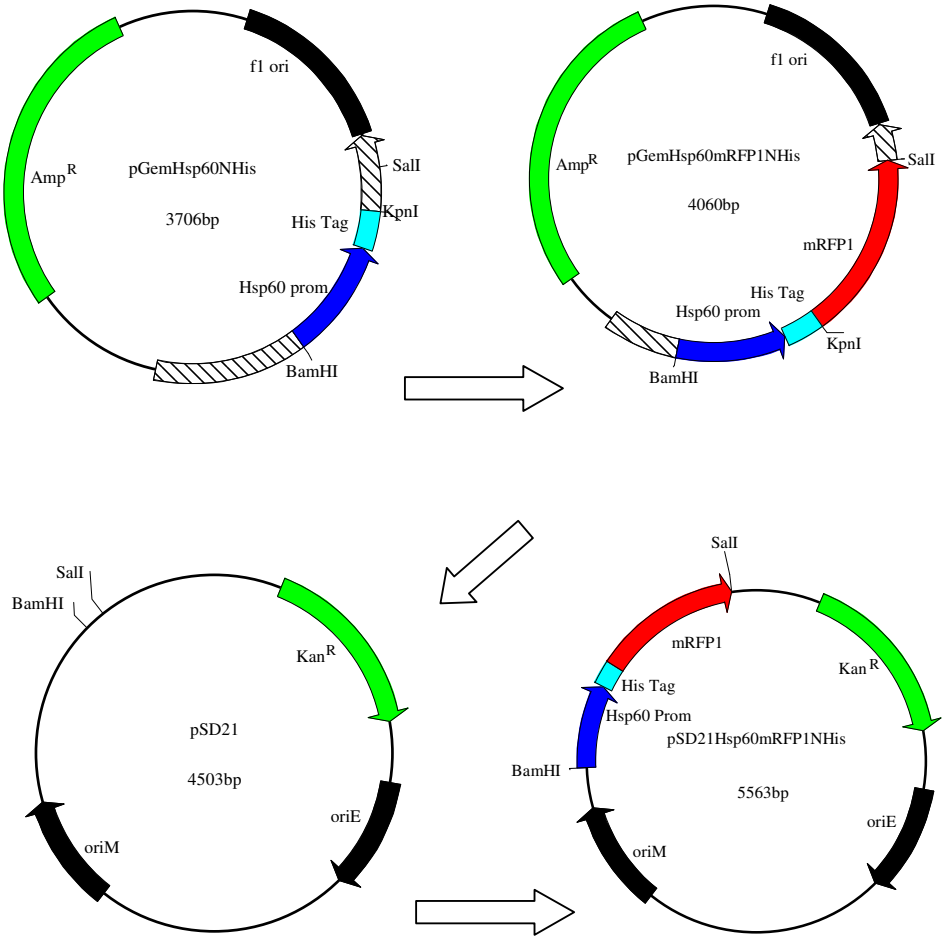

Figure 2.7b Construction of pSD21Hsp60mRFPNHis (continued). The mRFP1 gene was removed by digestion with *Kpn*I and *Sal*I and was ligated to *Kpn*I and *Sal*I digested pGemHsp60NHis (Section 2.6.3.1) to create pGemHsp60mRFP1NHis. The pGemHsp60mRFP1NHis vector was digested with *Bam*HI and *Sal*I and the liberated insert was cloned into pSD21 to create pSD21Hsp60mRFP1NHis.

2.6.3.8 Construction of p19KProp19KmRFPNHis

The mRFP1 gene was PCR amplified (Section 2.2.2) to add an N-terminal His-tag. The PCR template used was vector pRSETB (Appendix B.11) with primer pairs that add RE sites *Hind*III and *Cla*I named P19Kmrfpf2 and P19Kmrfpr3 (Table 5, Section 2.2.1). The PCR product was run on a gel and the 848bp band was purified (Section 2.3.3) and cloned into pGem-T-Easy (Section 2.6.1) to create pGemmRFPNHis. The PCR product was removed from pGemmRFPNHis by digesting with *Hind*III and *Cla*I (Section 2.3.9), the digested products were run on a gel and the 704bp band was purified. Vector p19Kpro (Appendix B.1) was digested with *Hind*III and *Cla*I, run on a gel, the large molecular band was purified and dephosphorylated (Section 2.3.11). The 704bp *Hind*III and *Cla*I digested insert from pGem5mRFPNHis was ligated to the *Hind*III and *Cla*I digested and dephosphorylated p19Kpro to create P19KproP19KmRFPNHis. The ligation was electroporated into *E. coli* (Section 2.3.12.1) and a PCR colony screening (Section 2.2.3) was done using primers P19Kmrfpf2 and P19Kmrfpr3. The colonies testing positive for the plasmid containing the correct insert size (corresponding to the 848bp band) were grown in liquid media (Section 2.1.2.1) containing kanamycin (Appendix A.1.2). A plasmid DNA extraction was done (Section 2.3.5) and the construct was sequence verified (Section 2.3.8) using primers P19Kmrfpf2 and P19Kmrfpr3. Figure 2.8 is a visual illustration of the main cloning steps involved to create the construct P19KmRFPNHisP19Kpro.

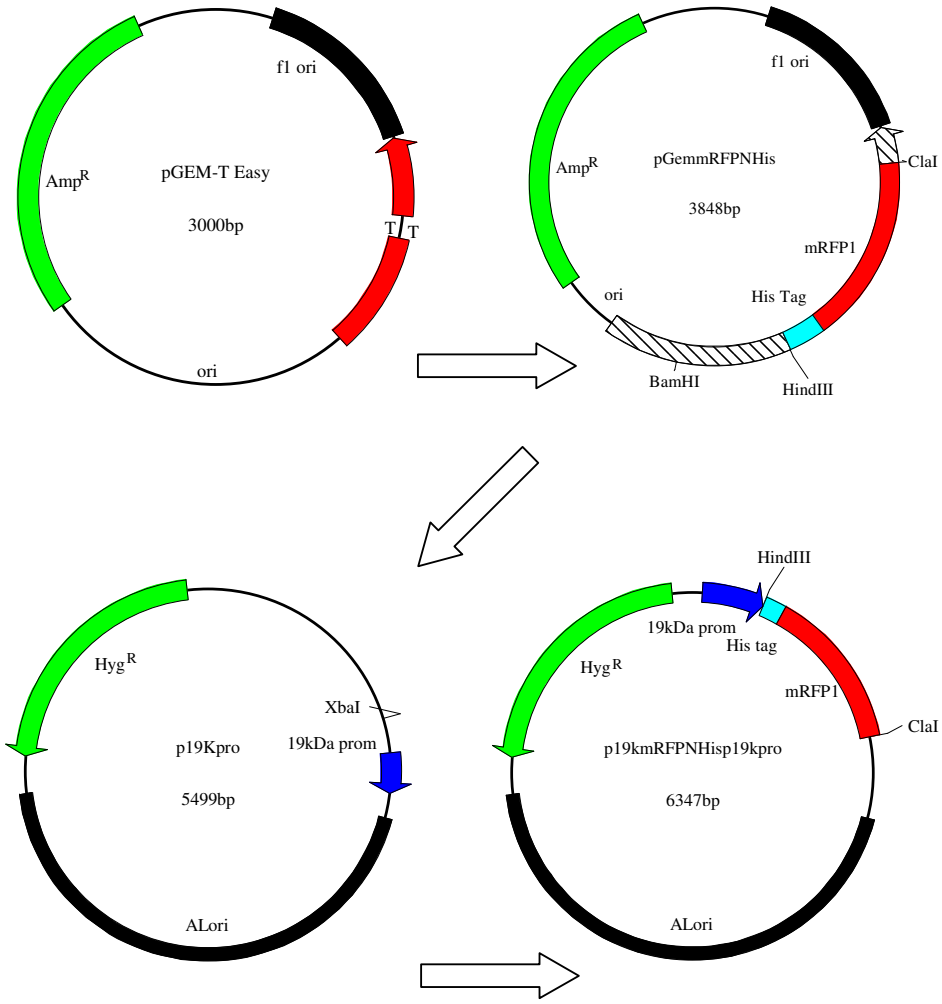

Figure 2.8 Construction of P19KproP19KmRFPNHis. A His-tagged mRFP1 gene was PCR amplified and cloned into pGem-T-Easy to create pGemmRFPNHis. The His-tagged mRFP gene was removed by digestion with *Hind*III and *Cla*I and cloned into *Hind*III and CaII digested P19Kpro to create P19KmRFPNHisP19Kpro.

2.6.3.9 Construction of pSD21Int1mRFPNHis

pGemInt1NHis (Section 2.6.3.5) was sequenced (Section 2.3.8) with the primers SP6 and T7 (Table 5, Section 2.2.1) to verify the orientation of the Int1 promoter and His tag in pGem-T-easy. Constructs with an orientation such that it places the His tag coding orientation towards the SP6 side were digested (Section 2.3.9) with *Kpn*I and *Sal*I, run on a gel (Section 2.3.1), the large molecular band was purified (Section 2.3.3) and dephosphorylated (Section 2.3.11). The *Kpn*I and *Sal*I digested pGemInt1NHis was then ligated (Section 2.3.12) to the *Kpn*I and *Sal*I digested pGemmRFP1 from Section 2.6.3.8 and was named pGemInt1mRFPNHis. The ligation was electroporated into *E. coli* (Section 2.3.13.1) and a PCR colony screening (Section 2.2.3) was done using primers Int1F and Hsp60mRFP1r2 (Table 5, Section 2.2.1). Colonies testing positive for the plasmid containing the correct insert size (corresponding to the 989bp band) were then picked and grown in liquid media (Section 2.1.2.1) containing ampicillin (Appendix A.1.1), followed by plasmid DNA extraction (Section 2.3.5). The purified plasmid DNA was then digested with *Bam*HI and *Sal*I, run on a gel and the 983bp band was then purified. pSD21 (Appendix B.12) was digested with *Bam*HI and *Sal*I, run on a gel, the large molecular band was purified and dephosphorylated. The digested pSD21 vector was then ligated to the *Bam*HI and *Sal*I digested 983bp fragment from pGemInt1mRFPNHis and was named pSD21Int1mRFPNHis. The ligation was electroporated into *E. coli* and a PCR colony screening was done using primers Int1F and Hsp60mRFP1r2, colonies testing positive for the plasmid containing the correct insert size (corresponding to the 989bp band) were grown in liquid culture containing kanamycin (Appendix A.1.2) and their plasmid DNA was extracted and sequence verified using primers Int1F and Hsp60mRFP1r2. Figure 2.9 is a visual illustration of the main cloning steps to create pSD21Int1mRFPNHis**.**

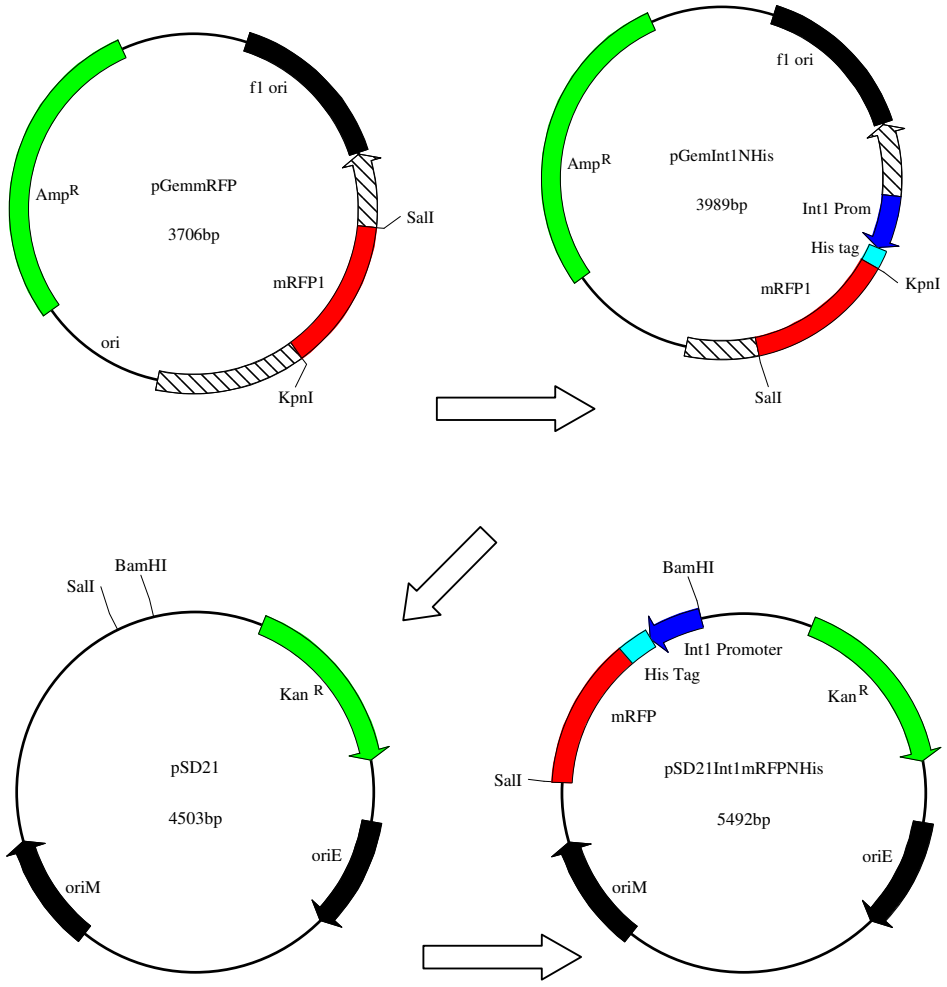

Figure 2.9 Construction of pSD21Int1mRFPNHis. The mRFP1 gene was removed from pGemmRFP and was cloned into the *Kpn*I/*Sal*I sites of pGemIntNHis. The Int1 promoter as well as His-tagged mRFP1 gene was removed by *Kpn*I and *Sal*I and cloned into the *Kpn*I and *Sal*I sites of pSD21.

2.6.3.10 Construction of pDMN1mRFPNHis

Expression vector pDMN1 (Appendix B.10) was digested with *Hind*III and *Hpa*I (Section 2.3.9), run on a gel (Section 2.3.1) and the large molecular size band was purified from a gel (2.3.3) and dephosphorylated (Section 2.3.11). The mRFP1 gene was PCR amplified (Section 2.2) from pRSETB (Appendix B.11) using primers RCMRFPF and RCMRFPR (Table 5, Section 2.2.1) and subsequently cloned into pGem-T-Easy (Section 2.6.1). The insert was removed from pGem-T-Easy by digestion with *Hind*III and *Hpa*I (Section 2.3.9), was run on a gel, the 708bp mRFP1 gene was purified from the gel. The *Hind*III and *Hpa*I digested mRFP1 gene was then ligated (Section 2.3.12) to the *Hind*III and *Hpa*I digested pDMN1 vector. The ligation was electroporated into *E. coli* (Section 2.3.13.1) and plated on LB agar (Appendix A.2.8) containing kanamycin (Appendix A.1.2). A PCR colony screening (Section 2.2.3) was performed using the above-mentioned primer pair. Colonies positive for the plasmid containing the crorrect insert size (corresponding to the 706bp band) were selected and grown in LB media containing kanamycin and used for plasmid DNA extraction (Section 2.3.5). Figure 2.10 is a visual illustration of the main cloning steps to construct pDMNmRFPNHis.

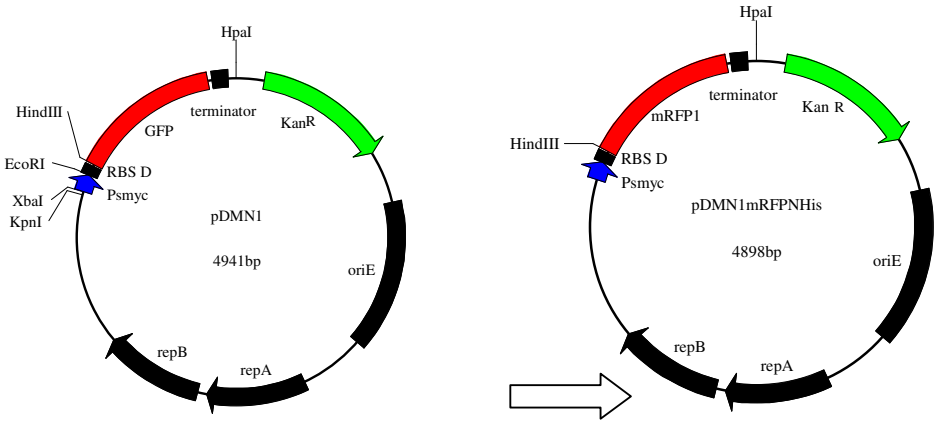

Figure 2.10 Construction of pDMN1mRFPNHis. The mRFP1 gene was PCR amplified from pRSETB to add RE sites *Hind*III and *Hpa*I and was cloned into the *Hind*III and *Hpa*I RE sites of pDMN1.

2.6.3.11 Construction of pJem15Hsp60NHis

pJem15 (Appendix B.6) was digested with *Bam*HI and *Kpn*I (Section 2.3.9), run on a gel (Section 2.3.1) and the large molecular band was purified (Section 2.3.3) and dephosphorylated (Section 2.3.11). The digested vector was then ligated (Section 2.3.12) to the 361bp *Bam*HI and *Kpn*I digestion product from pGemHsp60NHis (Section 2.6.3.1), consisting of the Hsp60 promoter and a sequence for an N-term His tag. The ligation was then electroporated into *E. coli* (Section 2.3.13.1) and a PCR colony screening (Section 2.2.3) was done using primers ESAT6-f and Hsp60PromHisr3 (Table 5, Section 2.6.3.1). Colonies testing positive for the plasmid containing the correct insert size (corresponding to the 361bp band) were then grown in liquid media (Section 2.1.2.1) containing ampicillin (Appendix A.1.1). A plasmid DNA extraction was then done (Section 2.3.5) and the constructs were sequence verified (Section 2.3.9) using the above-mentioned primer pair. Figure 2.11a is a visual illustration of the main cloning steps to create pJem15Hsp60NHis.

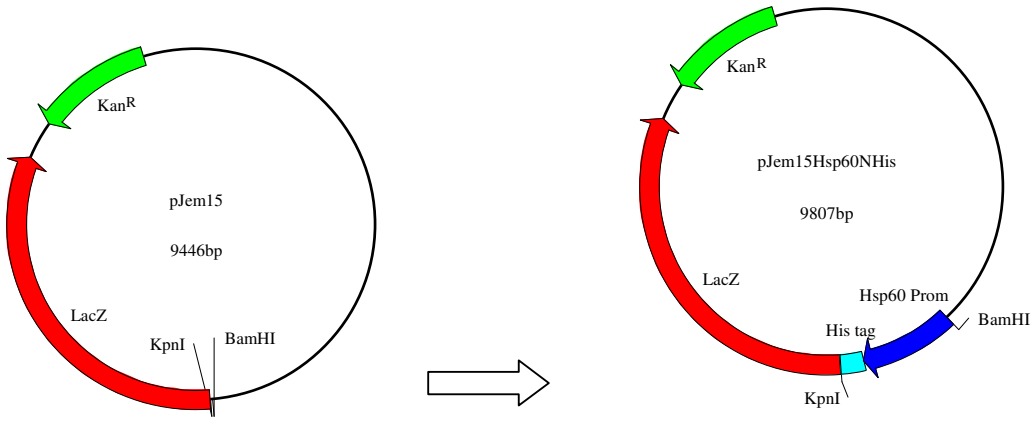

Figure 2.11a Construction of pJem15Hsp60NHis. The Hsp60 promoter and His tag was removed from pGemHsp60NHis3 (Section 2.6.3.1) using *Bam*HI and *Kpn*I and was ligated into *Bam*HI and *Kpn*I digested pJem15.

A second cloning method was applied to create pJem15Hsp60NHis due to cloning difficulties which made use of PCR to replace a stop codon (situated between the *LacZ* gene and promoter start codon) with a start codon in order to allow N-term His-tag fusions. A portion of the *LacZ* gene as well as a region upsteam of the *LacZ* gene was PCR amplified from pJem15 (Appendix B.6) using primers LacZFusion-f2 and LacZFusion-r (Table 5, Section 2.2.1). The PCR product, named "LacZfusion", was run on a gel (Section 2.3.1), the 1195bp band was purified (Section 2.3.3) and cloned into pGem-T-Easy (Section 2.6.1). The 1187bp insert was excised by RE digest with *Kpn*I and *Eco*RV and was cloned into the *Kpn*I and *Eco*RV sites of pJem15 to restore N-Term His tag fusion to the *LacZ* Gene. Figure 2.11b is a visual illustration of the cloning method to construct pJem15Hsp60NHis2:

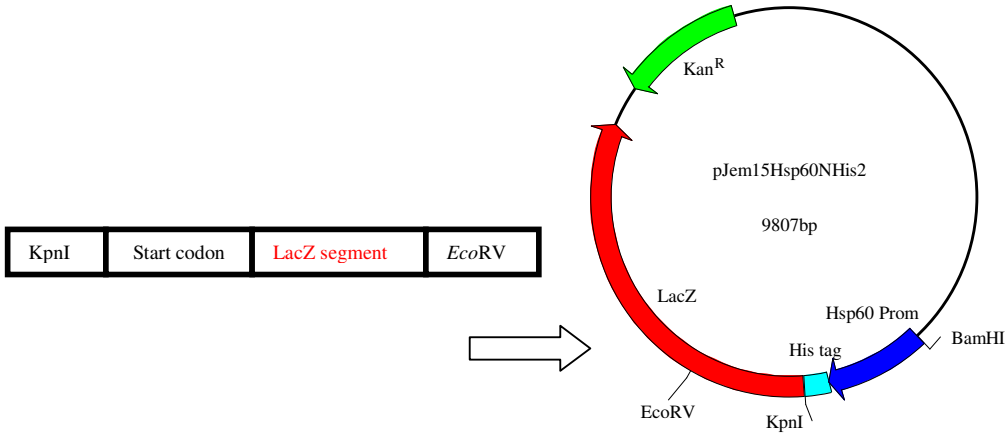

Figure 2.11b. Construction of pJem15Hsp60NHis2. A portion of the LacZ gene was PCR amplified, replacing a stop codon with a start codon on the PCR product. This product was then cloned into pGem-T-Easy, excised with *Kpn*I and *Eco*RV and subsequently cloned into *Kpn*I and *Eco*RV digested pJem15Hsp60NHis to create pJemHsp60NHis2.

2.6.3.12 Construction of pJem15Int1NHis

The Int1 promoter was PCR amplified (Section 2.2.2) from *M. tb* H37Rv DNA using primers Int1LacZF and Int1LacZR (Table 5, Section 2.2.1) which add *Bgl*II RE sites to the product. The product was run on a gel (Section 2.3.1) and the 289bp band was excised and purified (Section 2.3.3). The purified PCR product was cloned into pGem-T-Easy (Section 2.6.1) and excised with *Bgl*II (Section 2.3.9). pJem15 (Appendix B.6) was partially digested with *Bgl*II (Section 2.3.10), run on a gel (Section 2.3.1), the large molecular band was purified (Section 2.3.3) and dephosphorylated (Section 2.3.11). The digested Int1 promoter was ligated to the *Bgl*II digested pJem15 vector (Section 2.3.12) and electroporated into *E. coli* (Section 2.3.13.1). A PCR colony screening (Section 2.2.3) was done using the above-mentioned primer pair, colonies testing positive for the plasmid containing the correct insert size (corresponding to the 289bp band) were grown in liquid media (Section 2.1.2.1) containing kanamycin (Appendix A.1.2). A plasmid DNA extraction was then done (Section 2.3.5) and the constructs were sequence verified (Section 2.3.8) using the above-mentioned primer pair. Figure 2.12 is a visual illustration of the main cloning steps to create pJem15Int1NHis.

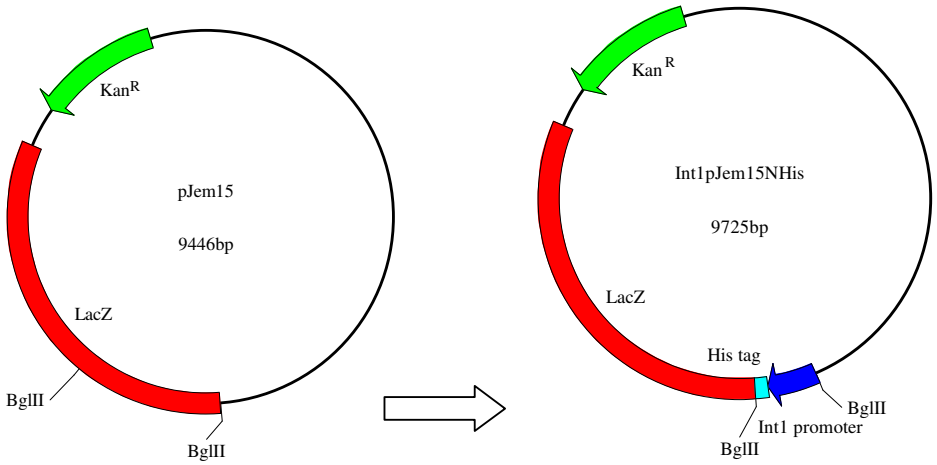

Figure 2.12 Construction of pJem15Int1NHis. The Int1 promoter was PCR amplified, cloned into pGem-T-Easy and subsequently cloned into pJem15 to create Int1pJem15NHis.

2.6.4 Construction of reporter cosmids

The optimal promoter/reporter protein/affinity tag combination (as constructed in Section 2.6.3) was to be ligated to mycobacterial genomic DNA with the end goal of creating a recombinant reporter mycobacteriophage. The methodology of lambda packaging was chosen due to the vast increase in transformation efficiency in comparison to electroporation. Selected reporter constructs were thus transferred to cosmids to allow for lambda packaging as well as antibiotic selection of recombinant clones. Several cloning approaches were attempted to ensure the successful creation of a suitable reporter cosmid for cloning into a mycobacteriophage. The N-term His-tagged reporter gene mRFP1 and the promoter pSmyc was chosen to be transferred into a suitable cosmid and the various cloning methodologies are listed in Table 18.

Table 18. List of reporter cosmids constructed.

Construct Name	Reporter Gene	Promoter	Affinity tag	Expression vector	Antibiotic resistance	Section
pYub412mRFPPvuI	mRFP1	pSmyc	N-term His tag	pYub412.2	Ampicillin	2.6.4.1
pYub412mRFPSapI	mRFP1	pSmyc	N-term His tag	pYub412.2	Ampicillin	2.6.4.2
pYub412mRFPNdeIBglII	mRFP1	pSmyc	N-term His tag	pYub412.2	Ampicillin	2.6.4.3
pDMN1LCosmRFP	mRFP1	pSmyc	N-term His tag	pDMN1	Kanamycin	2.6.4.4
pYubmRFP	mRFP1	pSmyc	N-term His tag	pYub412	Ampicillin	2.6.4.5
pYubSSmRFP	mRFP1	pSmyc	N-term His tag	pYub412	Ampicillin	2.6.4.6
pYub412LacZ	LacZ	pSmyc	N-term His tag	pYub412	Ampicillin	2.6.4.7

2.6.4.1 Construction of pYub412mRFPPvuI

The pSmyc promoter along with the mRFP1 gene was PCR amplified from the vector pDMN1mRFPNHis (Section 2.6.3.10) with the primer pair mRFPreporter1-f and mRFPreporter1-r (Table 5, Section 2.2.1) which add *Pvu*I RE sites to the PCR product. The PCR product was run on a gel (Section 2.3.1) and the 955bp band was purified (Section 2.3.3) The PCR product was cloned into pGem-T-Easy (Section 2.6.1) and named pGempSmycmRFP. The insert was removed by digesting with *Pvu*I (Section 2.3.9), the digested DNA was run on a gel and subsequently the 949bp band was purified from the gel. pYub412 (Appendix B.14) was digested with *Bcl*I to remove a previous insert from the cosmid, the digested DNA was then run on a gel, the 8.5kb DNA band was removed and purified and then self-ligated (Section 2.3.12). The self-ligated DNA was then electroporated into *E.*

coli (Section 2.3.13.1) and plated onto LB agar plates (Appendix A.2.8) containing both ampicillin and hygromycin (Appendix A.1.1 and A.1.3 respectively). A single colony was selected and grown in LB media (Section 2.1.2.1) containing ampicillin and hygromycin, thereafter a cosmid DNA extraction was done (Section 2.3.6). The purified DNA was then digested with *Pvu*I, run on a gel, the digested molecular size band (as determined by running controls along the digest) was purified and dephosphorylated (Section 2.3.11). The 949bp *Pvu*I liberated DNA fragment from pGempSmycmRFP was then ligated to *Pvu*I digested pYub412, electroporated into *E. coli* and plated out on LB agar containing hygromycin (ampicillin was no longer added since the cloning site used was situated inside the ampicillin resistance gene coding sequence). The resulting colonies were PCR screened using the above-mentioned primer pair and the cosmid DNA was extracted (Section 2.3.6). Figure 2.15 is a visual illustration of the main cloning steps involved to create the construct pYub412mRFP*Pvu*I.

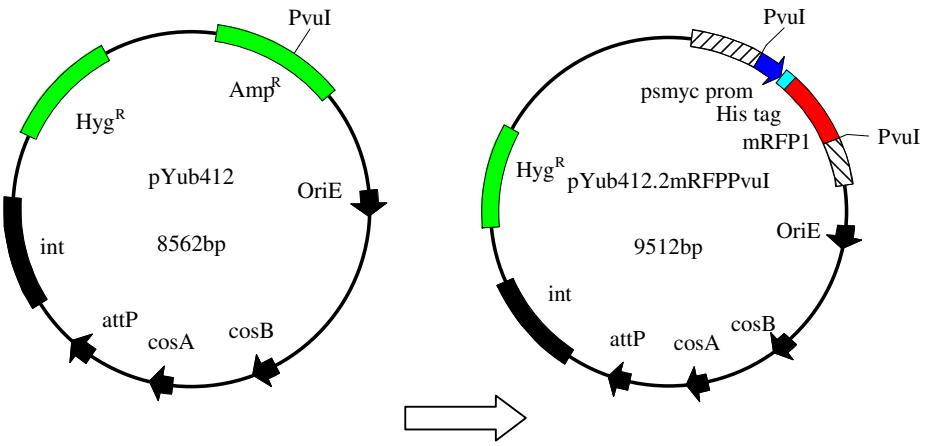

Figure 2.15. The pSmyc promoter as well as the mRFP1 gene was PCR amplified from pDMNmRFPNHis to add *Pvu*I RE sites and this was cloned into *Pvu*I digested pYub412 to create pYub412mRFP*Pvu*I

2.6.4.2 Construction of pYub412mRFPSapI

The cloning steps for construction of pYub412mRFPSapI were the same as for pYub412mRFPPvuI (Section 2.6.4.1) except that the primer set used was RepTm4SapF2 and RepTm4SapR which add *Sap*I RE sites to the PCR product. The PCR product size, however, differed and was 967bp. Additionally the resultant SapI digest was 955bp. This places the reporter construct downstream of the *E. coli* origin of replication and inserted it into the beginning of the first cos site ("cos B"). Subsequent digests thus made use of *Sap*I instead of *Pvu*I and the sequencing similarly made use of RepTm4SapF2 and RepTm4SapR. Figure 2.16 is a visual illustration of the final cloning product pYub412mRFP*Sap*I.

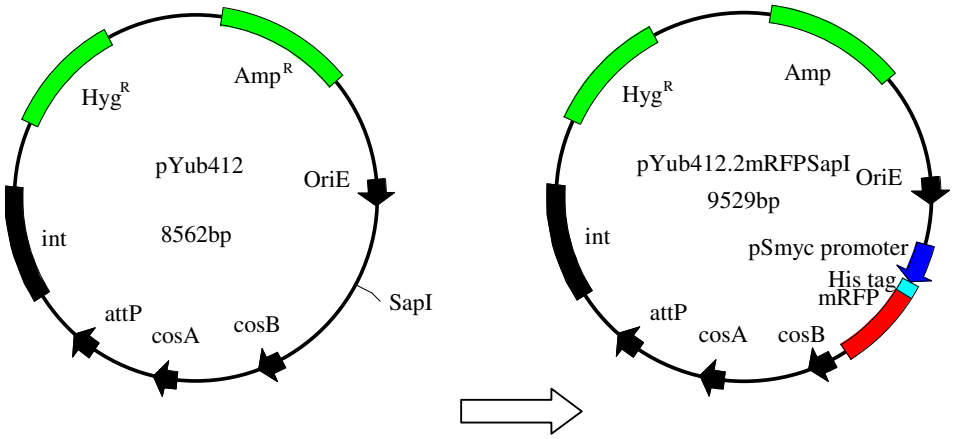

Figure 2.16 Construction of pYub412mRFPSapI. The pSmyc promoter as well as the mRFP1 gene was PCR amplified from pDMNmRFPNHis to add *Sap*I RE sites and this was cloned into *Sap*I digested pYub412 to create pYub412mRFP*Sap*I.

2.6.4.3 Construction of pYub412mRFPNdeIBglII

The pSmyc promoter along with the *mRFP1* gene was PCR amplified (Section 2.2) from the vector pDMNmRFPNHis (Section 2.6.3.10) with the primer pair RepNdeFixF and RCMRFPR (Table 5, Section 2.2.1) which PCR amplifies a product with *Hind*III and *Hpa*I RE sites but without an *Nde*I site, simplifying downstream cloning that make use of these RE sites. The product was cloned into pGem-T-easy (Section 2.6.1) and named pGemmRFPNdeFix and subsequently excised by RE digestion using *Hind*III and *Hpa*I (Section 2.3.9). The digestion was then run on a gel (Section 2.3.1) and the 716p product was excised and purified from the gel (Section 2.3.3). The purified fragment was then ligated to *Hind*III and *Hpa*I digested pDMN1 (as derived from Section 2.6.3.10). The ligation product, named pDMNmRFPNHisNdeIfix, was electroporated into *E. coli* (Section 2.3.13.1) and a PCR colony screening (Section 2.2.3) was done using the above-mentioned primer pair. Colonies testing positive for the plasmid containing the correct insert size (corresponding to the 716bp band) were grown in liquid media (Section 2.1.2.1) containing kanamycin (Appendix A.1.2) and subsequently their plasmid DNA was extracted (Section 2.3.5). The purified DNA was then used as template for a PCR reaction using primer pairs D29repF and D29repR (which add *Nde*I and BglII RE sites to the PCR product). The PCR product was cloned into pGem-T-Easy and named pGemmRFPNHisNdeIfix. The insert was removed by digesting with *Nde*I and BglII. The digest was run on a gel and purified from the gel. pYub412 (Appendix B.14) was digested with *Nde*I and BglII, run on a gel, and the large molecular band was purified and dephosphoylated (Section 2.3.11). The 713bp *Nde*I and BglII digest fragment liberated from pGemmRFPNdeFix was then ligated to the *Nde*I and BglII digested pYub412 and electroporated into *E. coli.* The electroporation was plated out on LB agar containing ampicillin and hygromycin. Colonies were PCR screened using the primer pair D29repF and D29repR, and positive colonies were grown in LB media containing ampicillin and hygromycin. A cosmid DNA extraction was subsequently performed (Section 2.3.6). Figure 2.17a and 2.17b is a visual illustration of the main cloning steps to create pYub412mRFPNdeIBglII.

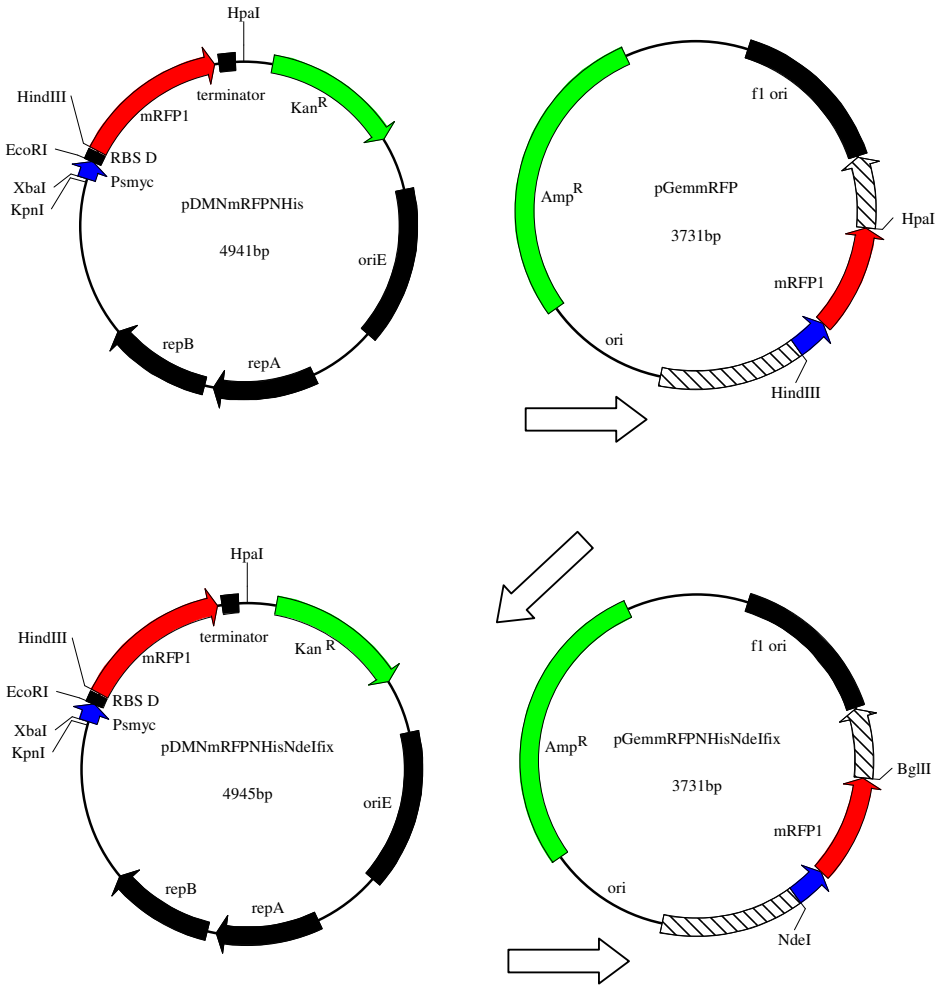

Figure 2.17a Construction of pYub412mRFPNdeIBglII. The pSmyc promoter and *mRFP* gene was PCR amplified from pDMNmRFPNHis to add *Hind*III and *Hpa*I sites as well as to remove an *Nde*I site in the mRFP1 gene. This insert was then cloned into the HindIII and HapI sites of pDMN1 to create pDMNmRFPNHisNdeIfix. The pSmyc promoter and *mRFP* gene was PCR amplified from pDMNmRFPNHisNdeIfix to add NdeI and BglII RE sites to the product and cloned into pGem-T-Easy to create pGemmRFPNHisNdeIfix.

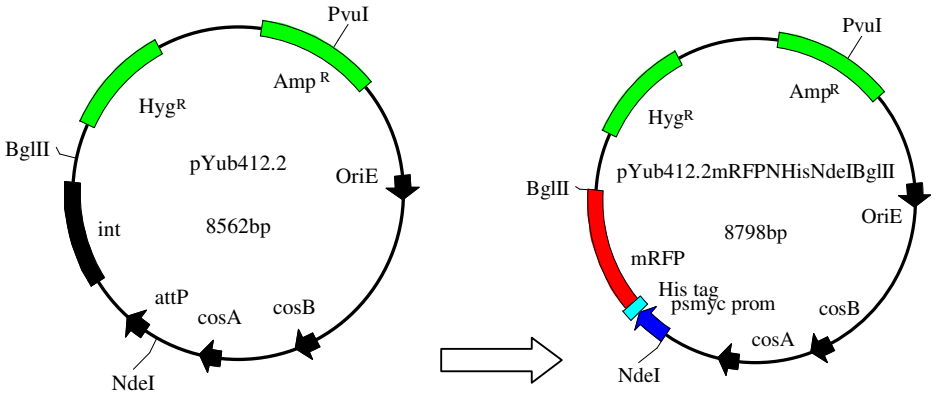

Figure 2.17b Construction of pYub412mRFPNdeIBglII (continued). The pSmyc promoter and *mRFP* gene was excised from pGemmRFPNHisNdeIfix (Figure 2.17a) and cloned into the NdeI and BglII sites of pYub412 to create pYub412mRFPNHisNdeIBglII.

2.6.4.4 Construction of pDMNLCosmRFP

Due to cloning technicalities using pYub412, an additional vector was constructed to act as a shuttle cosmid. Vector pDMNmRFPNHis (Section 2.6.3.10) was digested (Section 2.3.9) with *Mlu*I and *Xba*I to remove the mycobacterial origin of replication. The digest was run on a gel (Section 2.3.1) and the large molecular band was purified (Section 2.3.3) and dephosphorylated (Section 2.3.11). A lambda cos site was PCR amplified from pYub412 (Appendix B.13) using primer pair LambdaCosF and LambdaCosR (Table 5, Section 2.2.1) which added *Xba*I and Mlu sites as well as one *Pac*I site to the product. The PCR product was then cloned into pGem-T-Easy (Section 2.6.1) and the lambda cos fragment was excised by *Xba*I and Mlu digestion. The digested DNA was run on a gel and the 330bp fragment was excised and purified form the gel. The digested lambda cos fragment was then ligated (Section 2.3.12) to the digested pDMNmRFPNHis DNA and electroporated into *E. coli* (Section 2.3.13.1). A PCR colony screening (Section 2.2.3) was done using the above-mentioned primer pair. Colonies testing positive for the plasmid containing the correct insert size (corresponding to the 330bp band) fragment were grown in liquid media (Section 2.1.2.1) containing kanamycin (Appendix A.1.2) and used for cosmid DNA extraction (Section 2.3.6). Figure 2.18 a visual illustration of the main cloning steps involved in creating pDMN1LCosmRFP.

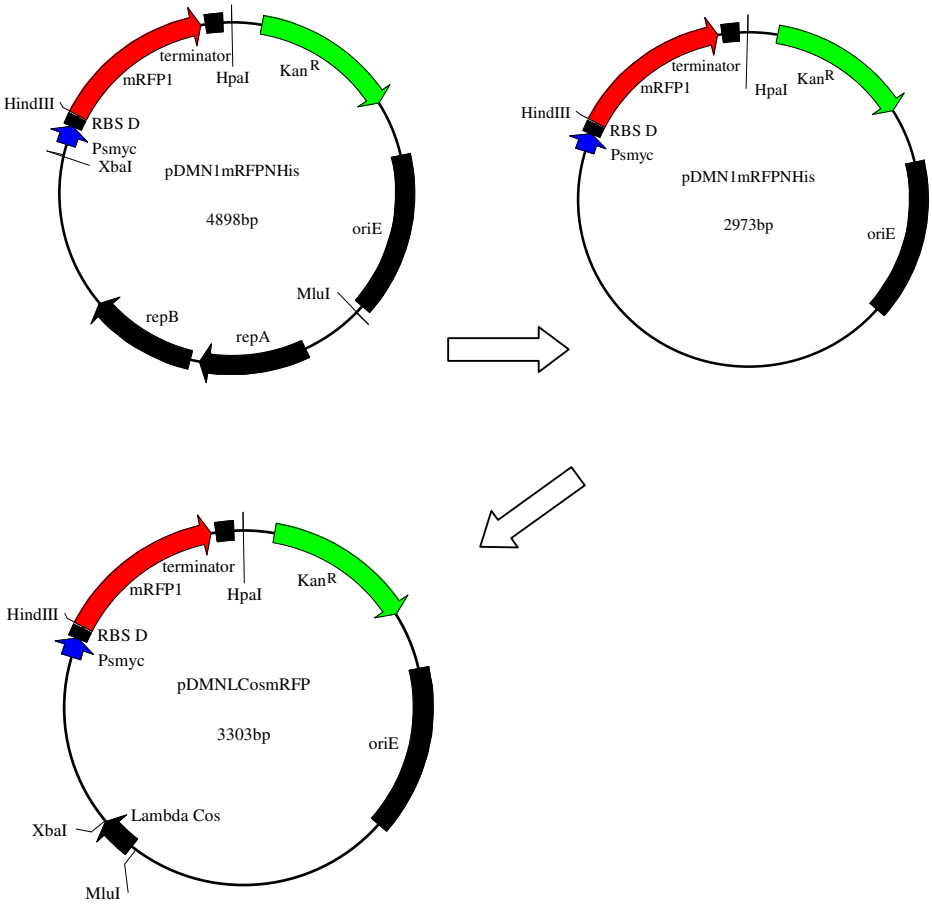

Figure 2.18 Construction of pDMNLCosmRFP. pDMNmRFPNHis was digested with Mlu and *Xba*I, removing the mycobacterial origin of replication genes repB and repA. The Lambda Cos site from pYub412 was PCR amplified to add RE sites *Mlu*I, *Xba*I and one *Pac*I site to the PCR. The PCR product was cloned into pGem-T-Easy, excised using *Xba*I and *Mlu*I and then cloned into *Mlu*I and *Xba*I digested pDMNmRFPNHis to create pDMNLCosmRFP.

2.6.4.5 Construction of pYubmRFP

pYub412 (Appendix B.14) was digested with *Sal*I and *Eco*RV (Section 2.3.9), the digest was run on a gel (Section 2.3.1) and the 4968bp fragment was purified (Section 2.3.3) and dephosphorylated (Section 2.3.11). This digest removes the integration site AttP and the integration gene *Int* as well as the Hygromycin resistance gene. The N-term His-tagged mRFP1 gene along with the pSmyc promoter was PCR amplified (Section 2.2) from pDMNmRFPNHis (Section 2.6.3.10) using the primer pair Rep4*Sal*IF and Rep4EcoRVR (Table 5, Section 2.2.1) which add *Sal*I and *Eco*RV RE sites to the PCR product. The 955bp PCR product was then cloned into pGem-T-easy (Section 2.6.1) and subsequently excised by digestion with *Sal*I (partial digest) and *Eco*RV. The digest was run on gel and the 951bp fragment was purified from the gel. The *Sal*I and *Eco*RV digested pYub412 was then ligated to the 951bp *Sal*I and *Eco*RV fragment from pGem-T-Easy. A PCR colony screening (Section 2.2.3) was done using the above-mentioned primer pair and colonies testing positive for the cosmid containing the correct insert size (corresponding to the 951bp band) were picked and cultured in liquid media (Section 2.1.2.1) and used for cosmid DNA extraction (Section 2.3.6). The cosmid was then sequenced (Section 2.3.9) using the above-mentioned primer pair. Figure 2.19 is a visual illustration of the main cloning steps to create pYubmRFP.

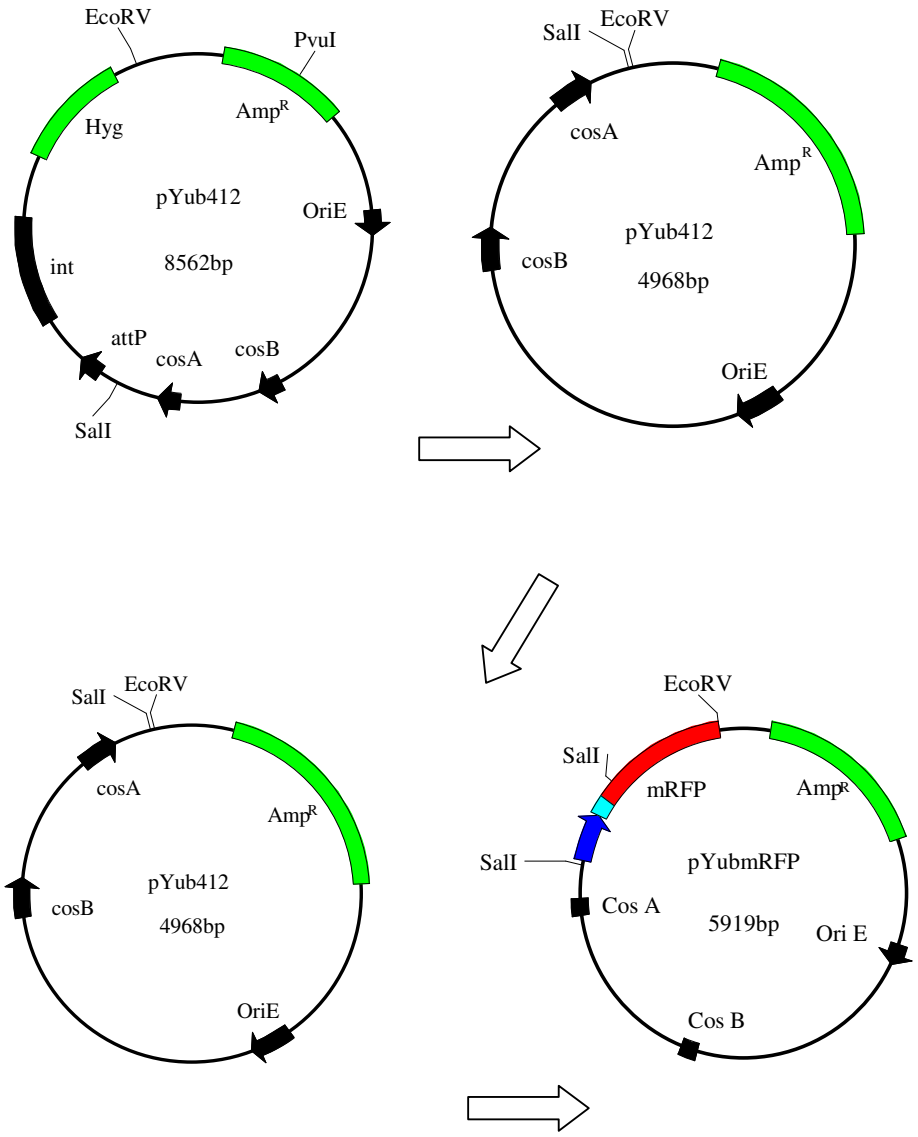

Figure 2.19 Construction of pYubmRFP. pYub412 was digested with *Sal*I and *Eco*RV to remove the hygomycin resistance and *int* genes as well as the attP integration site. The N-term His-tagged *mRFP1* gene and pSmyc promoter was PCR amplified to add *Sal*I and *Eco*RV RE sites. The PCR product was cloned into pGem-T-easy and subsequently removed by the same RE`s (partial digest using *Sal*I) and cloned into pYub412 (digested with *Sal*I and *Eco*RV) to create pYubmRFP.

2.6.4.6 Construction of pYubSSmRFP

pYubmRFP (Section 2.6.4.5) was further reduced in size by digesting with *Sap*I and *Mlu*I (Section 2.3.9) which removes non-essential DNA including one of the two lambda cos sites. Since these RE sticky ends were not compatible, Klenow enzyme was used to fill in the 5` overhangs and to remove 3` overhangs to form blunt ends suitable for ligation. One microgram of digested DNA, 2 µl 10X Klenow filling buffer (Appendix A.3.17) and 1U Klenow enzyme (roche) was incubated for 15 min at 37°C. The enzyme was then deactivated by incubation at 65°C for 10 min. The blunt ends were ligated (Section 2.3.12) and electroporated into *E. coli* (Section 2.3.13.1) and plated on LB agar media containing ampicillin (Appendix A.1.1). Colonies were picked and grown in liquid media (Section 2.1.2.1) containing ampicillin. The cosmid DNA was then extracted (Section 2.3.6) and run on a gel along with pYubmRFP DNA, which had not been cut with *Sap*I and *Mlu*I. Size reduction was indicative of successful digestion, Klenow filling and ligation. The new smaller cosmid was named pYubSSmRFP. The original pYubmRFP has a size of 5919bp whereas the modified pYubSSmRFP is 3887bp, giving a 2032bp size reduction, and yielding a reporter cosmid more suitable for lambda packaging when ligated to mycobacteriophage DNA. Figure 2.20 is a visual illustration of the main cloning steps to create pYubSSmrfp.

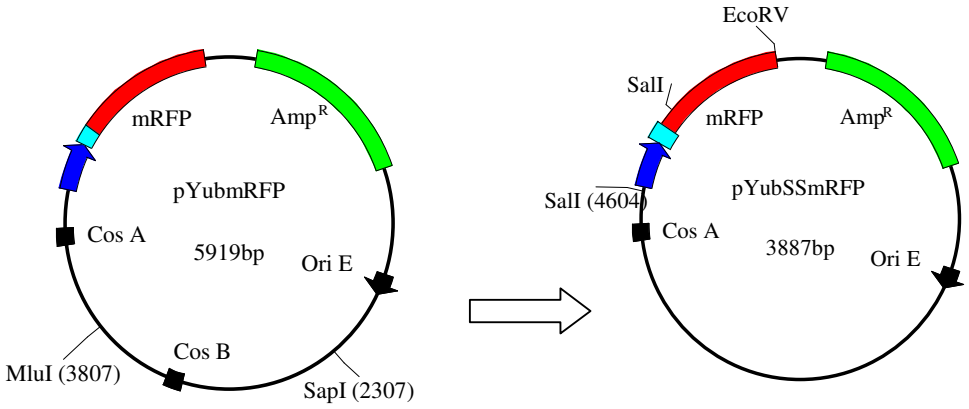

Figure 2.20 Construction of pYubSSmrfp. pYubmRFP was digested with *Sap*I and *Mlu*I and then treated with Klenow enzyme, followed by self-ligation to create the size-reduced pYubSSmRFP.

2.6.4.7 Construction of pYub412LacZ

Due to the large size of the *LacZ* gene, pYub412SSmRFP (Section 2.6.4.6) was chosen as cloning vector since it was maximally reduced in size (due to lambda packaging size constraints). The SalI and *Eco*RV RE sites were selected as cloning sites due to previous successful cloning into these RE sites (Section 2.6.4.5). However, due to the presence of multiple *Sal*I and *Eco*RV cutting sites in both the pSmyc promoter and *LacZ* PCR product, a three-way-cloning methodology was adopted.

pYub412SSmRFP was used as PCR template and the pSmyc promoter was PCR amplified (Section 2.2) with primer pair PromsmycF and PromsmycR (Table 5, Section 2.2.1) to add *Sal*I and *Not*I RE sites. The PCR removed an existing *Sal*I site and replaced it with a *Not*I site. The PCR product was then run on a gel (Section 2.3.1) and the 253bp band was purified (Section 2.3.3). The PCR product was then cloned into pGem-T-Easy (Section 2.6.1) and named pGempSmycSalINotI. Thereafter, it was excised by digesting with *Sal*I and *Not*I, the digest was run on a gel and the 246bp band was purified. The LacZ gene was then PCR amplified from pJem15 (Appendix B.6) using primer pair LacZNotIF and LacZEcoRVR, which added RE sites *Not*I and *Eco*RV to the product. The PCR product was run on a gel, the 3065bp fragment was purified, cloned into pGem-T-Easy and named pGemlacZNotIEcoRV. The *LacZ* gene was then excised by doing a partial digest (Section 2.3.10) and

the 3060bp fragment was purified. The 3060bp digested *LacZ* fragment was then ligated to the 253bp pSmyc fragment as described in Section 2.3.12, (however a ratio of 1:1 was used instead of 1:3) and the resulting product was named SalIpsmycNotILacZEcoRV. pYub412SSmRFP was then digested with *Sal*I and *Eco*RV, run on a gel, the large molecular size band was purified and dephosphorylated (Section 2.3.11). SalIpsmycNotILacZEcoRV was ligated into the digested pYub412SSmRFP and electroporated into *E. coli* (Section 2.3.13.1). A PCR colony screening was done using both primer sets listed above and colonies testing positive for the plasmid containing the correct insert sizes (corresponding to both the 253bp and 3060bp bands) were picked and grown in liquid media (Section 2.1.2.1) containing ampicillin (Appendix A.1.1). A plasmid DNA extraction was then done (Section 2.3.5) and the constructs were sequence verified using the above-mentioned primer pairs. Figure 2.21 is a visual illustration of the main cloning steps to create pYub412psmycLacZ.

The pSmyc promoter was PCR amplified to add *Sal*I and *Not*I RE sites and cloned into pGem-T-Easy (not shown), the LacZ gene was PCR amplified to add *Not*I and *Eco*RV sites (and cloned into pGem-T-Easy)

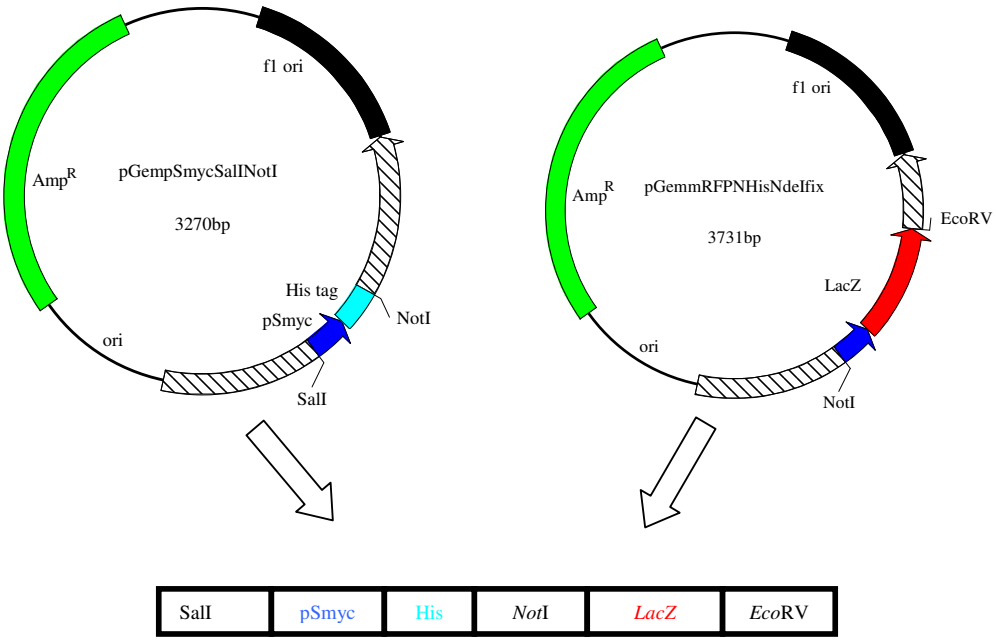

Figure 2.21a Construction of pYub412psmycLacZ. The pSmyc promoter and Histidine-tag was PCR amplified to add SalI and NotI sites to the product which was cloned into pGem-T-Easy to create pGempSmycSalINotI. Similarly, the *LacZ* gene was PCR amplified to add NotI and EcoRV sites to the product which was cloned into pGem-T-Easy and called pGemlacZNotIEcoRV. The two inserts were then excised and pGem-T-Easy and ligated to create SalIpsmycNotILacZEcoRV.

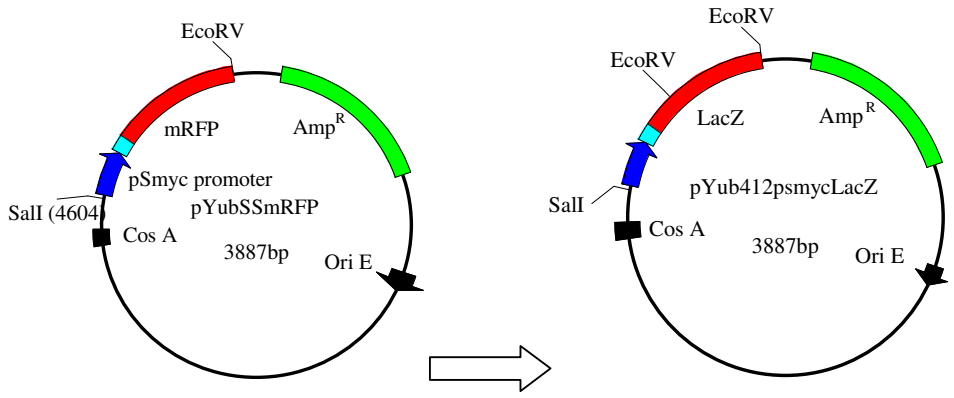

Figure 2.21b Construction of pYub412psmycLacZ (continued). pYub412SSmRFP was digested with *Sal*I and *Eco*RV to remove the pSmyc promoter and *mRFP* gene. The SalIpsmycNotILacZEcoRV ligation product (Figure 2.21a) was then cloned into the *Sal*I and *Eco*RV digested pYubSSmRFP to create pYub412psmycLacZ.

2.6.5 Construction of reporter mycobacteriophages

2.6.5.1 Selection of suitable mycobacteriophage for cloning

The choice of mycobacteriophage for cloning was limited due to several criteria. Firstly, the selected mycobacteriophage required *M. tb* in its natural host range so as to act as a TB diagnostic, as well as being able to infect *M. smegmatis* to allow the majority of amplification and cloning steps to take place outside of a biohazard level 3 facility. The selected mycobacteriophage genome also needs to be able to accommodate the additional space in its head capsid to allow for the reporter cosmid. This required the removal of non-essential DNA from the selected mycobacteriophage genome, as well as knowledge on potential cloning sites in the genome. Two approaches could be followed. (1) A novel mycobacteriophages could be screened for their host range, their genomes could be sequenced to obtain information on non-essential DNA and cloning sites, they could be size reduced and then used for cloning; (2) Previously constructed reporter mycobacteriophages could be utilized for cloning. Both these strategies were followed in this study to ensure that a suitable mycobacteriophage could be identified.

During this study, two mycobacteriophages were available to us for use as reporter mycobacteriophage - mycobacteriophage D29 and mycobacteriophage phAE142, kind gifts from Torin R. Weisbrod (HHMI, University of Pittsburgh, Department of Biological Sciences). Mycobacteriophage D29 is a well characterized mycobacteriophage of lytic nature which has been previously used both for plaque-based diagnostics as well as reporter mycobacteriophage-based diagnostics. Since mycobacteriophage D29 is wild-type it does not contain any excisable cosmids which would thus necessitate the removal of non-essential genomic DNA in order to accomodate a reporter cosmid as discussed in Section 2.6.5.2. A partial restriction enzyme digest method has previously been shown generate size reduced mycobacteriophages which retain their infectivity (Pearson,R.E. 1996). Mycobacteriophage phAE142 is a luciferase reporter mycobacteriophage which is derived from mycobacteriophage Tm4. Since the LRP contains an integrating luciferase cosmid, phAE142 offers the attractive cloning avenue of excising the existing luciferase cosmid and replacing it with a reporter cosmid from this study as discussed in Section 2.6.5.3.

In order to obtain novel mycobacteriophages, an isolation procedure was performed on various soil samples from regions in South Africa as described in Section 2.4.1. Large scale stocks were created of each isolated mycobacteriophage as described in Section 2.4.2. The large scale stocks were then ultracentrifuged as described in Section 2.4.3. The phage-bands resulting from the ultracentrifugation were then dialysed to purifiy the mycobacteriophage samples as described in Section 2.4.4. To document their morphology, transmission electron microscopy was used as in Section 2.4.5 and, finally, their genomic DNA was extracted and purified as in Section 2.4.6. In order to determine if the novel mycobacteriophages were suitable for use as reporter mycobacteriophage, a host range check was performed by means of a spot test. A grid was drawn on large petri-dish plates containing 7H10 (Appendix A.2.2). A lawn of *M. smegmatis* was created by spreading 120 µl of competent *M. smegmatis* (Section 2.1.2.8) onto the plate after which it was allowed to dry. The novel mycobacteriophages were then spotted onto the corresponding grid area by pippeting about 10-20 µl onto the plate and allowing the droplet to dry. This was done along with the controls of D29 and phAE142, which are known to infect both *M. tb* and *M. smegmatis*. The plates were sealed with parafilm and incubated for 12-24h at 37°C. If the novel mycobacteriophages lead to plaque production

on the lawn of *M. smegmatis* the titer was considered as suitable. Alternatively, a higher titer was prepared and the test was repeated. The process was repeated using both *M. bovis* BCG as well as with *M. tb* under P3 conditions. The culturing method was changed for *M. tb* and *M. bovis* BCG from that of *M. smegmatis,* due to the tendency of *M. tb and M bovis* BCG cells to clump. Tween-80 was added (0.05% (w/v) ,Sigma), and the culture time was extended to 14-20 days due to the slow growth rate of these species. Since Tween-80 interferes with the binding of mycobacteriophages to their mycobacterial hosts, the Tween was subsequently removed by a centrifugation step of the culture followed by resuspension in 7H9 media containing no Tween (repeated twice). The culture was then used for plaque testing as with *M. smegmatis* and the host range was recorded for the mycobacteriophages following 7-30 days incubation on 7H11 solid media (Appendix A.2.3).

2.6.5.2 Construction of D29 reporter mycobacteriophage

Wild-type mycobacteriophage D29 DNA was electroporated into *M. smegmatis* (Section 2.3.13.2). Plaques were purified and used for DNA extraction as described in Section 2.4. The linear mycobacteriophage D29 DNA was self-ligated at a concentration of 250 µg/ml to create concatemers. The ligase was then inactivated by incubation at 65°C for 20 min. DNA concatemers were partially digested with restriction enzyme *Sau*3AI (Section 2.3.10). The digest was then run on a gel (made as described in Section 2.3.1 except for using Cambridge Seakem low melting point agarose at 0.4% gel concentration). The digested DNA was mixed with 6X loading buffer (Fermentas) and loaded onto the gel. Field inversion gel electrophoresis (FIGE) (Section 2.3.2) was then used to separate the DNA using a Hoefer Scientific Instruments (HSI) PC750 Pulse Controller using the following settings: 60V, 18h, 50msec pulse, forward:reverse pulse ratio = 2.5:1. DNA fragments of 30-50kb were excised from the gel and phenol purified (Section 2.3.4). pYub412mRFP (Appendix B.14) was digested with *Bcl*I (Section 2.3.9) and ligated to the partially digested D29 DNA and used for lambda packaging (Section 2.3.14). The resulting colonies were then PCR screened (Section 2.2.3) using primers Rep4SalIF and Rep4EvoRVR (Table 5, Section 2.2.1). Positive colonies were picked and grown in 100 ml LB media (Section 2.1.2.1) containing ampicillin (Appendix A.1.1). The culture was then used for cosmid DNA extraction (Section 2.3.6). The purified cosmid DNA was electroporated into competent *M. smegmatis* (Section 2.1.2.8) incubated for 30 min at 37°C and then plated out with top agar (Appendix A.2.4) on 7H10 plates (Appendix A.2.7). The plates were observed for plaque formation after overnight incubation. Resulting plaques were picked and plaque purified (Section 2.4). Figure 2.22 illustrates the main cloning steps to create D29::pYub412mRFP. “lcos” indicates bacteriophage lambda cos recognition sites and “mcos” indicates mycobacteriophage cos recognition sites in the illustration below.

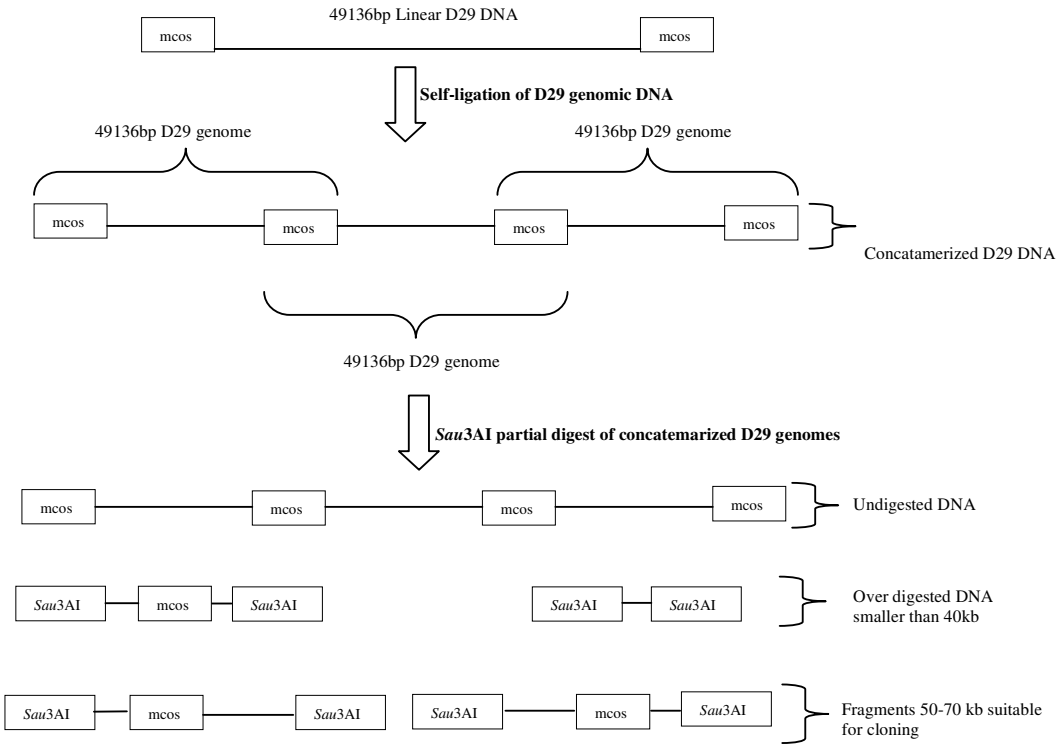

Figure 2.22a. Construction of D29 reporter mycobacteriophage. Mycobacteriophage D29 genomic DNA was self-ligated to create concatamers. The concatamers were partially digested with *Sau*3AI which yields various product sizes. The digested fragments were then separated on a FIDGE gel and fragments of 50-70kb were isolated and purified.

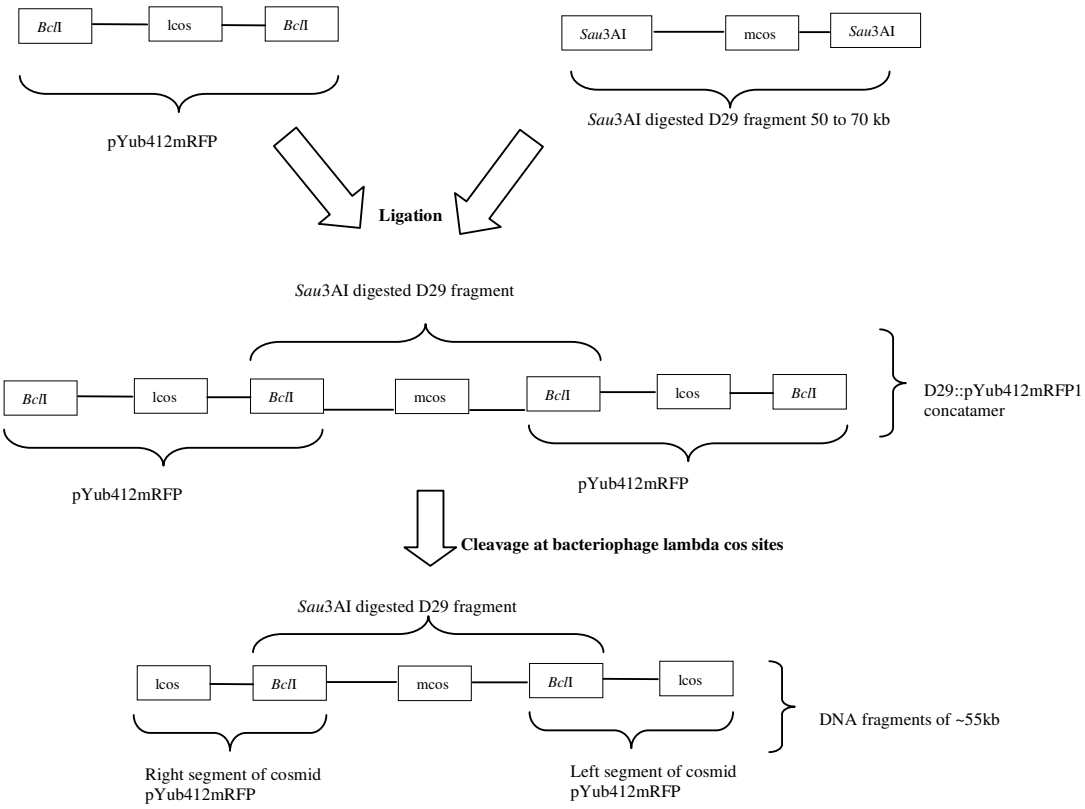

Figure 2.22b. Construction of D29 reporter mycobacteriophage (continued). D29 DNA (of 50 to 70kb in size) which was digested with *Sau*3AI and purified (Figure 2.22a) was ligated to *Bcl*I digested pYub412mRFP to create D29::pYub412mRFP concatamers (*Bcl*I and *Sau*3AI *o*verhangs are compatible*)*. The concatamerized D29::pYub412mRFP DNA was packaged into phage lambda, packaging all the DNA from one "lcos" site to the next "lcos" site.

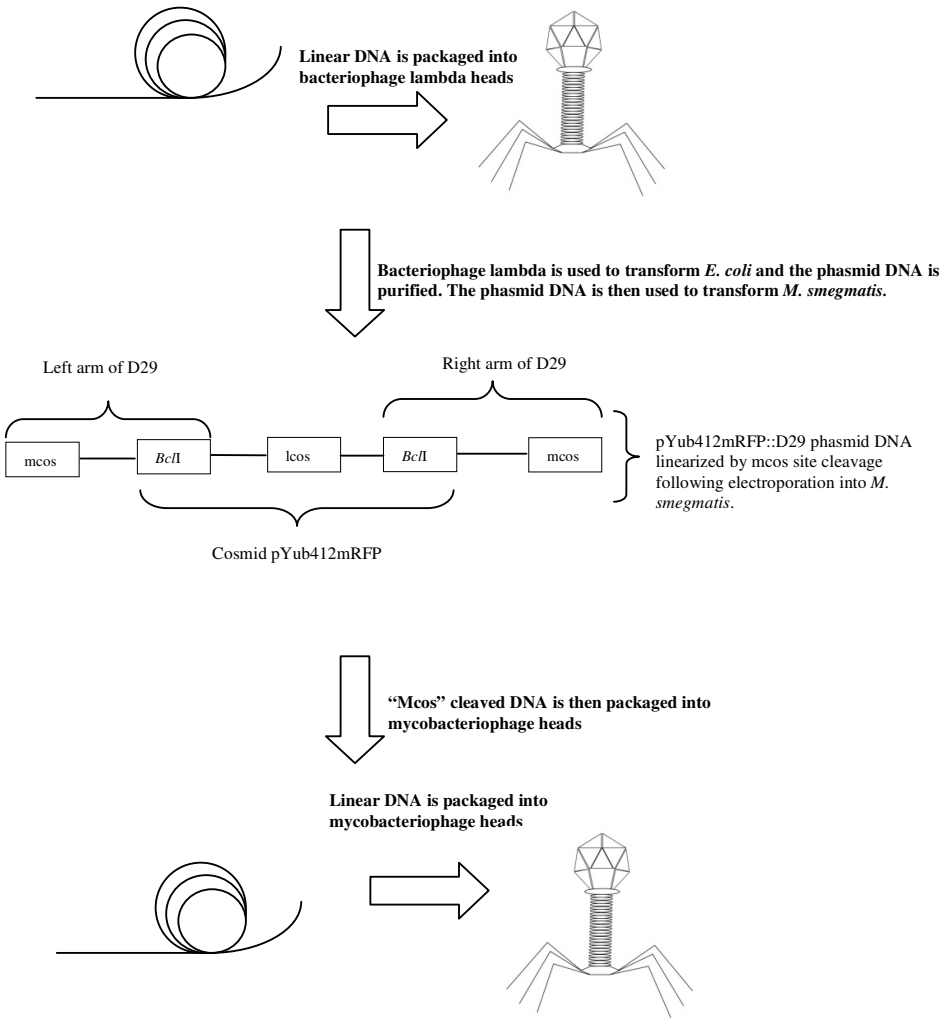

Figure 2.22c. Construction of D29 reporter mycobacteriophage. The D29::pYub412mRFP DNA which was linearized by cleavage at lambda cos sites (Figure 2.22b) was packaged into bacteriophage lambda heads and used to transform *E. coli.* Upon infecting *E. coli* the DNA was circularized and replicates as a circular phasmid. The phasmid DNA was then extracted and used to transform *M. smegmatis.* After infecting *M. smegmaits* the D29::pYub412mRFP DNA was linearized by cleavage at "mcos" sites (mycobacteriophage cos sites). Expression of mycobacteriophage D29 genes then lead to packaging of the recombinant DNA into mycobacteriophage heads and the production of a recombinant reporter mycobacteriophage. Resulting plaques were isolated and used for plaque purification yielding a high titer of recombinant mycobacteriophage D29::pYub412mRFP.

2.6.5.3 Construction of Tm4-based reporter mycobacteriophage

Three cloning strategies were followed in the construction of a recombinant reporter mycobacteriophage based on Tm4. In the first method, the starting material was mycobacteriophage phAE142 which, was reported to contain a luciferase reporter cosmid flanked by *Pac*I RE sites. However, no sequence data was available for this construct. DNA was extracted from the mycobacteriophage sample by means of phenol extraction (Section 2.3.4), the phAE142 DNA was then electroporated into competent *M. smegmatis* (Section 2.3.13.2). The cells were incubated at 37℃ for 20 min and then mixed with 4 ml top agar (Appendix A.2.4) and spread onto a 7H10 plate (Appendix A.2.2) and then incubated at 37℃ overnight. Resulting plaques were picked and used to produce large scale stocks (Section 2.4.2.), the mycobacteriophages were then purified by ultracentrifugation (Section 2.4.3) followed by dialysis (Section 2.4.4) and their DNA was extracted by phenol/chloroform/Isoamyl alcohol DNA extraction (Sections 2.4.6). The purified DNA was digested (Section 2.3.9) with restriction enzyme *Pac*I. The digested DNA was then run on a low melting point FIDGE gel (Section 2.3.2). The upper DNA band was extracted and purified from the gel by phenol extraction (Section 2.3.4). The purified phAE142 DNA was then ligated (Section 2.3.12) to reporter cosmids pDMNLCosmRFP (Section 2.6.4.4), pYubmRFP (Section 2.6.4.5), pYubSSmrfp (Section 2.6.4.6) and pYub412LacZ (Section 2.6.4.7) using a 2 µg phage DNA to 1 µg cosmid DNA ratio in a total volume of 10 µl. The 10 µl ligation mixtures were then packaged into phage lamba and used to infect *E. coli* (Section 2.3.14). The infected *E. coli* was and grown on LB agar plates containing the appropriate antibiotics (ampicillin was used for phAE142::pYubmRFP, phAE142::pYubSSmrfp and phAE142::pYub412psmycLacZ and kanamycin was used for phAE142::pDMNmRFP). Subsequent colonies were then picked, PCR screened (Section 2.2.3) using the corresponding primer pairs to construct the cosmids (see Sections 2.6.4.4, 2.6.4.5, 2.6.4,6 and 2.6.4.7 for primer details and PCR product sizes) grown in 100 ml LB (containing the appropriate antibiotics listed above), the cosmid DNA was extracted (Section 2.3.6) and 2 µg of the purified DNA was electroporated into competent *M. smegmatis*, incubated for 30 min at 37℃ and then plated out with top agar on 7H10 plates. The plates were observed for plaque formation after overnight incubation. Resulting plaques were purified and used to create high titer stocks of the recombinant mycobacteriophage.

In a second method designed to override the possible size constrains of phage lambda, (since the Tm4 capsid can accommodate more than bacteriophage lambda), the ligation of RE digested phAE142 DNA to reporter cosmid was performed at high DNA concentrations (5 µg cosmid DNA : 10 µg phAE142 DNA), precipitated by adding sodium acetate (Appendix A.3.38) and 95% ethanol (Appendix A.3.13) to the sample as described in Section 2.4.6, and resuspended in H_20. Between one and two micrograms of the ligation was then electroporated into competent *M. smegmatis* as described in Section 2.3.13.2. Figure 2.23a, Figure 2.23b and Figure 2.23c is a visual illustration of the first cloning method to create phAE142::pYubmRFP, phAE142::pYubSSmrfp and phAE142::pYub412psmycLacZ, and phAE142::pDMNmRFP where “lcos” indicates bacteriophage lambda cos recognition sites and “mcos” indicates mycobacteriophage cos recognition sites. Similarly, Figure 2.24 is a visual illustration of the second cloning method.

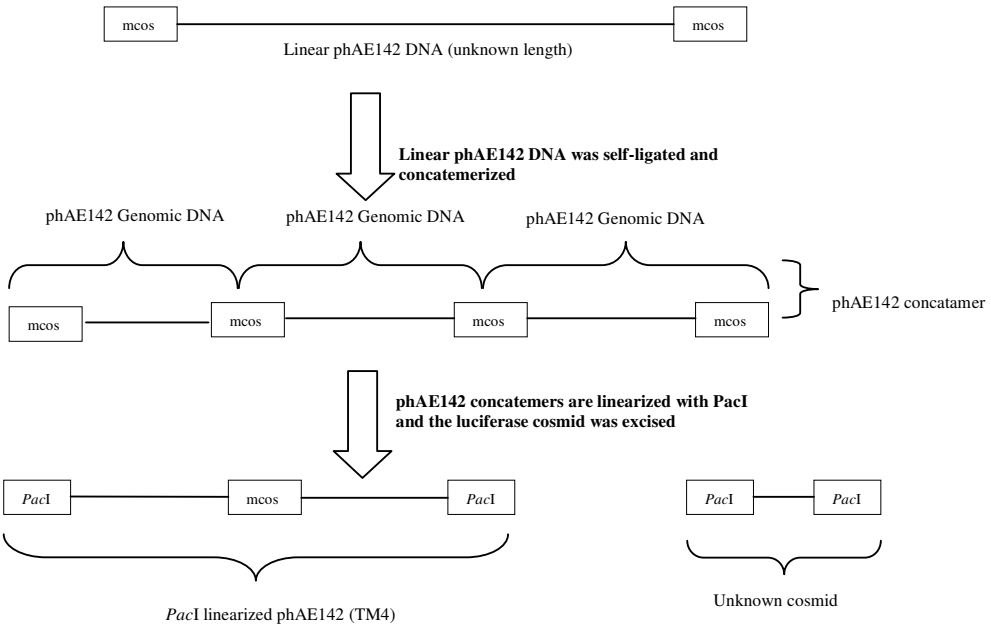

Figure 2.23a Construction of Tm4-based reporter mycobacteriophage (method 1). phAE142 DNA was electroporated into *M. smegmatis* and a plaque purification was performed to obtain large quantities of purified phAE142 DNA suitable for RE digests. The linear phAE142 DNA was concatamerized by self-ligation. The concatamerized DNA was digested with *Pac*I (several other REs were also tried in separate instances) to remove the unknown cosmid from phAE142 to liberate Tm4. The large molecular band was purified following FIDGE and ligated to reporter cosmids to create concatamerized phAE142::pYubmRFP, phAE142::pYubSSmrfp, phAE142::pYub412psmycLacZ and phAE142::pDMNmRFP.

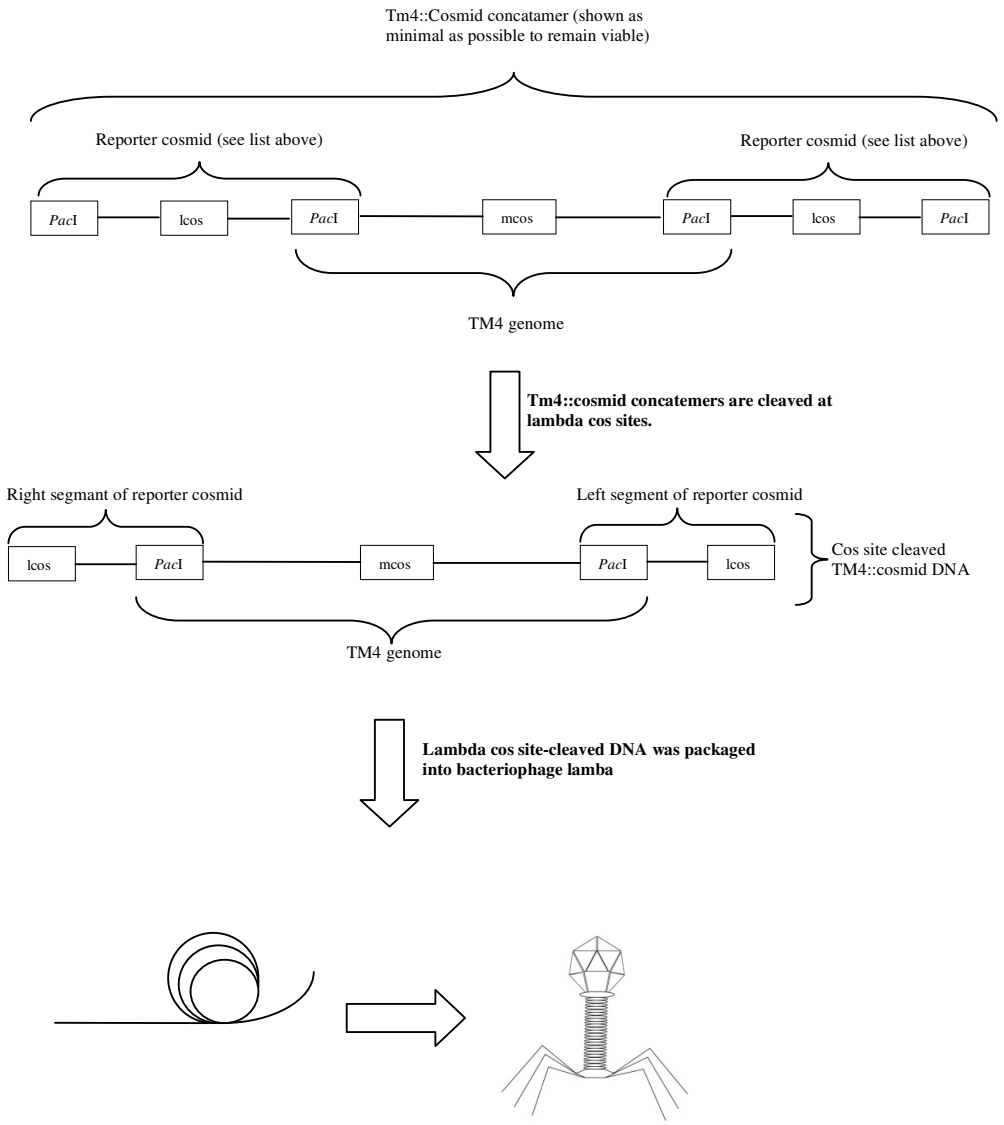

Figure 2.23b Construction of Tm4-based reporter mycobacteriophage (method 1, continued). The Tm4::cosmid concatemer DNA was cleaved at the lambda cos sites. The lambda cleaved DNA was then packaged into bacteriophage lambda. The recombinant bacteriophage lambda was then used to transform *E. coli.* Phasmid DNA was extracted from resultant colonies and used to transform *M. smegmatis* by electroporation.

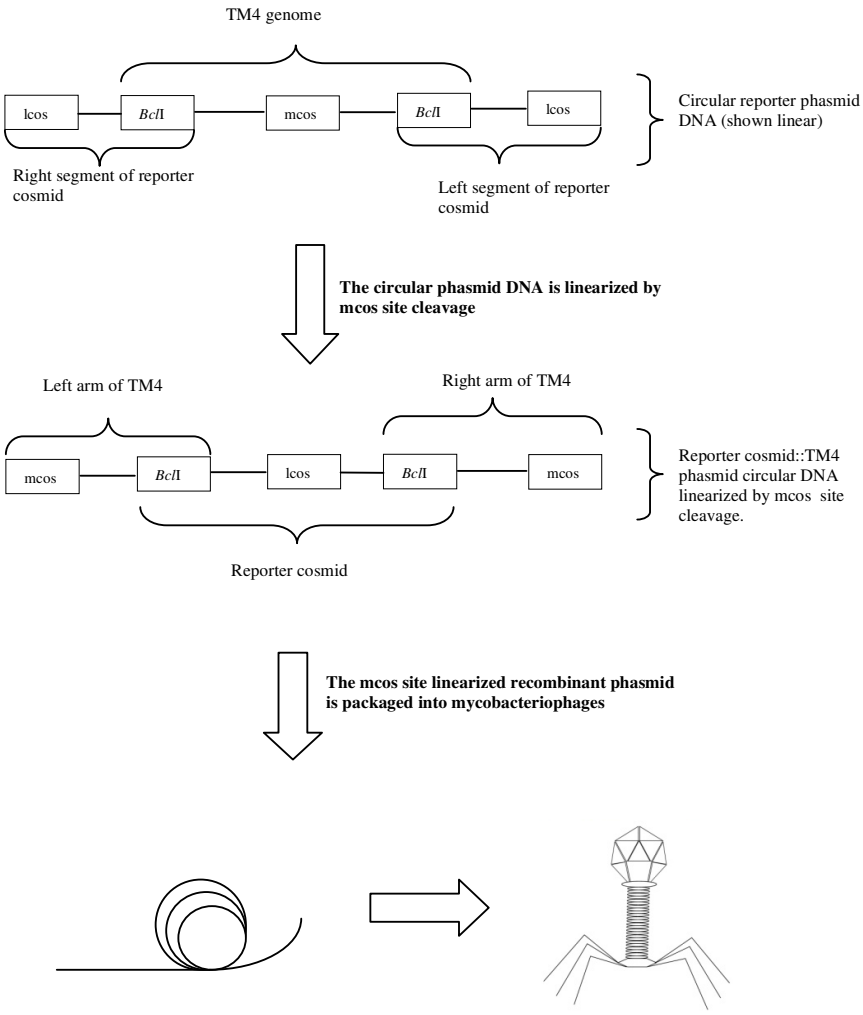

Figure 2.23c. Construction of Tm4-based reporter mycobacteriophage (method 1, continued). Following electroporation into *M. smegmatis* the phasmid DNA circularizes and mycobacteriophage Tm4 lytic genes are expressed. This leads to expression of genes that lead to the recombinant mycbacteriophage cleaving its genome at mycobacteriophage cos sites and packages its DNA into mycobacteriophages to result in propagation of recombinant Tm4 mycobacteriophages. Plaques were then used to create high titer stocks of the recombinant mycobacteriophages.

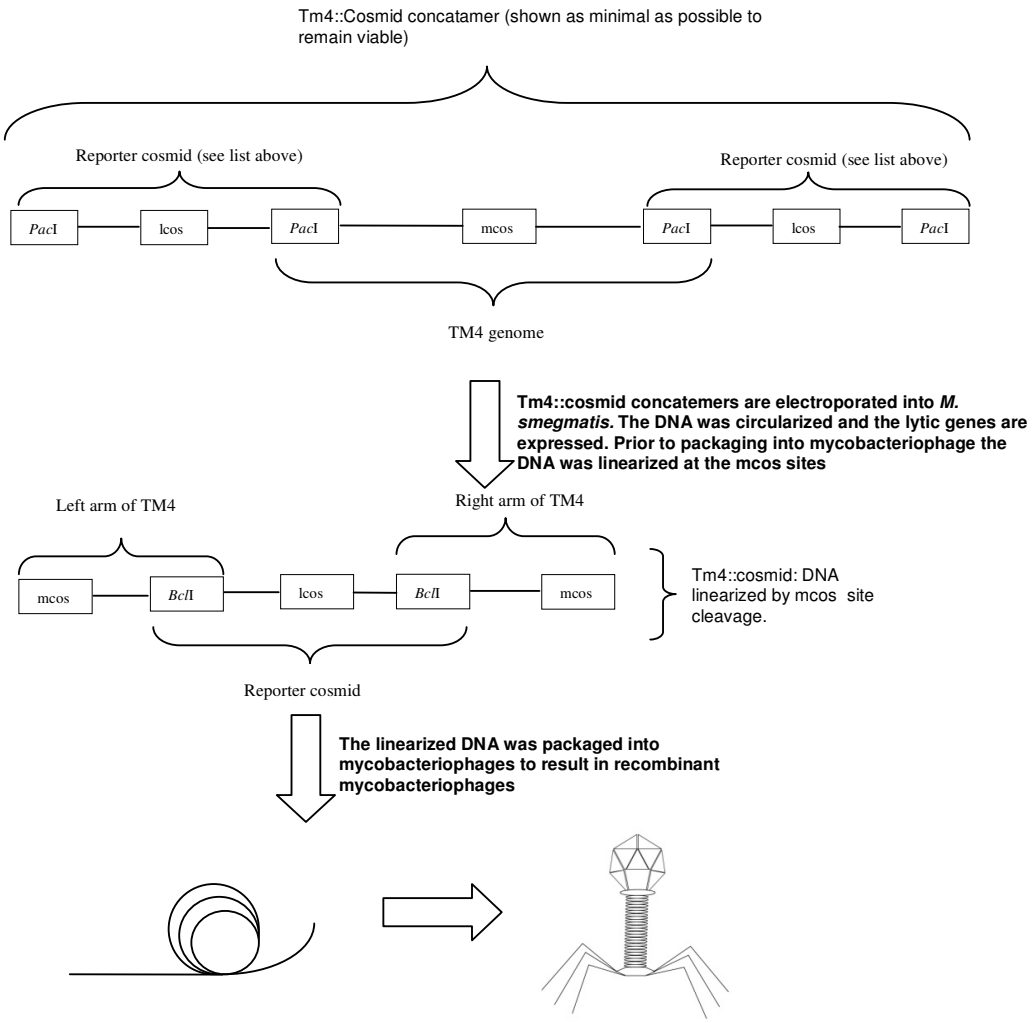

Figure 2.24. Construction of Tm4-based reporter mycobacteriophage (method 2). Resitriction enzyme digested Tm4 DNA was ligated to digested cosmid DNA to create concatemers. The DNA was electroporated into *M. smemgmatis* where the DNA is circularized. The mycobacterial genes are expressed which leads to cleavage of the DNA at the mcos sites and packaging into mycobacteriophages to result in recombinant mycobacteriophages. A plaque purification is then performed to produce a high titer stock of the recombinant mycobacteriophages.

In the third method phAE142 DNA (phenol purified DNA from the first method was used) was first self-ligated (Section 2.3.12) to form concatamers, 10 µl of the ligation was then used for lambda packaging (Section 2.3.14). Subsequent colonies were then picked and grown overnight (Section 2.1.2.1) in 100 ml LB media containing ampicillin (Appendix A.1.1). The phasmid DNA was then extracted (Section 2.3.6) and digested (Section 2.3.9) with *Pac*I. The digested DNA was run on a low melting point FIDGE gel (Section 2.3.2) and the upper large molecular weight band was excised and phenol purified (Section 2.3.4). Reporter cosmids pDMNLCosmRFP (Section 2.6.4.4), pYubmRFP (Section 2.6.4.5), pYubSSmrfp (Section 2.6.4.6) and pYub412LacZ (Section 2.6.4.7) was then digested (Section 2.3.9) with *Pac*I, run on a gel (Section 2.3.1), the large molecular size band was purified (Section 2.3.3), and ligated to the *Pac*I digested and purified phasmid DNA using a 2 µg phage DNA to 1 µg cosmid DNA ratio in a total volume of 10 µl. The 10 µl ligation was then packaged in to phage Lamba and used to infect and transform *E. coli* (Section 2.3.14). Subsequent colonies were then PCR screened (Section 2.2.3) using the primer pairs listed in Sections 2.6.4.4, 2.6.4.5, 2.6.4.6 and 2.6.4.7. Positive colonies were picked and grown in 100 ml LB containing ampicillin at 37°C overnight (Section 2.1.2.1). The cosmid DNA was then purified (Section 2.3.6) and 2 µg of the purified DNA was electroporated into competent *M. smegmatis* (Section 2.3.13.2). Resulting plaques were then picked and placed into 50 µl MP buffer again. A serial dilution was made of the selected plaques by placing 10 µl of the 50 µl plaque sample into 990 µl MP buffer (to create a 10^{-2} dilution) followed by sequentially placing 100 µl of the preceding sample into the next tube containing 900 µl MP buffer to create ten-fold dilutions down to 10^{-6}. Five microliters of each titer was mixed with 250 µl competent *M. smegmatis* (Section 2.1.2.8) for 20 min at 37°C and plated out on 7H10 plates along of 4 ml top agar (Appendix A.2.4) followed by overnight incubation at 37°C. The plate with the most confluent plaque pattern was then flooded with 4 ml MP buffer and incubated at 4°C overnight. The buffer was siphoned off with a syringe and filtered through a Millipore MILLEX®-GV polyvinylidene difluoride (PVDF) 0.22 µm filter unit. The filtered stock was then titered again (by testing various titers of the freshly filtered stock with 250 µl competent *M. smegmatis* as above) to determine the optimal titer for confluence on a large Petri dish. This titer was used to create large scale stocks of the recombinant mycobacteriophage (Section 2.4.2). The amplified recombinant mycobacteriophage stock was ultra-centrifuged (Section 2.4.3) and dialysed (Section 2.4.4) against MP buffer (Appendix A.3.23). The titer of the dialysed

mycobacteriophage was determined as described above. The recombinant mycobacteriophage was then used to test its expression in *M. smegmatis and M. tb.* Figure 2.25a, b, c and d is a visual illustration of the main cloning steps to construct Tm4::pYubmRFP, Tm4::pYubSSmrfp and Tm4::pYub412psmycLacZ, and Tm4::pDMNmRFP where "Lcos" indicated bacteriophage lambda cos recognition sites and "Mcos" indicate mycobacteriophage cos recognition sites.

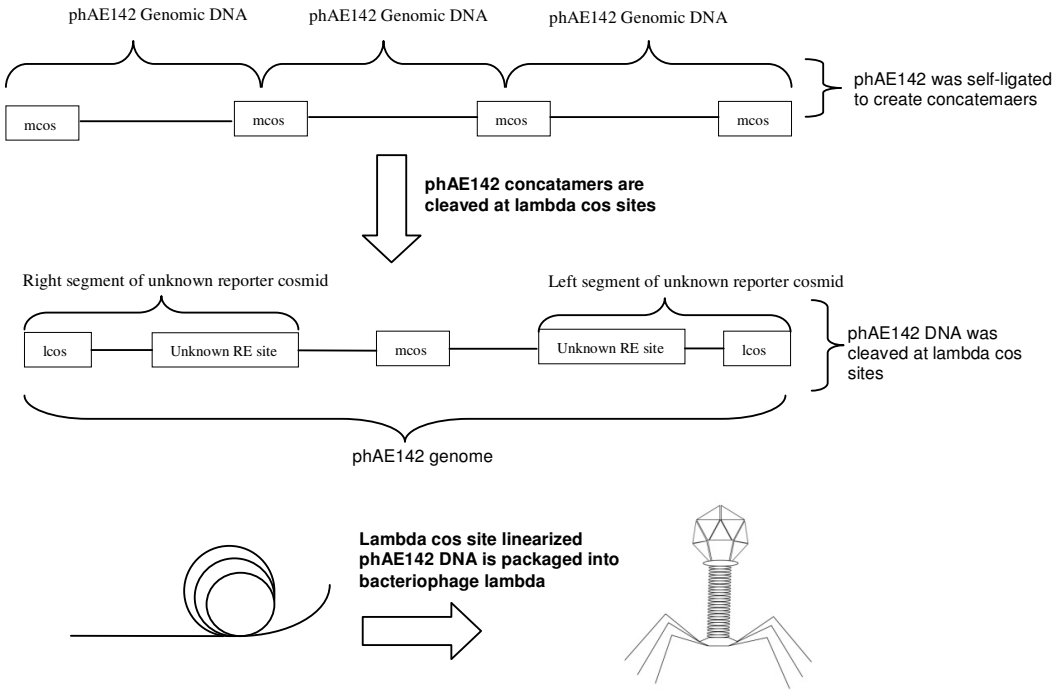

Figure 2.25a Construction of Tm4-based reporter mycobacteriophage (method 3). The linear phAE142 DNA was concatamerized by self-ligation. The phAE142 concatemers were cleaved at lambda Cos sites and packaged into bacteriophage lambda. The recombinant bacteriophage lambda was then used to transform *E. coli* and the phasmid DNA was extracted from resultant colonies.

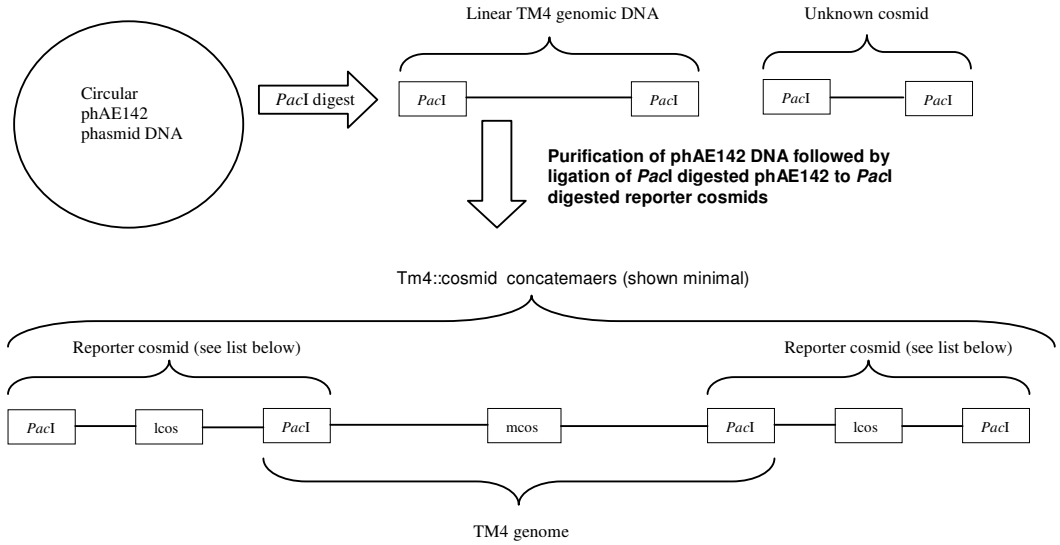

Figure 2.25b Construction of Tm4-based reporter mycobacteriophage (method 3, continued). The purified circular phAE142 phasmid DNA was digested with *Pac*I to linearize the DNA as well as to remove the unknown cosmid from phAE142 to yield the Tm4 genome with suitable RE sites for cloning. The large molecular weight band was purified following FIDGE and was ligated to reporter cosmids to create concatamerized Tm4::pYubmRFP, Tm4::pYubSSmrfp, Tm4::pYub412psmycLacZ and Tm4::pDMNmRFP.

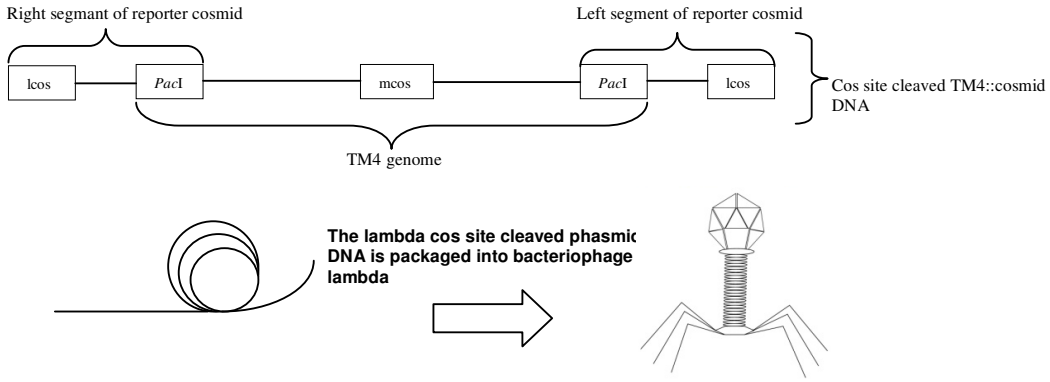

Figure 2.25c Construction of Tm4-based reporter mycobacteriophage (method 3, continued). The cosmid::mycobacteriophage DNA concatamer was cleaved at lambda cos sites, packaged into bacteriophage lambda and used to transform *E. coli*. Colonies were PCR screened following packaging into *E. coli,* cosmid DNA extraction was performed on subcultures of colonies testing positive.

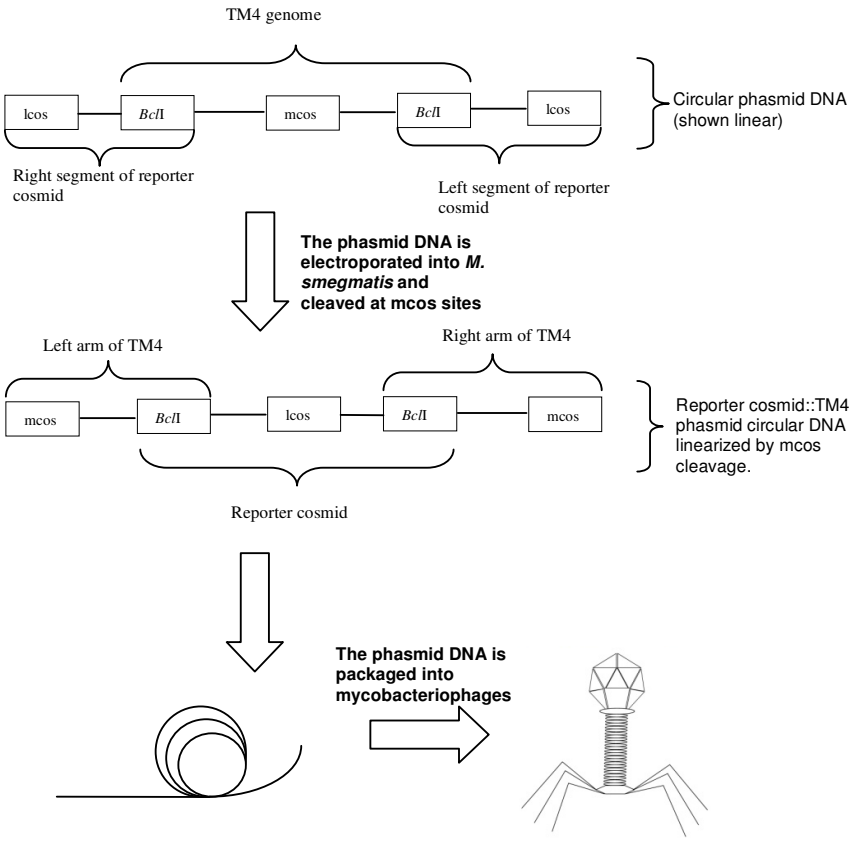

Figure 2.25d Construction of Tm4-based reporter mycobacteriophage (method 3, continued). The purified phasmid DNA was used to transform *M. smegmatis* after which the phasmid DNA was circularized. The lytic mycobacteriophage genes were then expressed followed by mcos site cleavage and packaging of the linear phasmid DNA into mycobacteriophages to result in recombinant mycobacteriophages. Resulting plaques were isolated and used for plaque purification yielding a high titer of recombinant mycobacteriophage.

2.7 Construct evaluation

2.7.1 Evaluation of promoter probe constructs

The ten promoter probe constructs (Section 2.6.2), construct pJem15Hsp60NHis (Section 2.6.3.11) as well as a positive control (using mycobacterial *Pan* promoter) was expressed in *M. smegmatis* in order to determine their relative promoter strengths. Each construct was electroporated into *M. smegmatis* (Section 2.3.13.2) and plated on solid media containing X-gal (Appendix A.3.49) and kanamycin (Appendix A.1.2). Single colonies were picked and used in a β-galactosidase assay (Section 2.5.5) to compare promoter strengths. The most suitable promoter was selected and used as promoter for certain reporter constructs (Sections 2.6.3.5, 2.6.3.6, 2.6.3.9 and 2.6.3.12).

2.7.2 Evaluation of reporter constructs

Each reporter construct (Sections 2.6.3.1 to 2.6.3.12) was expressed in *M. smegmatis* and protein expression was analysed. Each construct was electroporated into *M. smegmatis* (Section 2.3.13.2) and plated on solid media containing the appropriate antibiotics (Table 18, Section 2.6.3). Single colonies were picked and grown up in liquid media (Section 2.1.2.3) containing the appropriate antibiotics (Table 18, Section 2.6.3). Cell cultures were centrifuged at 4000 rpm in an Eppendorf Centrifuge 5810R for 15 min and the cell pellet was redissolved in 500 µl PBS (Appendix A.3.26). The cell resuspension was then ribolysed (Section 2.5.1) and 200 µl of the ribolysed sample was used in a His-Tag purification (Section 2.5.2) for constructs that have His-tags (Table 18, Section 2.6.3). Pictures were taken of the His-tag purified protein eluates and the His-tag purified proteins were also used to obtain their quantitative fluorescence readings using a fluorescence plate reader (Section 2.5.3) The protein concentrations were determined (Section 2.5.4) of the ribolysed and His-tag purified samples were determined and equal amounts of protein was then used in a SDS-PAGE gel separation (Section 2.5.6), Western blot (Section 2.5.6.4) followed by His-tag detection with His-tag specific antibodies (Section 2.5.6.6).

2.7.3 Evaluation of reporter mycobacteriophages

The performance of reporter mycobacteriophage Tm4::pYubSSmRFP was initially tested using *M. smegmatis* as host to investigate the role that several variables play on fluorescence expression. The first variable investigated was the possible nucleotide sequence variation between the progeny mycobacteriophage Tm4::pYubSSmRFP, particularly in the pSmyc promoter region. 20 progeny plaques were purified and used to create stocks. The stocks used in this instance were made by creating one plate with confluent plaque formation (as described in Section 2.4.2) per mycobacteriophage. 5 ml of MP buffer was added to each plate and incubated overnight at 4°C. The supernatant was then siphoned off and filter sterilized. The primer pair Rep4SalIF and Rep4EcoRVR (Table 5, Section 2.2.1) was used to PCR amplify the pSmyc promoter and mRFP1 gene which was subsequently sequenced for each of the 20 stocks. The 20 progeny mycobacteriophages were also tested to determine variations in fluorescence levels. The stocks of the 20 progeny mycobacteriophages were diluted to an equal concentration of 10^6 pfu/ml of which 200 µl from each was used to infect 200 µl of competent *M. smegmatis* followed by 24h incubation at 37°C. 300 µl of the infected cells were then transferred to a fluorescence micro-well plate and their relative fluorescence levels were measured as described in Section 2.5.3. Additionally 25 µl of each sample was pipetted onto a microscope slide, covered with a cover slip and visualized using a fluorescence microscope under oil immersion. The four best performing mycobacteriophages were then selected for further testiing, named mycobacteriophage Tm4:pYubSSmRFP 1.3, 6.3, R and 12 (referred to henceforth as mycobacteriophage 1.3, 6.3, R and 12).

In order to determine the optimal concentration of mycobacteriophage to use, a large titer range was tested. The two mycobacteriophages (R and 12) were used to create a dilution range from undiluted to a 10^{-9} dilution. 100 µl of each dilution was used to infect 200 µl of competent *M. smegmatis* overnight and the resultant fluorescence was measured. After determining the optimal concentration of mycobacteriophge, these results were used to determine the optimal incubation time for maximal fluorescence by incubating the four mycobacteriophages (1.3, 6.3, R and 12) at their optimal concentration with 200 µl *M. smemgmatis* for 1h, 4h, 6h, 12h, 16h, 24h and 46h at 37°C followed by fluorescence measurement. Using the optimal incubation time and mycobacteriophage concentration,

infections consisting of 200 µl *M. smegmatis* and 70 µl of mycobacteriophage 1.3, 6.3, R and 12 were ribolysed and used for his-tag purification (Section 2.5.2) of the mRFP1 protein. The fluorescence levels of the eulates were compared with the euates derived from uninfected *M. smemgmatis* cultues. The reporter mycobacteriophages were then evaluated in *M. tb* as described below.

M. tb strain H37Rv was grown in 7H9 (Appendix A.1) with an added 2 ml 20% Tween-80 (Appendix A.3.48) per liter of 7H9 at 37℃ with shaking. A millilitre of culture was aliquoted into Eppendorf tubes, the cells were pelleted by centrifugation in an Eppendorf centrifuge 5415D for 5 min at 2000 rpm. The cells were then resuspended in 7H9 (without Tween). Two-hundred microliters from each tube was then transferred into a new Eppendorf tube and 100µl of a 10^9 pfu/ml reporter mycobacteriophage Tm4::pYubSSmRFP stock was added (except to the control tube) and incubated for 6 hours at 37℃. Three-hundred microliters of 4% paraformaldehyde (Appendix A.3.25) was added to each tube and incubated at room temperature for 90 min to ensure killing of the *M. tb* bacilli. The tubes were then centrifuged as above, resuspended in 300µl 7H9 and incubated at room temperature for 90 min. The tubes were centrifuged as above, resuspended in 400µl PBS (Appendix A.3.26). The tubes were centrifuged again, resuspened in 25µl PBS and then spread onto a labeled glass microscope slide, covered with a cover slip and examined using a fluorescence microscope with a 1000x magnification using oil immersion. To test the viability of His-tag purification to amplify the reporter signal, 200 µl competent *M. smegmatis* cells were incubated overnight at 37℃ with 100 µl of the recombinant mycobacteriophage at various titers. The tube contents were then ribolysed (Section 2.5.1), His-tag purified (Section 2.5.2) and eluted in 100 µl elution buffer. The fluorescence of the eluates were tested by loading 100 µl of the eluates onto a 96-well fluorescence plate and thereafter measuring it using a modulus microplate reader as above (with ribolysed and His-tag purified *M. tb* as negative control).

Chapter 3 Results

3.1 Promoter screening

In order to identify additional mycobacterial promoters to be considered for reporter gene expression, 10 potential promoter regions of the ESAT-6 gene cluster region 5 were investigated and their expression levels were compared with the Hsp60 promoter. Two promoter prediction software programs were used to analyze the ten selected regions designated “Int1” to “Int10” in the *M. tb* H37Rv ESAT-6 gene cluster 5. The bioinformatics programs NNPP and BPPROM (Section 2.6.2) generated results for these regions as shown in Table 20.

Table 20. Promoter prediction results for NNPP and BPPROM software. “X” indicates that the software predicted the presence of a promoter.

Intergenic region	NNPP	BPPROM
Int1	X	X
Int2		X
Int3	X	
Int4	X	X
Int5	X	
Int6	X	
Int7	X	X
Int8	X	X
Int9		
Int10	X	X
Hsp60	X	X

NNPP predicted promoters for intergenic regions Int1, Int3, Int4, Int5, Int6 Int7, Int8 and Int10 as well as the known Hsp60 promoter. BPPROM predicted promoters for intergenic regions Int, Int2, Int4, Int7, Int8, Int10 as well as the known Hsp60 promoter. The ten intergenic regions were successfully PCR amplified, cloned into pGem-T-easy and subsequently cloned into the promoter probe vector pJem15. Figure 3.1.1a shows the PCR amplification results for a subsection of the intergenic regions (Int2 Int3, Int4 and Int5, not all are shown for reasons of conciseness). Subsequent sequencing of the products revealed that the primer set for Int3 and Int9 amplified the incorrect sequences and new primer sets were thus designed for these regions. The correct PCR products can be seen in Figure

3.1.1b and c (utilizing new primer sets). Figure 3.1.2a and 3.1.2b show the excision of the intergenic regions from pGem-T-Easy and Figure 3.1.3 shows the successful PCR colony screening following the ligation of the intergenic regions into pJem15. A sub-section of intergenic regions are shown for reasons of conciseness.

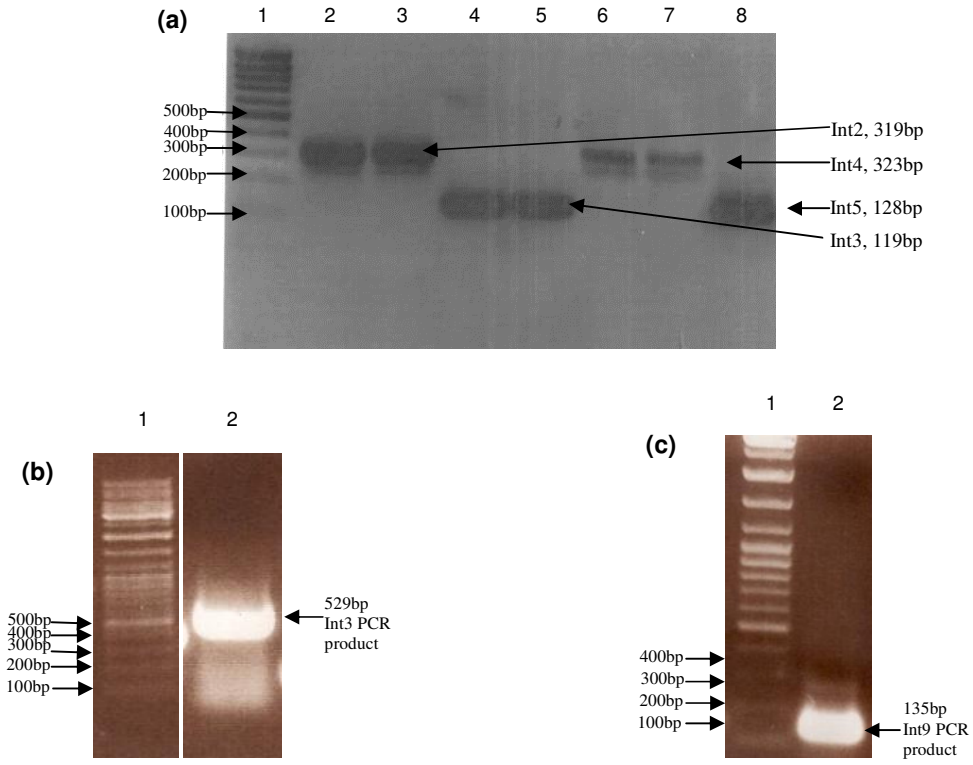

Figure 3.1.1 PCR amplification of intergenic regions from *M. tb* H37Rv ESAT-6 gene cluster 5. **(a)** Lane 1: GeneRuler 100bp DNA ladder plus, Lane 2&3: Int2 PCR product, Lane4&5: Int3 PCR product, Lane 6&7: Int4 PCR product, Lane 8: Int5 PCR product. **(b)** Lane 1: GeneRuler DNA ladder mix, Lane 2: PCR amplification of Int3 (please note that an irrelevant section of the gel image was removed for purposes of clarity) **(c)** Lane 1: GeneRuler DNA ladder mix, Lane 2: PCR amplification of Int9.

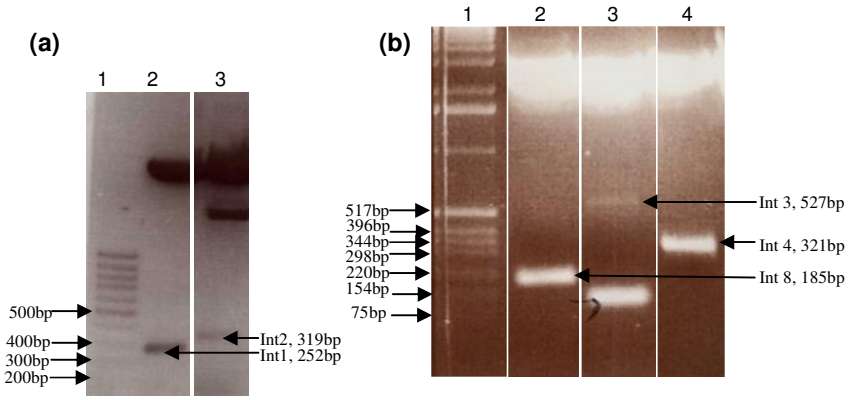

Figure 3.1.2 *Bam*HI and *Kpn*I excision of intergenic regions Int1, Int2, Int3, Int4$_{1}$, Int8$_{2}$ and Int9 from pGem-T-Easy. (a) Lane 1: GeneRuler 100bp DNA ladder, Lane2: Int1 digest product, Lane 3: Int2 digest product. **(b)** Lane 1: molecular marker X, Lane 2: Int8 digest product, Lane 3: Int3 digest product as well as an unknown amplification product from the *M. tb* H37Rv genome due to use of primers with less than ideal specificity, Lane 4: Int4 digest product. (Please note that irrelevant sections of the gel images were removed for purposes of clarity)

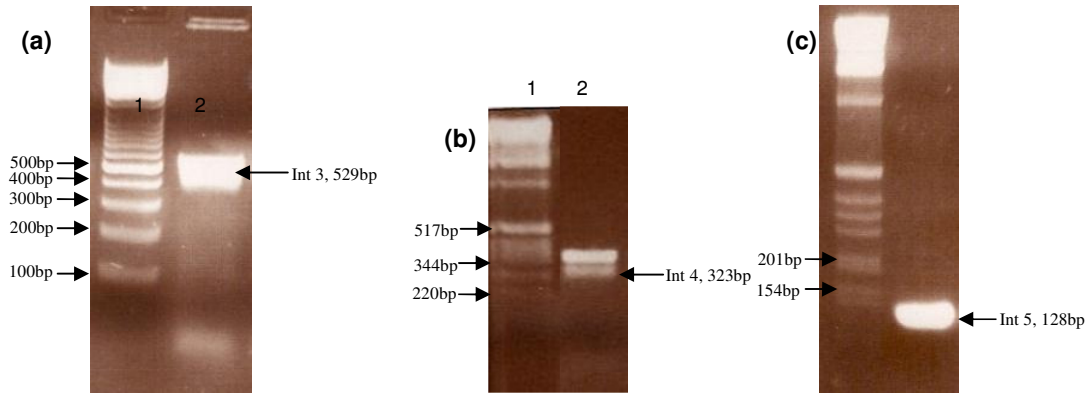

Figure 3.1.3 PCR colony screening for intergenic promoters following ligation to pJem15 and electroporation into *E. coli*. (a) Lane 1: GeneRuler DNA ladder mix, Lane 2: PCR product for Int3. **(b)** Lane 1: molecular marker X, Lane 2: PCR product for Int4. Two products can be seen due to non-specific binding from one of the two primers, the lower band represents the correct size band. (Please note that irrelevant sections of the gel image was removed for purposes of clarity) **(c)** Lane 1: molecular marker X, Lane 2: PCR product for Int5.

Sequencing results for the intergenic regions cloned into the promoter probe vector pJem15 showed that the inserts match the ten intergenic regions in the H37Rv genome sequence. Appendix C.1 shows the sequence information for the ten intergenic regions. The 10 sequence-verified promoter constructs were electroporated into *M. smegmatis* and plated together with X-gal in order to perform a visual test of promoter activity. Plates with a blue/green appearance indicated promoter activity by metabolizing the X-gal into its substrate (as in Figure 3.1.4).

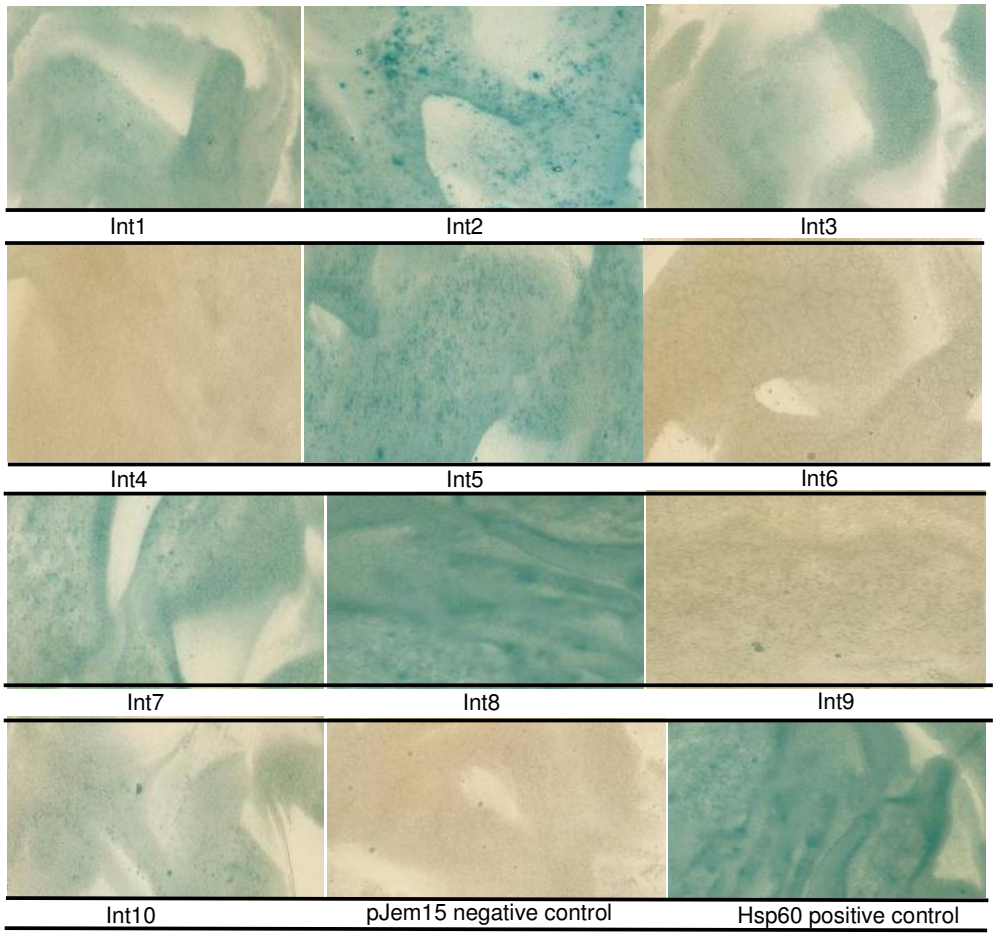

Figure 3.1.4. Int1 to Int10 plated onto solid media containing X-gal. Green/blue colonies indicate β- galactosidase activity and thus promoter activity. pJem15 was included as negative control and Hsp60 as positive control. Promoters Int1, Int2, Int3, Int5, Int7, Int8, Int10 and Hsp60 show promoter activity.

In order to quantify the promoter strengths of the intergenic regions a β-galactosidase assay was performed, which revealed the relative promoter strength of the 10 constructs. The promoter strengths of the 10 intergenic regions, relative to the Hsp60 promoter and a negative control (pJem15 lacking a promoter insert) are shown in Figure 3.1.5. The β-galactosidase assay indicated that Int1, Int2, Int5 and Int8 are strong promoters relative to the Hsp60 promoter (in *M. smegmatis).* Int5, Int8 and Int10 displayed variable promoter strength between β-galactosidase assays. In certain instances the Int10 promoter was weaker than Int1 and in others it was stronger indicating that the promoter is not always active despite the similar growth conditions between cultures as well as between β-galactosidase assays. However, Int1 and Int2 displayed constantly high promoter activity indicating its suitability to drive expression of reporter genes.

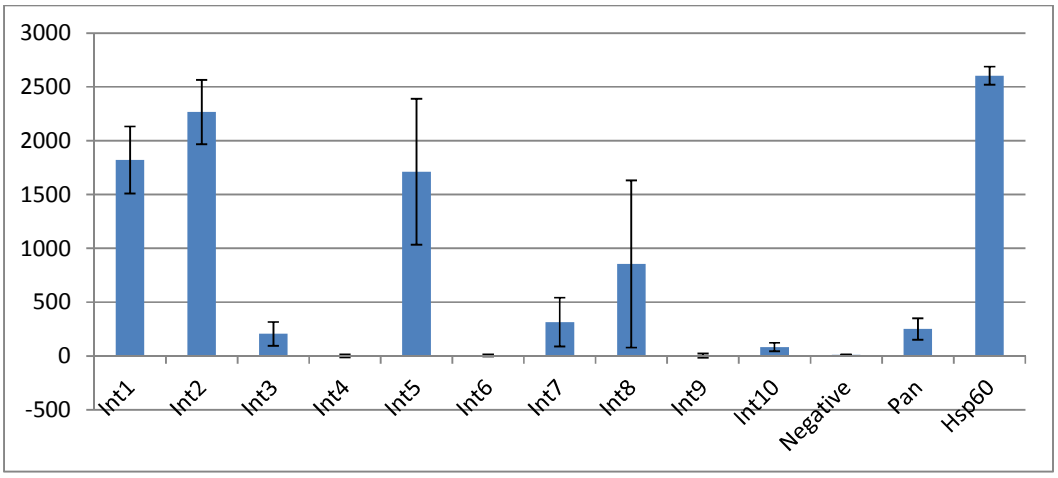

Figure 3.1.5. β-Galactosidase activity indicating the relative promoter strength of the ten intergenic regions. The relative promoter strength for intergenic region Int to Int10 can be se seen compared to the Hsp60 promoter, the mycobacterial Pan promoter and negative control (vector pJem15 without a promoter element) and represents the data from 3 separate assays. The figure shows that Int1, Int2, Int5 and Int8 are strong promoters relative to the Hsp60 promoter.

3.2 Reporter constructs

3.2.1 pSD21Hsp60DsRedNHis

Construct pSD21Hsp60DsRedNHis was constructed to test expression of N-term His-tagged DsRed driven by the Hsp60 promoter in expression vector pSD21. Vector pSD21Hsp60DsRedNHis was constructed by cloning the N-term His-tagged *DsRed* gene, driven by the Hsp60 promoter, into expression vector pSD21 (as described in Section 2.6.3.1). The Hsp60 promoter was PCR amplified (the PCR product is shown in Figure 3.2.1.1), cloned into pGem-T-Easy and excised using *Bam*HI and *Kpn*I. Figure 3.2.1.1b shows the RE excision from pGem-T-Easy. The digested DNA was then cloned into the *Bam*HI and *Kpn*I digested pDsRed vector. Figure 3.2.1.2 shows the RE digest of pDsRed.

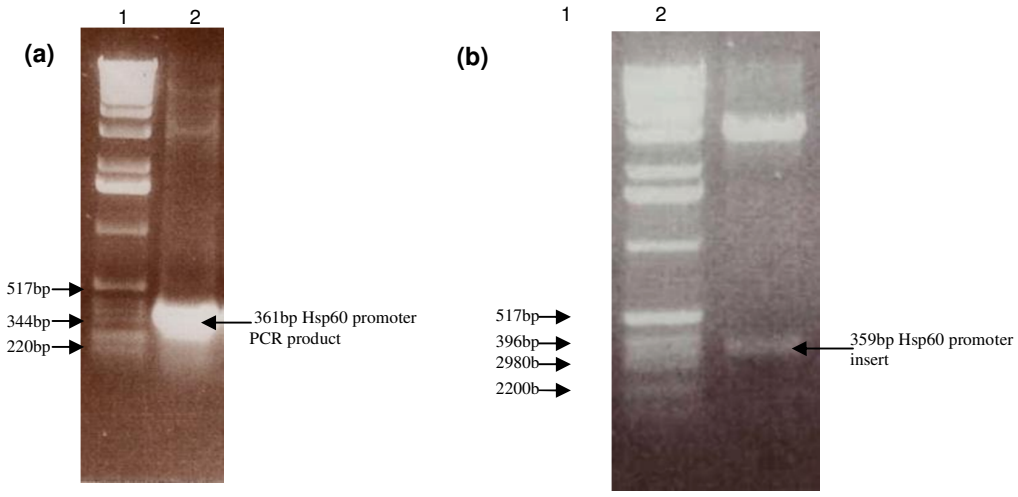

Figure 3.2.1.1. PCR amplification and RE excition of the Hsp60 promoter from pGem-T-Easy. **(a)**: PCR amplificiation of Hsp60 promoter with primers ESATRFPF and Hsp60PromHisR3 (Section 2.2.1), Lane 1: molecular marker X, Lane 2: Hsp60 promoter PCR product. **(b)** RE excision of the Hsp60 promoter from pGem-T-Easy. Lane1: molecular marker X, Lane 2: *Bam*HI and *Kpn*I digest excision of the Hsp60 promoter from pGem-T-easy.

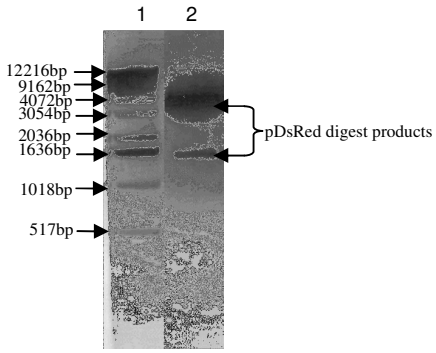

Figure 3.2.1.2 *Bam*HI and *Kpn*I digest of vector DsRed. Lane 1: molecular marker X, Lane 2: digest of pDsRed (Please note that irrelevant sections of the gel image were removed for purposes of clarity).

The Hsp60 promoter was sucsesfully cloned into pDsRed and sequence analysis confirmed the correct sequence of the promoter. The Hsp60 promoter and DsRed gene was excised from pDsRed as shown in Figure 3.2.1.3a and cloned into the XbaI RE site of pSD21. Figure 3.2.13b shows a digest screening for the Hsp60 promoter and DsRed gene insert following cloning into pSD21.

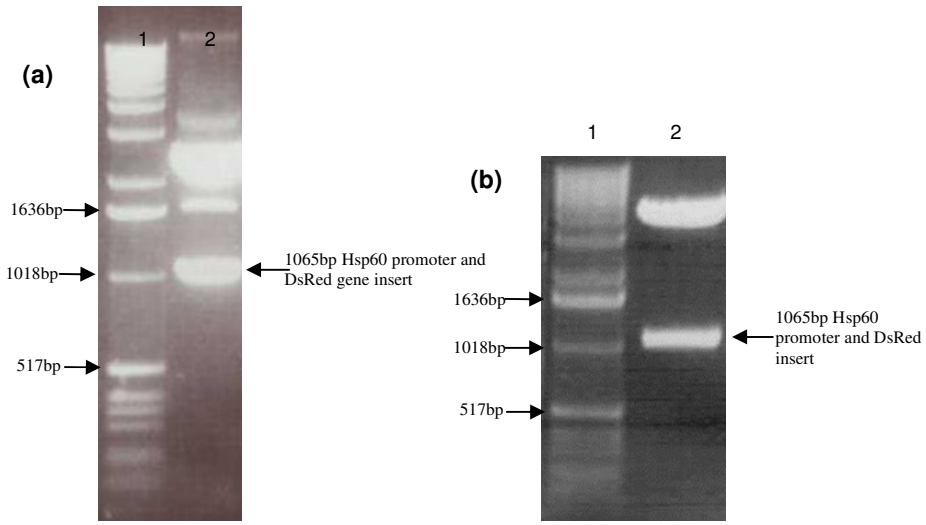

Figure 3.2.1.3 RE excision of the Hsp60 promoter and *DsRed* gene from pDsRed and pSD21. (a) RE excision of the Hsp60 promoter and *DsRed* gene from pDsRed. Lane 1: molecular marker X, Lane 2: *Xba*I digested pDsRedHsp60NHis (Section 2.6.3.1) excising the Hsp60 promoter and DsRed gene from pDsRed. **(b)** RE excision of the Hsp60 promoter and *DsRed* gene from pSD21. Lane 1: molecular marker X, Lane 2: *Xba*I digest screening of pSD21Hsp60DsRedNHis (Section 2.6.3.1) confirming the Hsp60 promoter and *DsRed* insert.

Vector pSD21DsRedHsp60NHis was subsequently sequence verified and electroporated into *M. smegmatis* to test the expression of the affinity-tagged DsRed fluorescent protein. Figure 3.2.1.4a shows the visible red signal from a cell pellet of a *M. smegmatis* culture containing the vector pSD21Hsp60DsRedNHis. Figure 3.2.1.4b shows the purified eluate following affinity chromatography performed on the cell pellet shown in Figure 3.2.1.4a. The visible signal from the eluate proves the functionality of the N-term His-tag to the DsRed protein. Figure 3.2.1.5 shows a fluorescence microscopy photograph of *M. smegmatis* cells containing the construct. The expression of the DsRed gene was however variable among transformants. Picking of colonies with a red appearance (as well as PCR positive for the Hsp60 promoter and DsRed gene) led to subcultures with unpredictable expression profiles ranging from good expression to none.

(a) **(b)**

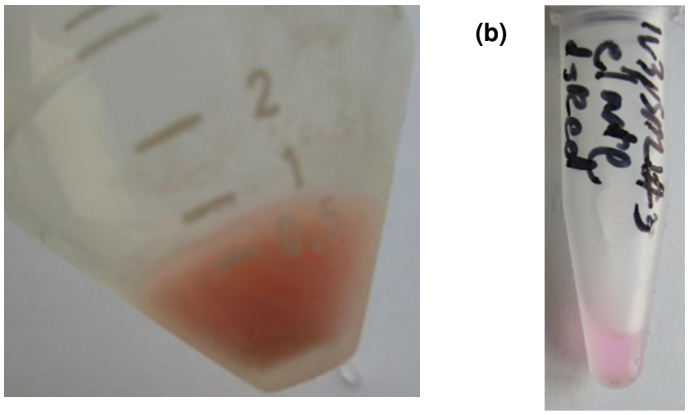

Figure 3.2.1.4. DsRed expression in *M. smegmatis* cells containing pSD21Hsp60DsRedNHis. (a) *M. smegmatis* cell pellet of a culture containing pSD21Hsp60DsRedNHis following overnight expression **(b)**. Eluate of a His-tag purification performed on cell pellet shown in (a).

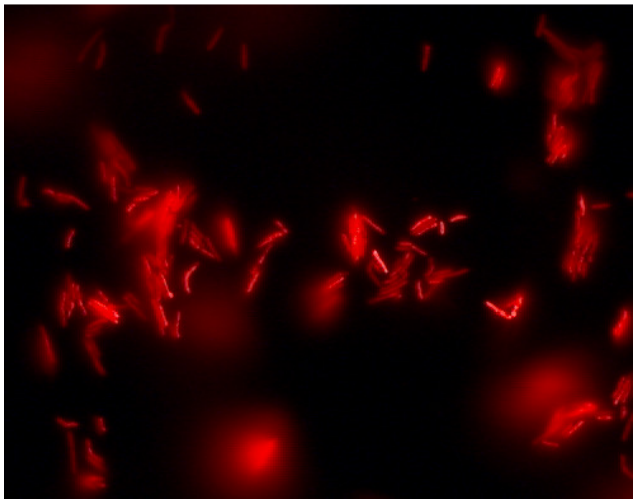

Figure 3.2.1.5. Fluorescence microscopy of *M. smegmatis* cells containing pSD21Hsp60DsRedNHis. Individual cells are visible following overnight expression of affinity-tagged DsRed.

3.2.2 p19KProHsp60DsRedNHis

Construct p19KProHsp60DsRedNHis was constructed to test expression of N-term His-tagged DsRed driven by the Hsp60 promoter using expression vector p19KPro. Construct p19KProHsp60DsRedNHis was constructed by cloning the Hsp60 promoter and N-Term His-tagged *DsRed* gene into vector p19KPro. The 1052bp fragment from Figure 3.2.1.3, consisting of the Hsp60 promoter and DsRed gene, was cloned into the *Xba*I RE site of p19KPro. Construct p19KProHsp60DsRedNHis did not display visibly red cell pellets, however the majority of the cultures had a supernatant which were visibly red. Despite the low level of red signal visible in the cell pellet, cells were easily detected using fluorescence microscopy as in Figure 3.2.2.

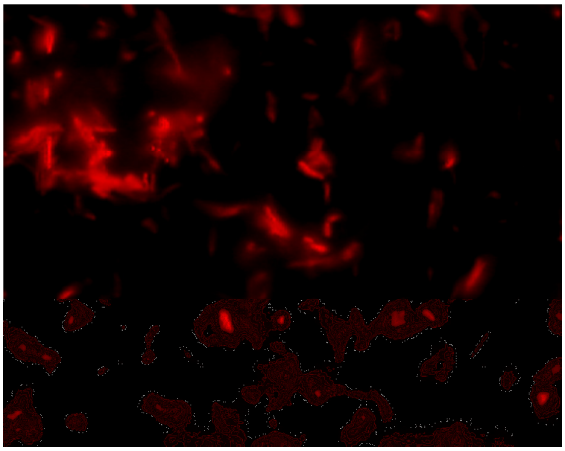

Figure 3.2.2. Fluorescence microscopy of a *M. smegmatis* culture containing vector p19KProHsp60DsRedNHis. Single cells are shown to be visible.

3.2.3 pSD21Hsp60DsRedCHis

Construct pSD21Hsp60DsRedCHis was constructed to test expression of C-term His-tagged DsRed driven by the Hsp60 promoter using expression vector pSD21. The construct pSD21Hsp60DsRedCHis consisted of the Hsp60 promoter, C-term His-tagged *DsRed* gene cloned into pSD21. The Hsp60 promoter and *DsRed* gene was PCR amplified to add a C-term His-tag to the *DsRed* gene and this was cloned into pGem-T-Easy and subsequently subcloned into pSD21. Figure 3.2.3a shows the restriction enzyme excision of the promoter and reporter gene from pGem-T-Easy, Figure 3.2.3b shows the PCR colony screening following ligation to pSD21 and Figure 3.2.3c shows a digest screening for the promoter and reporter gene insert following ligation to pSD21. Expression of the C-term His-tagged *DsRed* gene from transformants could not be detected for this construct in *M. smegmatis* despite being positive PCR for the insert (data not shown).

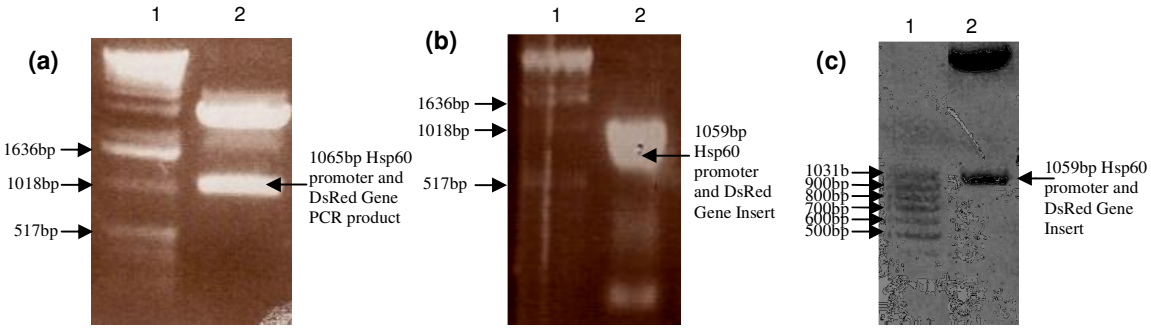

Figure 3.2.3. Cloning of the Hsp60 promoter and *DsRed* gene into pSD21 (a) *Bam*HI and *Xba*I excision of the Hsp60 promoter and *DsRed* gene from pGem-T-Easy. Lane 1: molecular marker X, Lane 2: RE digest product for the promoter and reporter gene. **(b)** PCR colony screening for the Hsp60 promoter and DsRed gene insert following ligation to pSD21. Lane 1: molecular marker X, Lane 2: PCR product for the promoter and reporter gene. **(c)** *Bam*HI and *Xba*I digest screening for the hsp60 promoter and DsRed gene following ligation to pSD21. Lane1: GeneRuler 100bp DNA ladder, Lane 2: promoter and reporter gene digest product (Please note that an irrelevant section of this gel image was removed for reasons of clarity).

3.2.4 pACEHsp60DsRedCHis

Construct pACEHsp60DsRedCHis was constructed to test expression of C-term His-tagged DsRed driven by the Hsp60 promoter using expression vector pACE. The construct pACEHsp60DsRedCHis was constructed by cloning the Hsp60 promoter and C-term His-tagged *DsRed* gene cloned into expression vector pACE (as described in Section 2.6.3.4). The Hsp60 promoter and C-term His-tagged *DsRed* gene was excised from pGem-T-Easy as in Figure 3.2.3a (Section 3.2.3) and cloned into *Bam*HI and *Xba*I digested pACE (Figure 3.2.4.1a). Figure 3.2.4.1b shows the PCR colony screening for the Hsp60 promoter and *DsRed* gene following ligation to pACE and electroporation into *E. coli.* Construct pACEHsp60DsRedCHis was successfully sequence verified and electroporated into *M. smegmatis*. Expression of *DsRed* was detectable compared to pSD21DsRedCHis, by means of fluorescence microscopy. Figure 3.2.4.2 shows a typical fluorescence microscopy result for *M. smegmatis* cells containing this construct.

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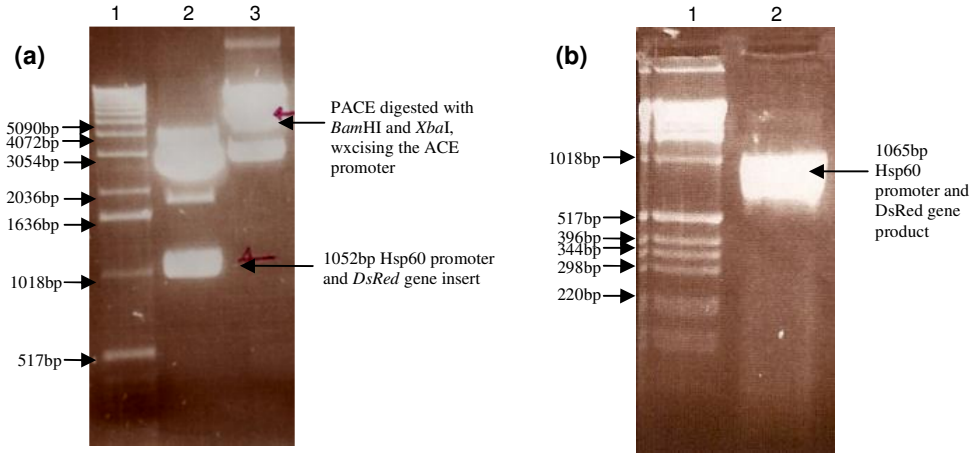


Figure 3.2.4.1. Cloning of the Hsp60 promoter and *DsRed* Gene into vector pACE. (a) Digest excision of th promoter and reporter gene from pGem-T-Easy and RE digest of vector pACE. Lane 1: molecular marker X. Lane 2: *Bam*HI and *Xba*I digest excising the Hsp60 promoter and *DsRed* gene fragment from pGem-T-Easy, Lane 3: *Bam*HI and *Xba*I digest of pACE removing the ACE promoter. **(b)** PCR colony screening of transformants following ligation of the promoter and reporter gene to vector pACE. Lane 1: molecular marker X. Lane 2: PCR colony screening for the Hsp60 promoter and *DsRed* gene insert following electroporation of *E. coli* with construct pACEHsp60DsRedCHis.

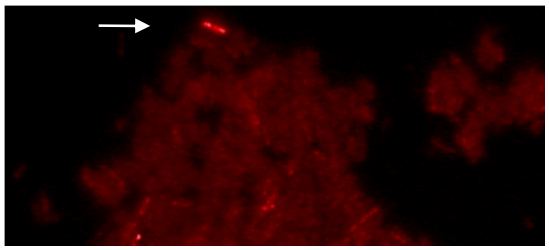


Figure 3.2.4.2. Fluorescent microscopy of *M. smegmatis* cells containing construct pACEHsp60DsRedCHis. A Large clump of cells can be seen with individual cells fluorescing brightly (one example shown by the arrow).

3.2.5 pSD21Int1DsRedNHis

Construct pSD21Int1DsRedNHis was constructed to test expression of N-term His-tagged DsRed driven by the Int1 promoter using expression vector pSD21. The construct pSD21Int1DsRedNHis was constructed by cloning the Int1 promoter and N-terminus His-tagged *DsRed* gene cloned into expression vector pSD21. The Int1 promoter was PCR amplified (the product is shown in Figure 3.2.5.1a), cloned into pGem-T-easy and subsequently excised using *Bam*HI and *Kpn*I (the digest product is shown in Figure 3.2.5.1b). The promoter was subsequently cloned into pDsRed after which both the promoter and reporter gene was excised and cloned into vector pSD21. Figure 3.2.5.1c shows the PCR colony screening results for the promoter and reporter gene following ligation into pSD21. This construct presented poor fluorescence visible in culture as well as under fluorescence microscopy (data not shown).

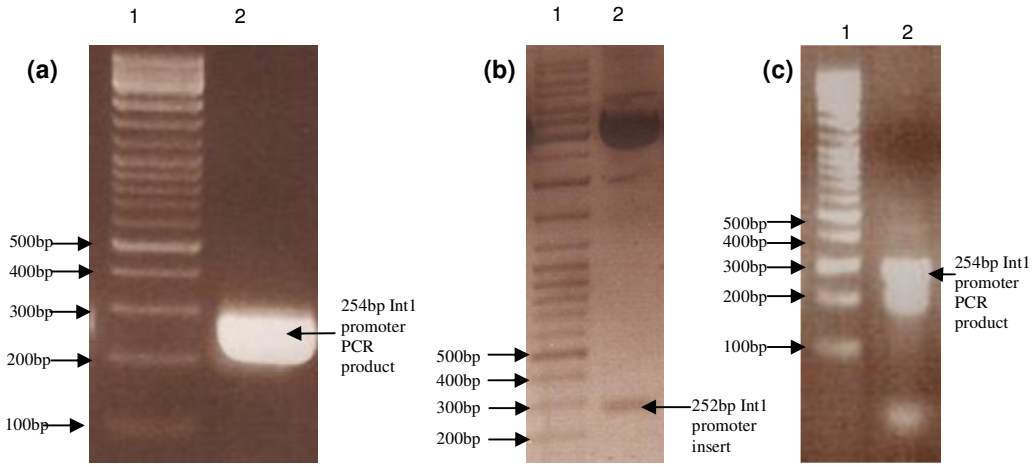


Figure 3.2.5.1 Cloning of the Int1 promoter and *DsRed* gene into pSD21. (a) PCR amplification of the Int1 promoter. Lane 1: GeneRuler 100bp DNA ladder plus, Lane 2: PCR product for the Int1 promoter. **(b)** *Bam*HI and *Kpn*I excision of promoter Int1 from pGem-T-Easy. Lane 1: Generuler DNA ladder Mix, Lane 2: digest product of the Int1 promoter from pGem-T-Easy. **(c)** PCR colony screening for the Int1 promoter following transformantion of *E. coli* with construct pSD21Int1DsRedNHis. Lane 1: GeneRuler DNA ladder mix, Lane 2: Int1 PCR product following PCR colony screening.

3.2.6 pACEInt1DsRedCHis

Construct pACEInt1DsRedCHis was constructed to test expression of C-term His-tagged DsRed driven by the Int1 promoter using expression vector pACE. Construct pACEInt1DsRedCHis was constructed by cloning the Int1 promoter and C-term His-tagged *DsRed* gene into vector pACE. The int1 promoter, which was previously cloned into pGem-T-Easy (Section 3.1), was excised with *Bam*HI and *Kpn*I and cloned into pACEHsp60DsRedCHis which replaces the Hsp60 promoter with the Int1 promoter. The PCR amplification product for the Int1 promoter can be seen in figure 3.2.6.1a. The *Bam*HI and *Kpn*I digest of pACEHsp60DsRedCHis can be seen in Figure 3.2.6.1a, lane 4. The PCR colony screening results can be seen for the completed construct using primers for the int1 promoter as in Figure 3.2.6.1b. Sequencing confirmed the successful cloning of the Int1 promoter and the construct was subsequently electroporated into *M. smegmatis*.

Figure 3.2.6.2 shows the fluorescence micrsoscopy results for pACEInt1DsRedCHis in *M. smegmatis*.

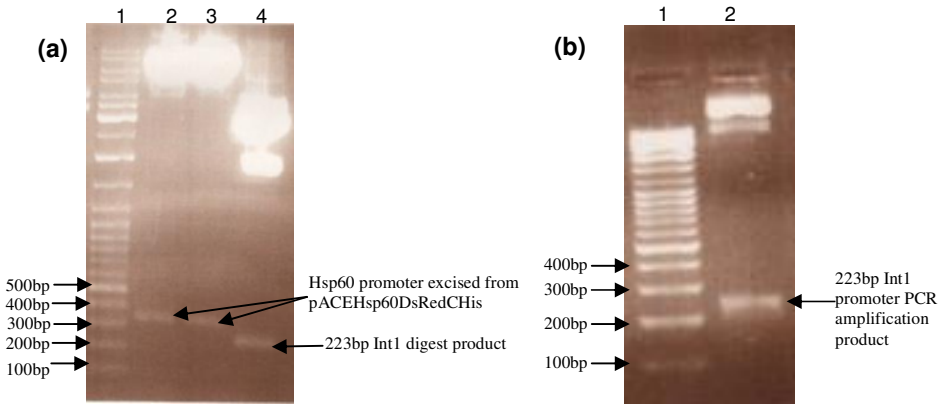


Figure 3.2.6.1. Cloning of the Int1 promoter into pACEHsp60DsRedCHis. (a) *Bam*HI and *Kpn*I digest of pACEHsp6-DsRedCHis and pGemInt1. Lane1: Generuler DNA ladder mix. Lane 2&3: RE digest of construct pACEHsp6-DsRedCHis excising the Hsp60 promoter, Lane 4: RE digest of construct pGemInt1 excising the Int1 promoter. **(b)** PCR colony screening for the Int1 promoter. Lane 1: GeneRuler 100bp DNA ladder plus, Lane 2: PCR colony screening for the Int1 promoter performed on tranformants containing construct pACEHsp60DsRedCHis.

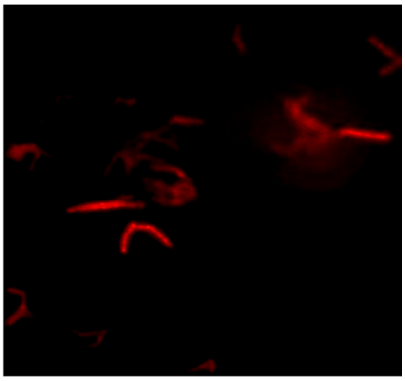


Figure 3.2.6.2. Fluorescence microscopy results for *M. smegmatis* containing vector pACEInt1DsRedCHis. Individual cells are visible.

3.2.7 pSD21Hsp60mRFPNHis

Construct pSD21Hsp60mRFPNHis was constructed to test expression of N-term His-tagged mRFP1 driven by the Hsp60 promoter using expression vector pSD21. The construct pSD21Hsp60mRFPNHis consisted of the Hsp60 promoter and N-term His-tagged *mRFP1* gene cloned into expression vector pSD21 (as described in Section 2.6.3.7). Figure 3.2.7a shows the PCR amplification *mRFP1*, Figure 3.2.7b shows the PCR colony screening for the mRFP1 gene following ligation to pGem-T-Easy and Figure 3.2.7c shows the successful excision of mRFP1 from pGem-T-Easy. The vector was sequence verified following cloning into pSD21, however mRFP1 expression was poor and variable. A small proportion of cells appeared fluorescent however the majority appeared non-fluorescent, the culture media however indicated fluorescence. Fluorescence microscopy could however not detect individual cells (results not shown).

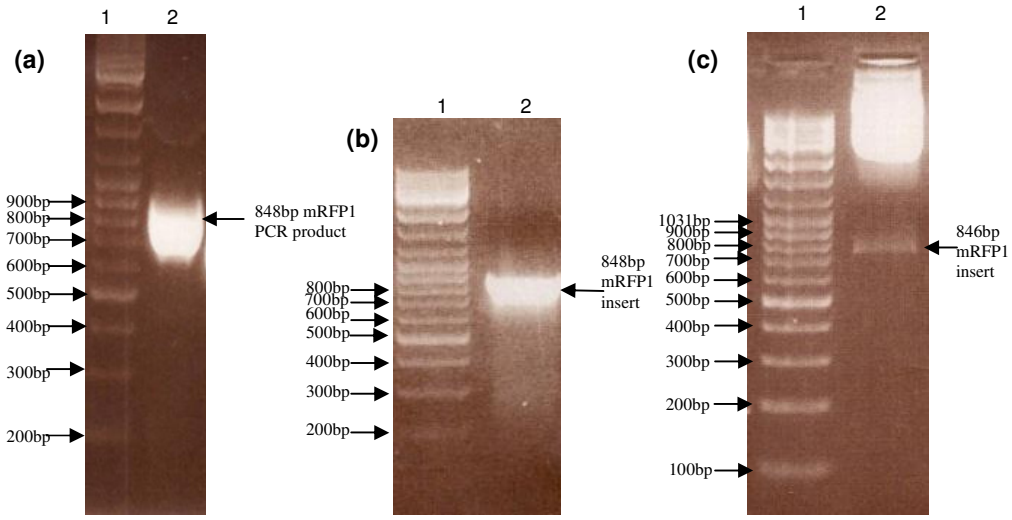


Figure 3.2.7 PCR amplification and cloning of the *mRFP1* gene. (a) PCR amplification of the *mRFP1* gene from pRSETB. Lane 1: GeneRuler DNA ladder. Lane 2: PCR amplification of mRFP1. **(b)** PCR colony screening for the *mRFP1* gene following ligation to pGem-T-Easy. Lane 1: GeneRuler DNA ladder mix, Lane 2: PCR colony screening product for the reporter gene. **(c)** *Kpn*I and *Sal*I excision of the mRFP1 insert from pGem-T-Easy. Lane 1: GeneRuler DNA ladder mix, Lane 2: Digest product consisting of the reporter gene.

3.2.8 p19KProp19KmRFPNHis

Construct p19KProp19KmRFPNHis was constructed to test expression of N-term His-tagged mRFP1 driven by the p19K promoter using expression vector pSD21. Construct p19KProp19KmRFPNHis consisted of the N-term His-tagged *mRFP1* gene cloned into vector p19KPro (as described in Section 2.6.3.8). The *mRFP1* gene was PCR amplified (as shown in Figure 3.2.9.1a), cloned into pGem-T-Easy and subsequently cloned into vector p19KPro. Figure 3.2.8.1b shows the PCR colony screening for the *mRFP1* gene following ligation into pGem-T-Easy and Figure 3.2.8.1c shows the *Hind*III and *Cla*I RE excision of the promoter and reporter gene from pGem-T-Easy. The construct was sequence verified and electroporated into *M. smegmatis.* Figure 3.2.8.2 shows the fluorescence microscopy for construct p19Kprop19KmRFP1NHis in *M. smegmatis.*

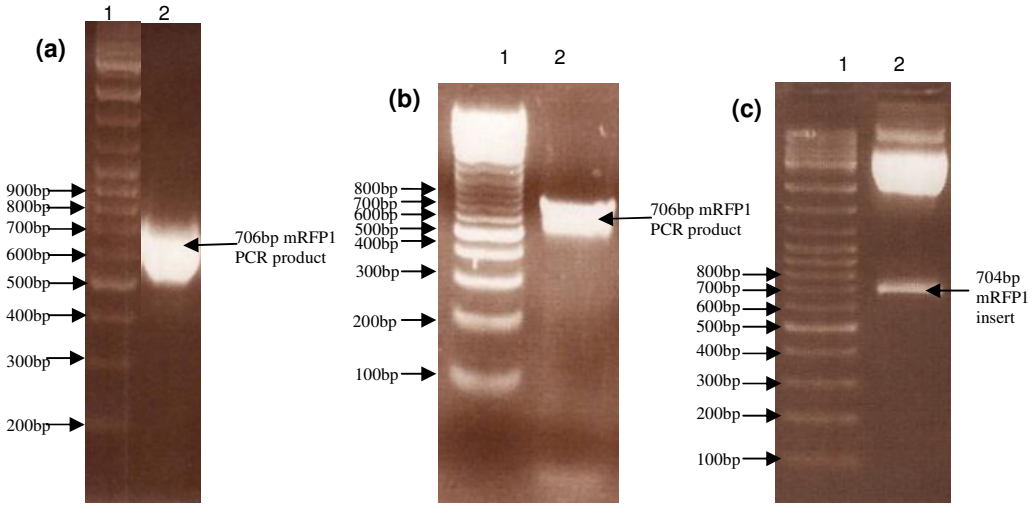


Figure 3.2.8.1. Cloning of the mRFP1 gene into pGem-T-Easy. (a) PCR amplification of the mRFP1 gene. Lane 1: GeneRuler DNA ladder mix, Lane 2: mRFP1 PCR product. (Please note that irrelevant sections of this gel image was removed for clarity) **(b)** PCR colony screening for the *mRFP1* gene following ligation to pGem-T-Easy. Lane 1: Generuler DNA ladder Mix, Lane 2: PCR colony screening PCR product for the *mRFP1* gene. **(c)** *Hind*III and *Cla*I excision of the mRFP1 gene from pGem-T-Easy. Lane 1 Generuler DNA ladder Mix, Lane 2: Digest product for *mRFP1.*

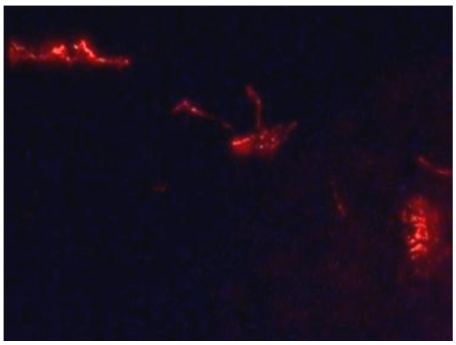


Figure 3.2.8.2. Fluorescence microscopy of *M. smegmatis* cells containing construct p19Kprop19KmRFPNHis. Individual cells are visible.

3.2.9 pSD21Int1mRFPNHis

Construct pSD21Int1mRFPNHis was constructed to test expression of N-term His-tagged mRFP1 driven by the Int1 promoter using expression vector pSD21. Construct pSD21Int1mRFPNHis was constructed by cloning the Int1 promoter and N-term His-tagged *mRFP1* gene into expression vector pSD21 (as described in Section 2.6.3.9). The *mRFP1* gene which was cloned into pGem-T-Easy was excised using *Kpn*I and *Sal*I and successfully cloned into pGem-T-Easy which contained the Int1 promoter and sequence for N-Term His-tag fusion (Figure 3.2.6.1, lane 4, shows the positive digest screening for the promoter). The promoter and *mRFP1* gene was successfully removed from pGem-T-Easy and cloned into pSD21 and confirmed by sequence analysis. Fluorescence was observed following electroporation into *M. smegmatis* as shown in 3.2.9.

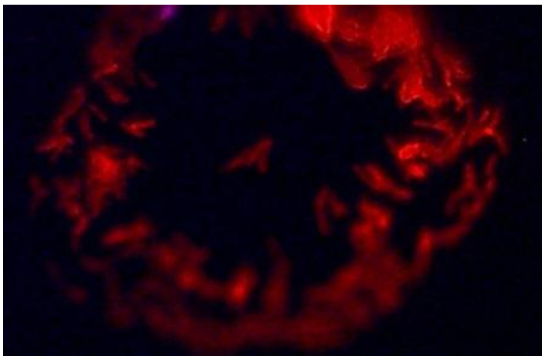


Figure 3.2.9. Fluorescence microscopy image of *M. smegmatis* cells containing construct pSD21Int1mRFPNHis. Individual cells are visible.

3.2.10 pDMN1pSmycMRFPNHis

Construct pDMN1pSmycMRFPNHis was constructed to test expression of N-term His-tagged mRFP1 driven by the pSmyc promoter using expression vector pDMN1. Construct pDMN1pSmycMRFPNHis was constructed by cloning the pSmyc promoter and N-term His-tagged *mRFP1* gene into expression vector pDMN1 (as described in Section 2.6.3.10). The *mRFP1* gene was successfully PCR amplified, cloned into pGem-T-Easy and successfully excised by RE *Hind*III and *Hpa*I. Vector pDMN1, which contains the pSmyc promoter, was digested with *Hind*III and *Hpa*I which removed the *GFP* gene as shown in Figure 3.2.10.1a. The *mRFP1* gene was cloned into pDMN1 which was confirmed by PCE colony screening as shown in Figure 3.2.10.2b. Figure 3.2.10.2 shows the fluorescence microscopy results of *M. smegmatis* cells containing construct pDMN1pSmycMRFPNHis. Constitutive and high

levels of *mRFP1* expression was observed from all cultures containing construct pDMN1pSmycRFPNHis which indicated that this construct was superior to others tested in this study.

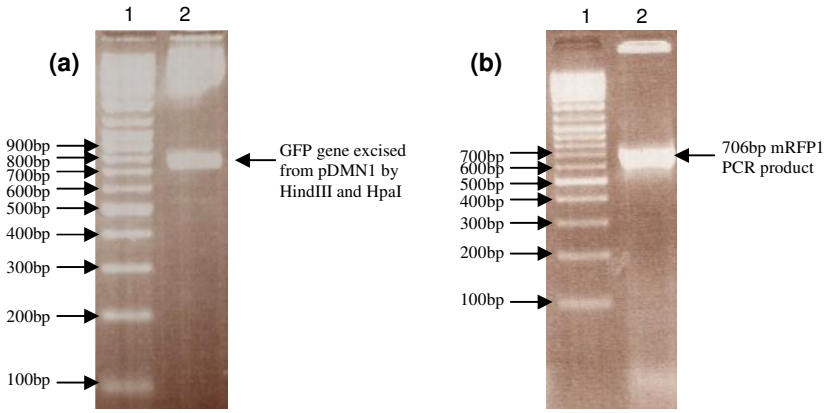


Figure 3.2.10.1. Cloning of mRFP1 into pDMN1. (a) RE digest of vector pDMN1. Lane 1: shows Generuler DNA ladder Mix, Lane 2: Digest product for pDMN1. (b) PCR colony screening for the mRFP1 gene following ligation to pDMN1. Lane 1: Generuler DNA ladder Mix, Lane 2: PCR product for *mRFP1*.

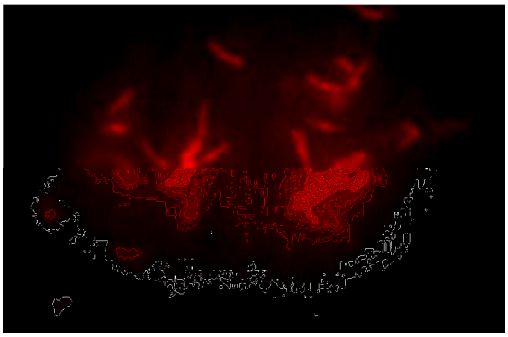


Figure 3.2.10.2. Fluorescence microscopy of *M. smegmatis* transformed with pDMN1pSmycmRFPNHis. Individual cells are detectable.

3.2.11 pJem15Hsp60LacZNHis

Construct pJem15Hsp60LacZNHis was constructed to test expression of the N-term His-tagged *LacZ* gene driven by the pSmyc promoter using promoter probe vector pJem15. Construct pJem15Hsp60LacZNHis was constructed to test expression of N-term His-tagged mRFP1 driven by the pSmyc promoter using expression vector pDMN1. Construct pJem15Hsp60LacZNHis was constructed by cloning the Hsp60 promoter and N-Term His-tagged *LacZ* gene into promoter probe vector pJem15 (as described in Section 2.6.3.11). The Hsp60 promoter was cloned into pGem-T-Easy, and subsequently subcloned into pJem15 using *Bam*HI and *Kpn*I. Figure 3.2.11a shows a PCR colony screening for the Hsp60 promoter following ligation to vector pJem15. This initial construct (pJem15Hsp60NHis, Section 2.6.3.11) required further cloning steps to remove an existing stop codon upstream of the Hsp60 promoter region in order to allow affinity-tag fusions to the *LacZ* gene (as described in Section 2.6.3.11). To achieve this goal, PCR was used to amplify the region containing the stop codon by means of a different primer set, which replaced the stop codon with a start codon. The PCR product was successfully PCR amplified (as shown in Figure 3.2.11b) and cloned into the *Eco*RV RE site of pJem15Hsp60NHis. Figure 3.2.11c shows the PCR colony screening for the insert. Construct pJem15Hsp60LacZNHis was sequence verified and electroporated into *M. smegmatis.* The cell lysate as well as his-tag purified eluate from *M. smegmatis* cells containing the construct resulted in a colorimetric reaction with X-gal substrate was added indicating the functionality of both the *LacZ* gene and affinity-tag. Figure 3.2.11d shows the visible colorimetric reaction.

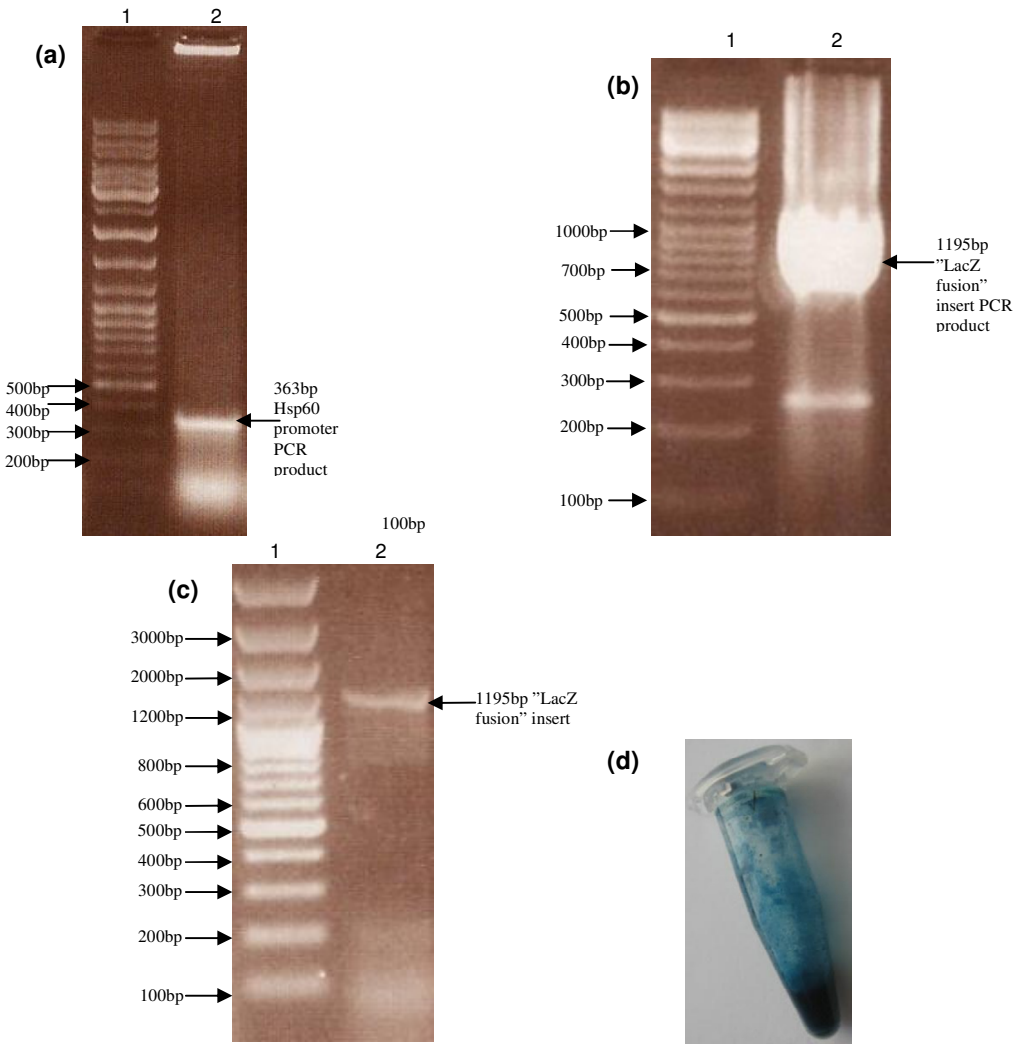


Figure 3.2.11. Construction of pJem15Hsp60LacZNHis in *M. smegmatis*. (a) PCR colony screening for the Hsp60 promoter following cloning into pJem15. Lane 1: Generuler DNA ladder Mix, Lane 2: PCR product for the promoter. **(b)** PCR amplification product for the stop codon region from pJem15Hsp60NHis. Lane 1: Generuler DNA ladder Mix, Lane 2: PCR amplification product for the region to be cloned into pJem15Hsp60NHis. **(c)** PCR colony screening for the insert following ligation to pJem15Hsp60LacZNHis. Lane 1: GeneRuler 100bp DNA ladder plus, Lane 2: PCR colony screening PCR product for the insert. **(d)** Eppendorf tube containing X-gal substrate mixed with his-tag purified eluate performed on the WCL of *M. smegmatis* cells containing pJem15Hsp60LacZNHis, 3h incubation.

Construct pJem15Int1LacZNHis was constructed to test expression of the N-term His-tagged *LacZ* gene driven by the Int1 promoter using promoter probe vector pJem15. Construct pJem15Int1LacZNHis was constructed by cloning the Int1 promoter into expression vector pJem15 (as described in Section 2.6.3.12). The Int1 promoter was PCR amplified to add *Bgl*II RE sites to the PCR product (as in Figure 3.2.12a). The PCR product was successfully cloned into pGem-T-Easy as shown by the PCR colony screening in Figure 3.2.12b. Figure 3.2.12c shows the partial digest products for pJem15 using *Bgl*II, which was ligated to the Int1 promoter. Following transformation of *M. smegmatis* with construct pJem15Int1LacZNHis, no detectable β-galactosidase activity could be observed by means of substrate addition to either the WCL or his-tag purification eluate (data not shown). It is thus possible that the partial digest performed on pJem15 excised DNA from the *LacZ* gene in turn removed functionality of the expressed enzyme.

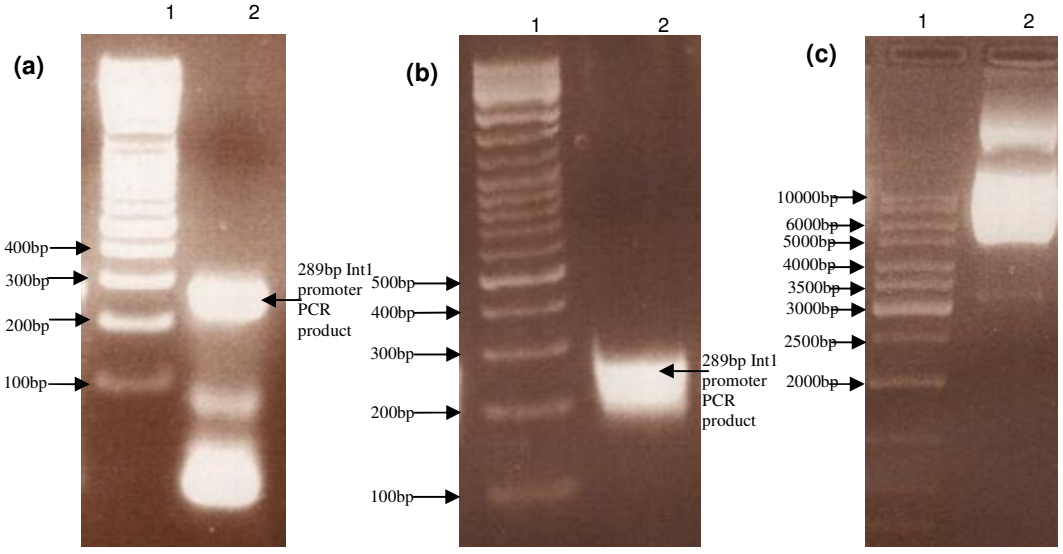


Figure 3.2.12. Cloning of pJem15Int1LacZNHis. (a) PCR amplification of the Int1 promoter adding flanking BglII RE sites to the product. Lane: Generuler DNA ladder Mix, Lane 2: PCR product for the Int1 promoter. **(b)** PCR colony screening for the Int1 promoter following ligation to pGem-T-Easy. Lane 1: Generuler DNA ladder Mix, Lane 2: PCR product for the Int1 promoter. **(c)** BglII partial digest of vector pJem15. Lane 1: Generuler DNA ladder Mix, Lane 2: Partial digest products for vector pJem15.

3.3 Reporter cosmids

3.3.1 pYub412mRFPPvuI

Construct pYub412mRFPPvuI was constructed to act as shuttle cosmid for transferral of the pSmyc promoter and mRFP1 gene to a suitable mycobacteriophage. Construct pYub412mRFP*Pvu*I was constructed by cloning the pSmyc promoter and N-term His-tagged *mRFP1* gene into the *Pvu*I RE site of pYub412 which clones the construct into the ampicillin resistance gene of pYub412. The pSmyc promoter and His-tagged *mRFP1* gene was successfully PCR amplified as in Figure 3.3.1a, cloned into pGem-T-Easy (Figure 3.3.1b shows the PCR colony screening result, however the gel image shown shows a smear, in addition to the correct band, due to a combination DNA overloading and incorrect imaging settings) and excised using *Pvu*I (as shown in Figure 3.3.1c). Cloning of the insert into *Pvu*I digested pYub412 led to no viable transformants on several attempts indicating that insertions into the *Pvu*I RE site of pYub412 disrupted expression of essential genes. The proximity of the RE site to the *E. coli* origin of replication suggests that this is possible.

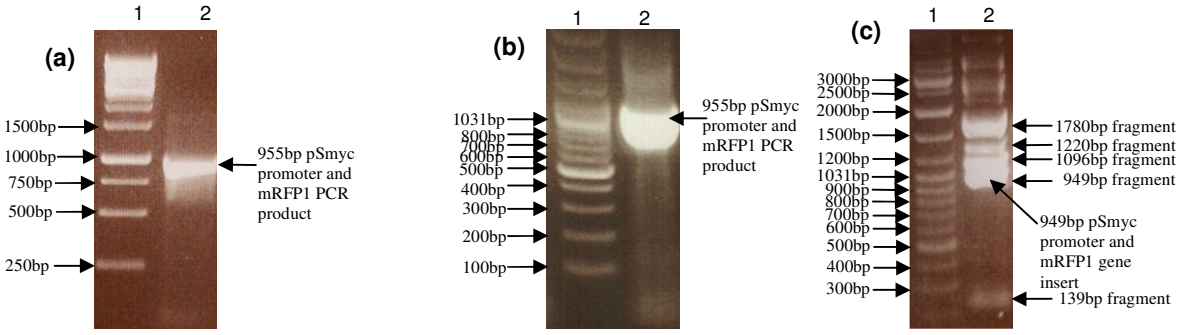


Figure 3.3.1. PCR amplification and cloning of the pSmyc promoter and mRFP1 gene into pYub412. (a) PCR amplification of the pSmyc promoter and His-tagged *mRFP1* gene insert from pSmycmRFPNHispDMN1. Lane 1 GeneRuler 1kb DNA ladder, Lane 2: PCR product for the promoter and reporter gene. **(b)** PCR colony screening for the pSmyc promoter and *mRFP1* gene insert following ligation to pGem-T-Easy. Lane 1: GeneRuler DNA ladder mix, Lane 2: PCR colony screening PCR product for the promoter and reporter gene. **(c)** *Pvu*I RE excision of the pSmyc promoter and *mRFP1* gene insert from pGem-T-Easy. Lane 1: GeneRuler DNA ladder mix, Lane 2: Digest products for pGemInt1mRFPNHis using *Pvu*I. Several bands (1780bp, 1220bp, 1096bp, 949bp and 139bp) can be seen due to either partial digest of the construct or due to the presence of multiple cutting sites in the construct, the 955bp band corresponds to the pSmyc promoter and mRFP1 gene insert as indicated.

3.3.2 Cloning of pYub412mRFPSapI

Construct pYub412mRFPSapI was constructed to act as shuttle cosmid for transferral of the pSmyc promoter and mRFP1 gene to a suitable mycobacteriophage. Construct pYub412mRFPSapI was constructed by cloning the pSmyc promoter and N-term His-tagged *mRFP1* gene into the *Sap*I RE site of pYub412 which places the reporter construct into the ampicillin gene (as described in Section 2.6.4.2). The pSmyc promoter and *mRFP1* gene was successfully PCR amplified (as shown in Figure 3.3.2a). The PCR product was cloned into pGem-T-Easy and excised using RE *Sap*I (as shown in Figure 3.3.2c). The promoter and reporter gene insert was cloned into the *Sap*I RE digested vector pYub412 (shown in Figure 3.3.2b). Electroporation of the pYub412mRFPSapI construct did not yield any viable transformants, which suggests that the insertion of the reporter construct disrupted essential genes.

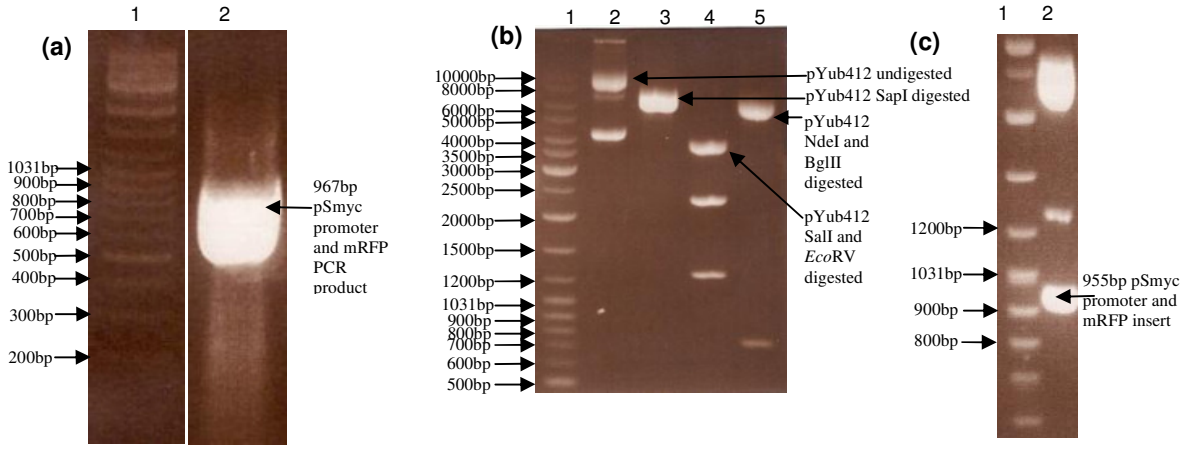


Figure 3.3.2. Cloning of the pSymc promoter and *mRFP1* gene into the *Sap*I RE site of pYub412.

(a) PCR amplification of the pSmyc promoter and mRFP1 gene adding *Sap*I RE sites to the product. Lane 1 GeneRuler DNA ladder Mix, Lane 2: PCR product for the pSmyc promoter and *mRFP1* gene. (Please note that an irrelevant section of this gel image was removed for clarity). **(b)** RE digest of pYub412. Lane 1: GeneRuler DNA ladder mix, Lane2: pYub412 undigested, Lane 3: pYub412 digested with *Sap*I, Lane 4: pYub412 digested with *Sal*I and *Eco*RV, Lane 5: pYub412 digested with *Nde*I and *Bgl*II **(c)**. *Sap*I RE excision of the pSmyc promoter and *mRFP1* gene from pGem-T-Easy. Lane 1: GeneRuler DNA ladder mix, Lane 2: *Sap*I excision of the promoter and reporter gene from pGem-T-Easy. The band above the 955bp band represents the digest product spanning the natural SapI RE site in pGem-T-Easy to the SapI RE site in the insert which is visible due to incomplete digest and represents a band of about 1400bp.

3.3.3 pYub412mRFPNdeIBglII

Construct pYub412.2mRFPNdeIBglII was constructed by cloning the pSmyc promoter and N-term His-tagged *mRFP1* gene into the *Nde*I and BglII RE sites of pYub412 (as described in Section 2.6.4.3). This removes a portion of the integrase gene and cloned the reporter construct between the hygromycin and lambda cos genes. The pSmyc promoter and *mRFP1* gene was successfully PCR amplified (as shown in Figure 3.3.3a) and cloned into pGem-T-Easy (Figure 3.3.3b shows the PCR colony screening result for the pSmyc promoter and *mRFP1* gene insert). The insert was subsequently excised with *Hind*III and *Hpa*I (as shown in Figure 3.3.3c) and cloned into vector pDMN1. The promoter and reporter gene insert was PCR amplified to add *Nde*I and BglII RE sites (as shown in Figure 3.3.3d) and cloned into pGem-T-Easy. Thereafter the promoter and reporter gene was cloned into the *Nde*I and *Bgl*II digested vector pYub412 (as shown in 3.2.2d and Figure 3.3.3e), however, no viable colonies were observed following electroporation into *E. coli,* indicating that essential genes were disrupted by means of this cloning method.

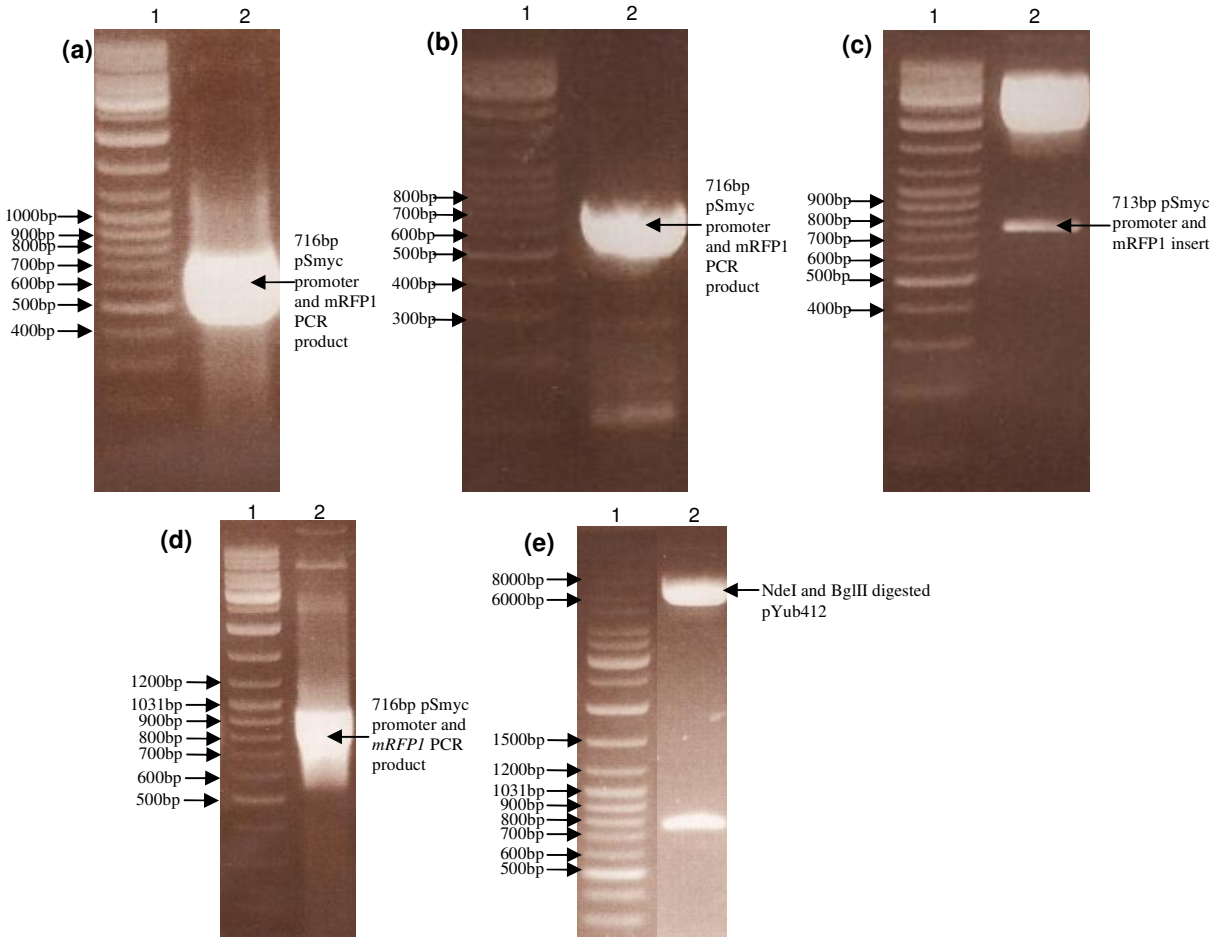


Figure 3.3.3. Construction of pYub412mRFPNdeIBglII. (a) PCR amplification of the pSmyc promoter and *mRFP1* gene adding *Hind*III and *Hpa*I RE sites to the product. Lane 1: GeneRuler DNA ladder mix, Lane 2: PCR product for the promoter and reporter gene. **(b)** PCR colony screening for the pSmyc promoter and *mRFP1* gene following ligation into pGem-T-Easy. Lane 1 GeneRuler DNA ladder mix, Lane 2: PCR colony screening product for the promoter and reporter gene. **(c)** *Hind*III and *Hpa*I RE digest excising the pSmyc promoter and *mRFP1* insert from pGem-T-Easy. Lane 1: GeneRuler DNA ladder mix, Lane 2: Digest product for the promoter and reporter gene. **(d)** PCR amplification of the pSmyc promoter and *mRFP1* gene following ligation to vector pDMN1 using primers to add *Nde*I and BglII RE sites. Lane 1 GeneRuler DNA ladder mix, Lane 2: PCR product for the promoter and reporter gene. **(e)** *Nde*I and BglII digest of vector pYub412. Lane 1: GeneRuler DNA ladder mix, Lane 2: Digest product for the pYub412. (Please note that an irrelevant section of this gel image was removed for clarity).

3.3.4 pDMNLCosmRFP

Construct pDMNLCosmRFP was constructed by cloning a lambda cos site from pYub412 into the *Xba*I and *Mlu*I RE sites of pDMN1 (as described in Section 2.6.4.4). The cloning method excised the mycobacterial origin of replication from pDMN1 and replaced it with a lambda cos site. This method created a novel reporter cosmid for cloning into mycobacteriophage. The lambda cos site from pYub412 was PCR amplified and cloned into pGem-T-Easy, excised by *Xba*I and *Mlu*I (as shown in Figure 3.3.4) and cloned into pDMN1pSmycmRFPNHis (as described in Section 2.6.3.10). The construct was sequence verified by using primers specific for the inserted cos site which revealed that the cos site was successfully cloned into the reporter construct. Lambda packaging of the cosmid ligated to mycobacteriophage DNA failed to produce viable transformants. The reason for the packaging failure was initially unclear; however excision of the luciferase containing cosmid from phAE142 (as shown in Figure 3.4.3.3a, Section 3.4.3) revealed that cosmid pDMNLCosmRFP is considerably larger than the luciferase containing cosmid. This suggests that the failure of pDMNLCosmRFP to be packaged together with mycobacteriophage DNA is due to size constraints of bacteriophage lambda and that a smaller reporter cosmid would be needed to successfully package. This was later confirmed by the successful packaging of a significantly smaller reporter cosmid (pYubSSmRFP1, section 3.3.6). Further investigation was halted due to success using other cosmid constructs.

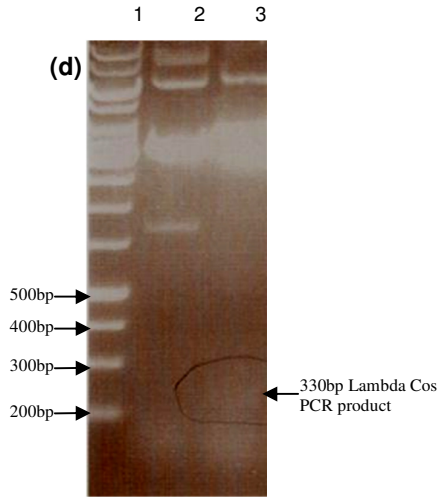


Figure 3.3.4. *Xba*I and *Mlu*I digest of the Lambda cos insert following ligation to pGem-T-Easy.

Lane 1: GeneRuler DNA ladder mix, Lane 3: Lambda cos site PCR product.

3.3.5 pYubmRFP

Construct pYubmRFP was constructed by cloning the pSmyc promoter and N-term His-tagged *mRFP1* gene into the *Sal*I and *Eco*RV RE sites of pYub412 which removes the hygromycin gene and integrase genes from pYub412 (as described in Section 2.6.4.5). The pSmyc promoter and *mRFP1* gene was successfully PCR amplified (as in Figure 3.3.5a) and cloned into pGem-T-Easy to add *Sal*I and *Eco*RV RE sites to the product. The insert was excised from pGem-T-Easy (as shown in Figure 3.3.5b) and cloned into *Sal*I and *Eco*RV digested pYub412 (as shown in Figure 3.2.2c, Section 3.3.2) which was verified by PCR colony screening (as shown in Figure 3.3.5c). The construct was sequence verified and used for cloning into mycobacteriophage phAE142.

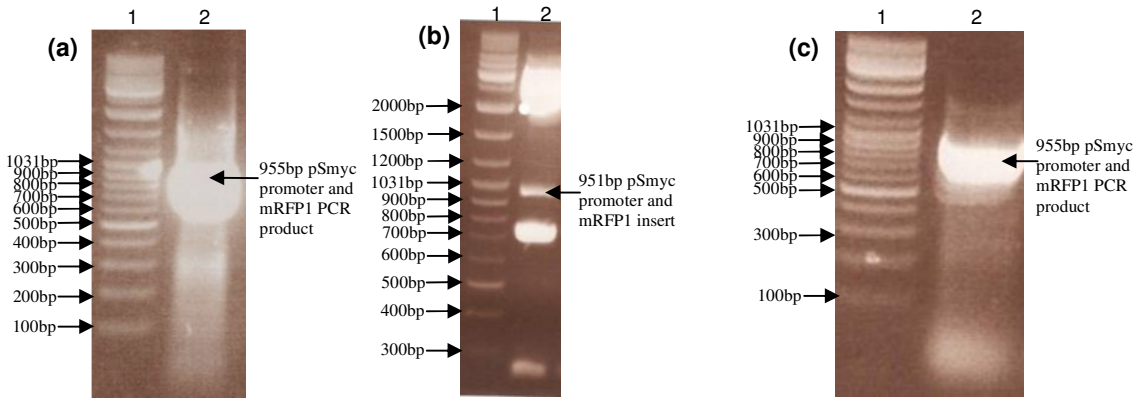


Figure 3.3.5. Cloning of the pSmyc promoter and *mRFP1* gene into pYub412. (a) PCR amplification of the pSmyc promoter and *mRFP1* gene from pDMN1 to add *Sal*I and *Eco*RV RE sites. Lane 1: GeneRuler DNA ladder mix, Lane2: PCR product for the promoter and reporter gene. **(b)** *Eco*RV and *Sal*I RE excision of the pSmyc promoter and *mRFP1* gene from pGem-T-Easy. Lane 1 GeneRuler DNA ladder mix, Lane 2: Promoter and reporter gene digest product. **(c)** PCR colony screening for the pSmyc promoter and *mRFP1* gene following ligation to pYub412. Lane 1: GeneRuler DNA ladder mix, Lane 2: PCR colony screening product for the promoter and reporter gene.

3.3.6 pYubSSmrfp

Construct pYubSSmrfp consisted performing a size reduction on construct pYubmRFP (Section 3.3.5).The pYubmRFP construct was successfully reduced in size by removing non-essential DNA by digesting with *Sap*I and *Mlu*I. The cosmid was treated with klenow enzyme (since the two RE sites used were not compatible), ligated and electroporated. A cosmid DNA extraction was performed on cultures of transformants. The new cosmid, designated pYubSSmrfp was 2032bp smaller than pYubmRFP and was used for cloning into mycobacteriophage phAE142. pYub412mRFP and pYub412SSmRFP was run on a gel together which confirmed the size reduction (as shown in Figure 3.3.6).

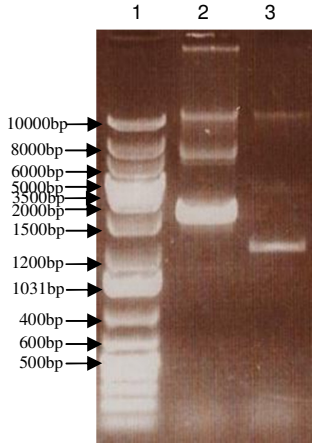


Figure 3.3.6. Gel electrophoresis of pYubmRFP and pYubSSmRFP. Lane 1: GeneRuler DNA ladder mix, Lane 2: pYubmRFP, Lane 3: pYubSSmRFP.

3.4 Construction of reporter mycobacteriophages

3.4.1 Selection of suitable mycobacteriophages for cloning

Twenty-one novel South African mycobacteriophages were evaluated for their ability to infect *M. tb*, as a surrogate for their usefulness in a TB diagnostic. Their capability to infect *M. tb* was tested, in three separate experiments, by means of a spot test followed by incubation of up to 2 weeks. It was, however, found that their host ranges did not include *M. tb* as suggested by the lack of clearing or plaque formation following the spot test. This was furthermore apparent, since the 3rd spot test made use of a high titer of each purified mycobacteriophage (obtained by means of CsCl ultracentrifugation) and included the addition of $CaCl_2$, a co-factor that influences the ability of certain mycobacteriophages to infect their hosts. Since mycobacteriophage D29 and phAE142 were available to us and were previously demonstrated to infect *M. tb,* they were selected for use in this study to create reporter mycobacteriophages.

3.4.2 Construction of D29 reporter mycobacteriophage

Mycobacteriophage D29 was obtained as wild type and required a size reduction in order to accommodate the additional DNA from a reporter cosmid to create a reporter mycobacteriophage. Several steps were taken to produce a size reduced mycobacteriophage D29. Mycobacteriophage D29 was propagated and its DNA was extracted (Section 2.4). The main cloning strategy for D29 (as discussed in Section 2.6.5.2) requires concatemerization of D29 genomic DNA followed by partial digestion to remove non-essential DNA from the D29 genome. This method was used to allow sufficient space to accommodate a reporter cosmid whilst maintaining a suitable size for lambda packaging. The D29 DNA was successfully self-ligated to form concatamers (as shown in Figure 3.4.2.1a). Phenol extraction and purification of the concatamerized DNA from agarose gels, followed by subsequent gel electrophoresis showed that both concatemers and linear genomic DNA was visible. Repeated isolation and purification (of up to three repeats) of the concatamerized DNA band consistently led to both linear and concatamers being visible following gel electrophoresis. This indicated that the purification step possibly shears some of the concatamerized DNA, or that a large amount of linear DNA fails to separate from the concatamerized DNA despite extensive a FIGE run time. The purified concatameric DNA was partially digested (as shown in Figure 3.4.2.1b) and DNA of

between 40 and 70Kb was purified from the gel. The purified DNA was ligated to *Bcl*I digested pYubmRFP DNA and lambda packaged into *E. coli.* This led to do production of a vast amount of PCR positive colonies when screening for the pYubmRFP cosmid (as showin in Figure 3.4.2.1c), however subsequent cosmid DNA extraction and electroporation into *M. smegmatis* led to no visible plaques. This indicated that the DNA which was packaged did not contain the essential genes required by D29 for infectivity and replication.

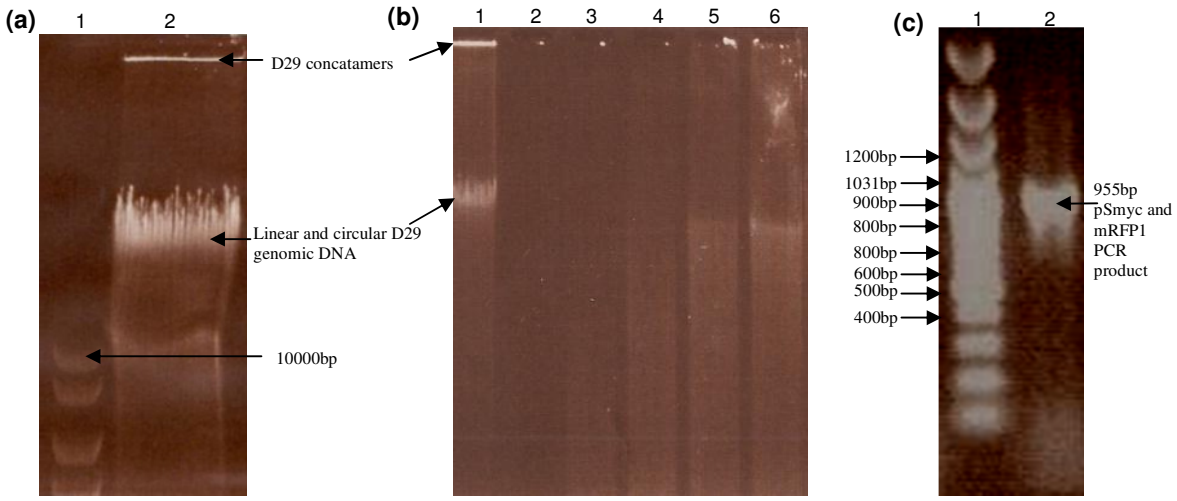


Figure 3.4.2.1. D29 self-ligation and partial digest. (a) FIDGE of self-ligated D29 genomic DNA displaying both concatamerized and linear DNA. Lane 1: GeneRuler DNA ladder mix, Lane 2: D29 DNA. **(b)** Partial digest of concatemerized D29 DNA with *Sau*3AI. Lane 1: Undigested D29 DNA, Lane 2-6: digested D29 DNA, 3µg of DNA per well, digested for 20 min with a serial dilution of RE *Sau*3AI from lane 2 to lane 6. **(c)** PCR colony screening for the pYubmRFP cosmid following ligation to partially digested D29 DNA and lambda packaging. Lane 1: GeneRuler DNA ladder mix, Lane 2: PCR colony screening product for the promoter and reporter gene from pYubmRFP.

3.4.3 Construction of Tm4 reporter mycobacteriophage

Mycobacteriophage Tm4 was available to us in the form of reporter mycobacteriophage phAE142 which contains a luciferase containing cosmid which was to be replaced with a cosmid from this study. Three cloning strategies were followed to create a Tm4-based reporter mycobacteriophage (as described in Section 2.6.5.3). In the first method, phAE142 was initially electroporated into *M. smegmatis* followed by plaque purification to obtain suitable quantities of phAE142 DNA for use. Electroporation of phAE142 DNA successfully led to plaque formation and enabled CsCl purification and DNA extraction. However, no RE map of the existing luciferase containing cosmid in phAE142 was available. A large number of RE digests were performed in order to excise the luciferase containing cosmid from phAE142, including REs that the creators of phAE142 commonly utilized in related publications (such as *Xba*I, *Not*I, *Nhe*I and *Pac*I). RE digest with these enzymes did not led to additional detectable DNA bands despite several attempts with varying concentrations as well as incubation times. Figure 3.4.3.1a shows *Pac*I RE digest products for phAE142 and Figure 3.4.3.1b shows a *Not*I RE digest of phAE142. These two gel images show no detectable difference between the digested and undigested DNA, independent of the concentration of genomic DNA used (which was tested using concentrations ranging from 0.5µg to 15µg). These results indicate that these cutting sites did not exist in the purified phAE142 DNA obtained as per the first method. However, following numerous DNA extractions from phAE142 and RE digests of the DNA, it was eventually observed that a feint band, possibly corresponding to the luciferase cosmid, was observed when RE *Pac*I and *Not*I was used (as shown in Figure 3.4.3.1c). The bands for both the *Pac*I and *Not*I digested phAE142 DNA were purified, ligated to pYubmRFP and lambda packaged. Subsequent PCR colony screening showed several colonies to be positive as in Figure 3.4.3.2a, however none of the colonies possessed DNA capable of producing plaques when electroporated into *M. smegmatis.* This reason for this was initially unclear. It was possible that the failure to package was due to size constrains during lambda packaging. It was also possible that the failure to package resulted from a failure of the DNA to ligate, possibly resulting from mutations or deletions in the phAE142 DNA. This was suspected to be the case since it has been reported that propagation of phAE142 requires a special strain of *M. smegmatis* in order to avoid mutations (Bardarov,S.,Jr 2003}. It could thus be that only a small subset of the phAE142 DNA did not undergo mutations or have deletions, which would correspond to the

poor digest efficiency and the feint band observed. The amount of successfully digested DNA would thus not be sufficient to outcompete the amount of pYubmRFP concatamer packaging leading to copious amounts of PCR positive colonies (using primers for the cosmid) containing no mycobacteriophage DNA. Despite screening more than 1000 colonies by means of PCR, the pooled PCR positive DNA did not result in any detectable plaque formation. Use of the first method thus proved inefficient and a second method was utilized in order to obtain a reporter mycobacteriophage.

In the second method, which was designed to override the size constraints of lambda packaging (as described in Section 2.6.5.3), direct electroporation of the ligation product of pYubmRFP and mycobacteriophage DNA was performed using large quantities of DNA (2µg). Plaques were subsequently observed and PCR plaque screening showed several positive plaques as in Figure 3.4.3.2b. However, subsequent plaque purification, DNA extraction and gel electrophoresis revealed that the ligation failed and that the PCR positive plaques were due to the excessive amount of DNA on the plate causing false positive PCR results. The failure of the second method to create a Tm4-based reporter mycoacteriophage suggested that the reason was either due to size constraints of the Tm4 mycobacteriophage head capsid or due to mutations or deletions in the phAE142 DNA which removes the required RE cutting sites.

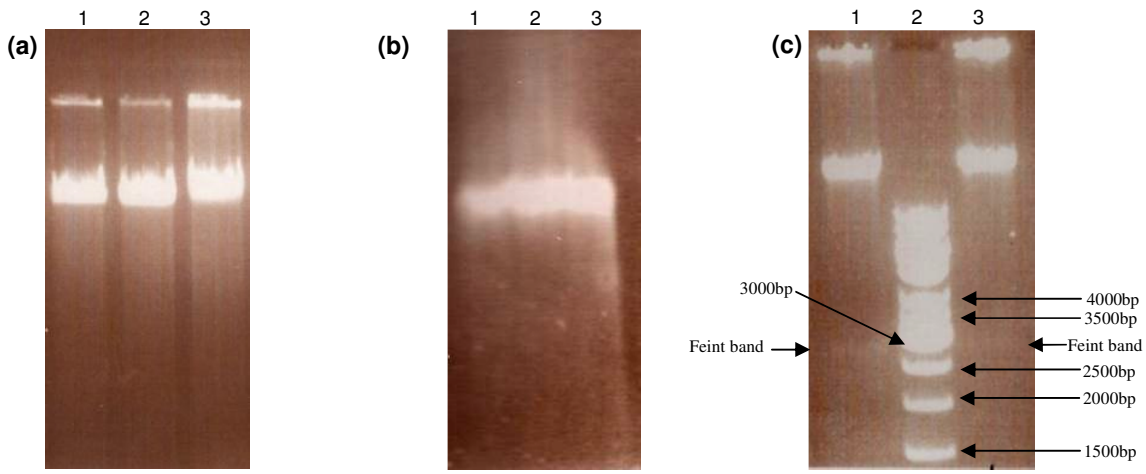


Figure 3.4.3.1. Mycobacteriophage phAE142 digests. (a): phAE142 DNA comparison of digested and undigested DNA. Lane 1: *Pac*I digested phAE142 DNA, Lane 2: Undigested DNA, Lane 3: self-ligated phAE142 DNA. **(b)** phAE142 digested to remove existing luciferase cosmid with *Not*I. Lane 1-3: Digested phAE142 DNA indicating no visible excised cosmid. **(c)** phAE142 digest with *Pac*I and *Not*I. Lane 1: phAE142 digested with *Pac*I, a feint band is visible corresponding to between 2500bp and 3500bp, Lane 2: GeneRuler DNA ladder Mix, Lane 3: phAE142 *Not*I digest, a feint band, similar to that in lane 1, is visible.

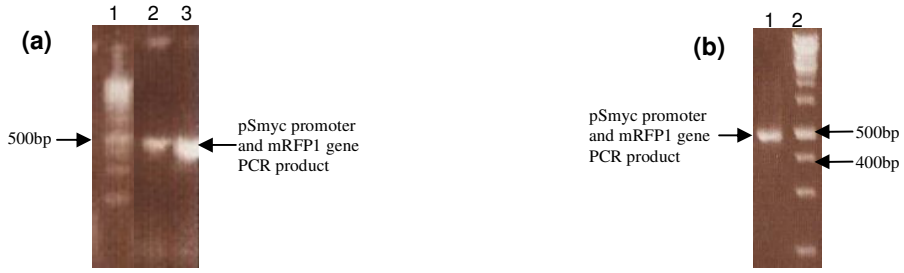


Figure 3.4.3.2. PCR colony and plaque screening for the pYubmRFP cosmid following method 1 and 2. (a) PCR plaque screening for the pYubmRFP cosmid using primers for the pSmyc promoter and *mRFP1* gene insert following ligation to phAE142 DNA and lambda packaging from method 1. Lane 1: Generuler DNA ladder mix, Lane 2-3: PCR plaque screening product for the promoter and reporter gene. **(b)** PCR plaque screening for pYubmRFP following ligation to phAE142 and electroporation into *M. smegmatis* for method 2.

The third cloning method to create a Tm4-based reporter mycobacteriophage was designed to circumvent the possibility of selective pressure and mutation of the necessary RE sites (as described in Section 2.6.5.3). Mycobacteriophage phAE142 DNA was self-ligated to create concatamers and was successfully lambda packaged into *E. coli.* Cosmid DNA was extracted from transformants and showed the correct size matching the genome size of phAE142 when run on a gel. Additionally the purified DNA led to plaque formation when electroporated into *M. smegmatis.* By propagating the mycobacteriophage DNA in *E. coli* and working with the DNA as a circular phasmid, instead of as a mycobacteriophage this circumvented the possible selective pressures leading to mutations or deletions when propagated in mycobacteria. The purified phAE142 DNA was digested with *Pac*I which successfully removed the existing luciferase cosmid from phAE142 to release the Tm4 genome (as show in in Figure 3.4.3.3a). The *Pac*I digested Tm4 DNA was successfully recovered from the gel, purified, ligated to pYubmRFP and lambda packaged. Several colonies were PCR positive for the reporter cosmid (as shown in Figure 3.4.3.3b), however electroporation of the purified DNA into *M. smegmatis* did not led to plaque formation. A possible reason for the failure to package the pYubmRFP and phAE142 ligation could be due to size constrains of lambda packaging. This was apparent since the cosmid which was excised from phAE142 by *Pac*I RE digest (as in shown in Figure 3.4.3.3a) is considerably smaller than pYubmRFP. For this reason pYubSSmRFP was created which was further size reduced by 2031bp compared to pYubmRFP. Subsequent ligation of *Pac*I digested pYubSSmRFP to phAE142 (named Tm4::pYubSSmRFP) followed by lambda packaging led to a substantial increase in PCR positive colonies as in Figure 3.4.3.3c. A phasmid DNA extraction was performed on cultures of the PCR positive colonies which led to the successful formation of plaques when electroporated into *M. smegmatis*. Subsequent plaque purification yielded a high titer stock of the novel reporter mycobacteriophage Tm4::pYubSSmRFP.

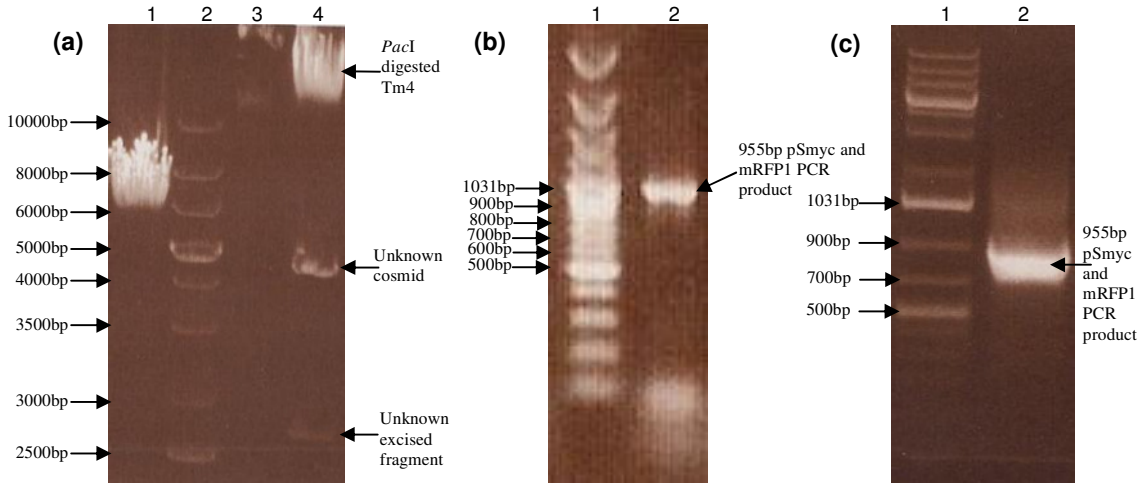


Figure 3.4.3.3a. Cloning of Tm4::pYubSSmRFP. (a) *Pac*I digest of mycobacteriophage phAE142. Lane 1: pYub412 DNA (high concentration), Lane 2: GeneRuler DNA ladder mix, Lane 3: Undigested self-ligated phAE142 DNA, Lane 4: *Pac*I digest of self-ligated phAE142 DNA, the luciferase containing cosmid can be seen excised from the genomic DNA. **(b)** PCR colony screening for the pYubmRFP cosmid following ligation to *Pac*I digested phAE142 DNA and lambda packaging. Lane 1: GeneRuler DNA ladder mix, Lane 2: PCR colony screening product for the promoter and reporter gene. **(c)** PCR colony screening for the pYubSSmRFP cosmid following ligation to *Pac*I digested phAE142 DNA and lambda packaging. Lane 1: GeneRuler DNA ladder mix. Lane 2: PCR colony screening product for the promoter and reporter gene.

3.5. Evaluation of reporter mycobacteriophage Tm4::pYubSSmRFP

The preliminary evaluation Tm4::pYubSSmRFP made use of a 96-well format fluorescence plate reader as well as fluorescence microscopy to test three aspects of the reporter mycobacteriophage important for its function and suitability as a reporter mycobacteriophage. These aspects were (1) variation in *mRFP1* expression between isolates, (2) the optimal titer, and (3) change in fluorescence levels as a function of time. In order to test for variation in expression between isolates of Tm4::pYubSSmRFP, 20 isolates were purified and investigated for fluorescence expression variation. Fluorescence measurements performed on *M. smegmatis* cells infected with Tm4::pYubSSmRFP revealed that there was some level of variation as shown in Figure 3.5.1. It should be noted that these results represent a single experiment and results need to be interpreted with caution. However, each sample had fluorescence levels considerably higher than the negative control consisting of uninfected *M. smegmatis* cells allowed to grow for a period equal to the infection and incubation time.

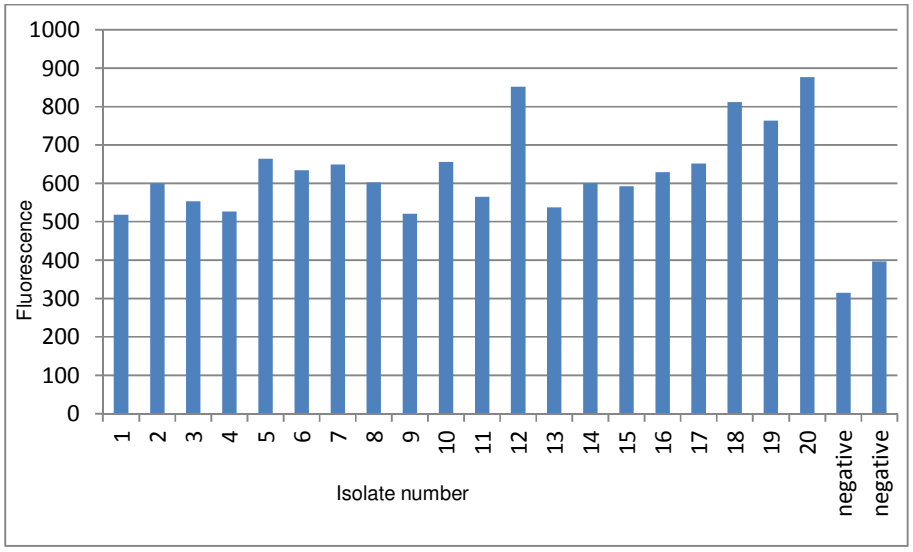


Figure 3.5.1. Relative fluorescence as detected by fluorescence plate reader for 20 isolates of reporter mycobacteriophage Tm4::pYubSSmRFP following infection of *M. smegmatis* cells compared to uninfected *M. smegmatis* allowed *a* replicating time equal to the infection and incubation time for the reporter mycobacteriophage. The X-axis shows the Isolate name and the Y-axis shows the fluorescence level. Mycobacteriophage isolates 12 and 18 to 20 show the highest levels of fluorescence.

In order to determine if the level of variation observed between the 20 isolates was due to mutations in the pSmyc promoter or *mRFP1* gene, the promoter and reporter gene was PCR amplified and sequenced. However, sequencing results revealed that there were no mutations or deletions in the amplified and sequenced regions. *M. smegmatis,* when cultured in the absence of Tween-80, has a high tendency to clump, which in turn makes accurate pipetting difficult. It is possible that the observed variations in the fluorescence levels for the 20 isolates could be to inadvertently using varying amounts of *M. smegmatis* due to pipetting inaccuracy. In order to investigate this, varying amounts of *M. smegmatis* was infected with a set amount of Tm4::pYubSSmRFP and the fluorescence levels were observed as shown in Figure 3.5.2 (note that this represents a single experiment and these results should be considered with caution). The result from Figure 3.5.2 appear to indicate a trend between fluorescence levels and the amount of *M. smegmatis* used for the infected cells, which suggests that inaccurate pipetting is likely to be the reason for the variations observed between the 20 isolates as in Figure 3.5.1. Figure 3.5.2 also indicates that, although *M. smegmatis* has some background fluorescence levels, the fluorescence levels do not substantially increase as the amount of cells increase.

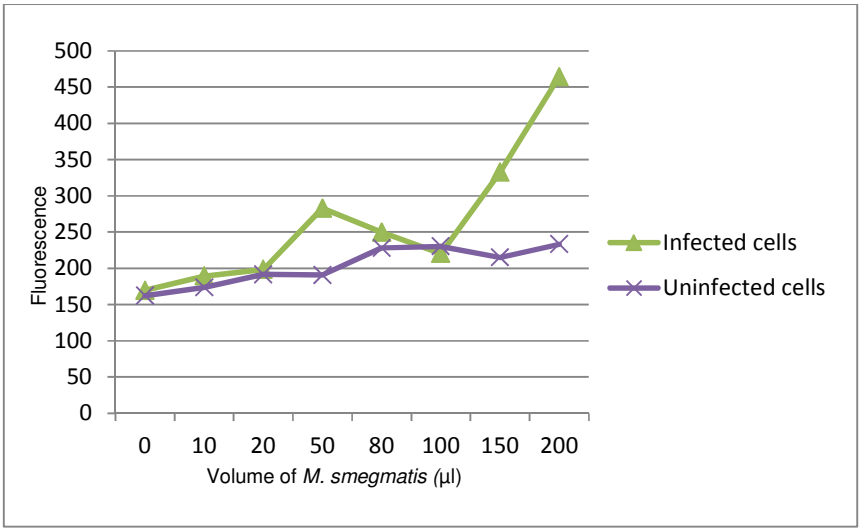


Figure 3.5.2. Fluorescence levels resulting from increasing amounts of *M. smegmatis* infected with a constant amount of Tm4::pYubSSmRFP. The X-axis indicates the amount of microliters of competent *M. smegmatis* which is infected by 20 µl of a 10^7 pfu/ml sample of Tm4::pYubSSmRFP. The Y-axis indicates the fluorescence level.

The second aspect that was investigated was the optimal titer to produce maximum fluorescence. To this goal, a large titer range was tested. Two mycobacteriophage isolates (12 and 20, see Figure 3.5.1) were used to create a dilution range from 10^9 pfu/ml to 1 pfu/ml. 100 µl of each dilution was used to infect 200 µl of competent *M. smegmatis* cells with overnight incubation and the resultant fluorescence. The resultant fluorescence is shown in Figure 3.5.3. These results indicate that the highest concentration, corresponding to 10^9 pfu/ml, correspond the highest fluorescence levels observed.

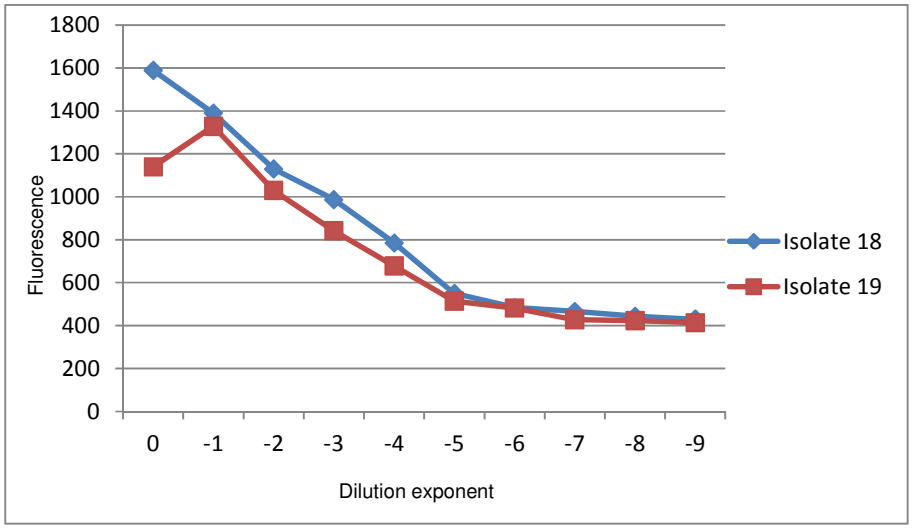


Figure 3.5.3. *M. smegmatis* fluorescence levels following 24h incubation with a titer range of mycobacteriophage Tm4::pYubSSmRP (Isolate 18 and 19). The X-axis indicates the dilution exponent used of a 10^9 pfu/ml stock of the two isolates. The Y axis shows the fluorescence level corresponding to the dilution used (where 0 indicates undiluted or 10^9 pfu/ml and -9 indicates 1pfu/ml. Note that these results indicate data from a single experiment and needs to be considered with caution.

The third aspect that was investigated was the optimal incubation time for maximal fluorescence. This was determined using four mycobacteriophages (12, 18, 19. and 20). A total of 70 µl for each 10^9 pfu/ml stock was used to infect 200 µl of *M. smegmatis* cells for 3, 5, 8, 9 and 46h at 37°C, after which fluorescence readings were taken using a fluorescence plate reader. Figure 3.5.4 shows the results for this time-frame, which show detectable fluorescence levels as early as 3h and with a peak of more

than 46h. It was also possible to detect fluorescence from individual cells as early as 5h using fluorescence microscopy as shown in Figure 3.5.5

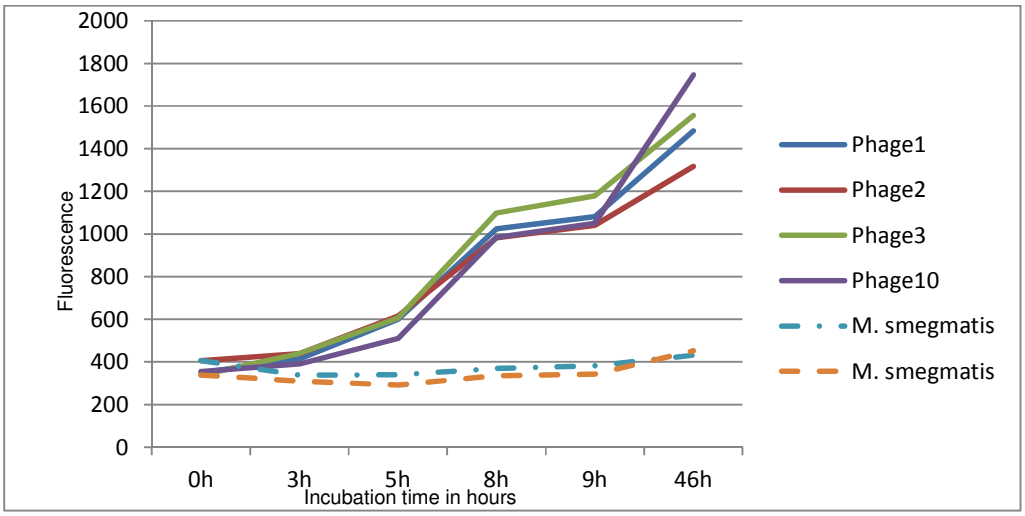


Figure 3.5.4. Fluorescence levels over time following infection of *M. smegmatis* by mycobacteriophage Tm4:pYubSSmRFP. Mycobacteriophage 1, 2, 3 and 10 was used to infect 300 µl of *M. smegmatis* and incubated for 0h, 3h, 5h, 8h, 9h and 46h followed by fluorescence measurement as described in Section 2.5.3, the dotted lines indicate uninfected *M. smegmatis* as negative controls. Note that these results were generated from a single experiment and need to be interpreted with caution.

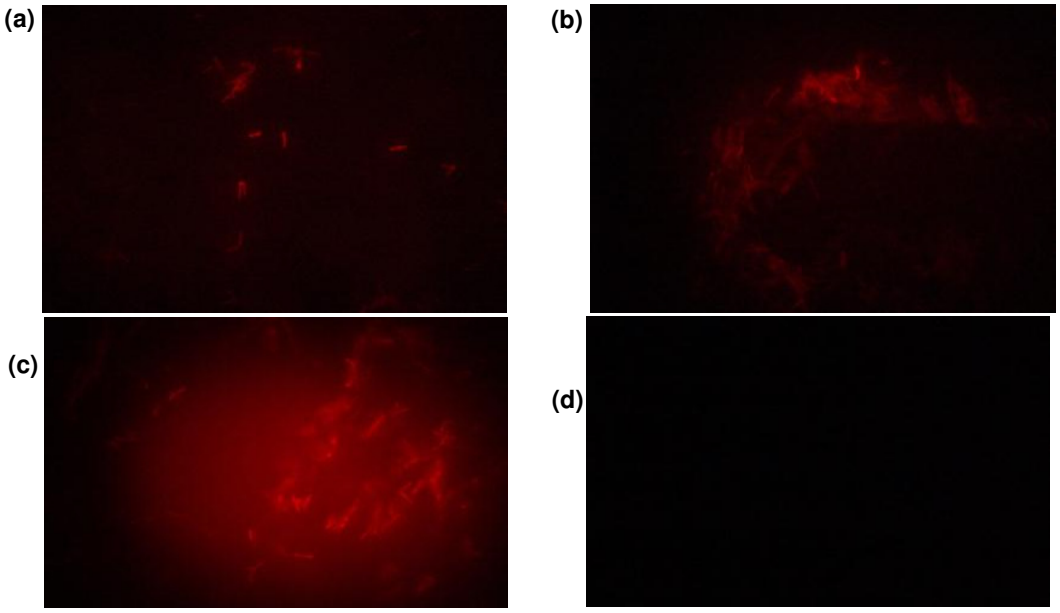


Figure 3.5.5 Fluorescence microscopy of *M. smegmatis* cells following 5h infection and incubation with Tm4::pYubSSmRFP. (a), (b) and (c) *M. smegmatis* cells infected with three different isolates of Tm4::pYubSSmRFP, individual cells are visible on each slide. **(d)** Uninfected *M. smegmatis* cells.

Using the optimal parameters from Figures 3.5.3 and 3.5.4, a 24h infection and incubation time together with a titer of 10^9 pfu/ml was tested on *M. smegmatis* using four mycobacteriophages together with uninfected *M. smegmatis* as negative control. Figure 3.5.6 shows the resulting fluorescence measurements, which indicate that the fluorescence levels resulting from the infected cells were clearly discernible from the uninfected cells. It also shows that 24h of *M. smegmatis* proliferation does not result in a very large increase in background fluorescence. However, it should be kept in mind that the *M. smegmatis* cells in the infected cells would not be proliferating to such an extent as the uninfected cells due to cellular lysis due to mycobacteriophage infection and replication. It can also be seen from Figure 3.5.6 that the reporter phage infected samples have up to 3 to 8 times the expression levels of the negative samples, suggesting that Tm4::pYubSSmRFP may perform favourably for use as a diagnostic.

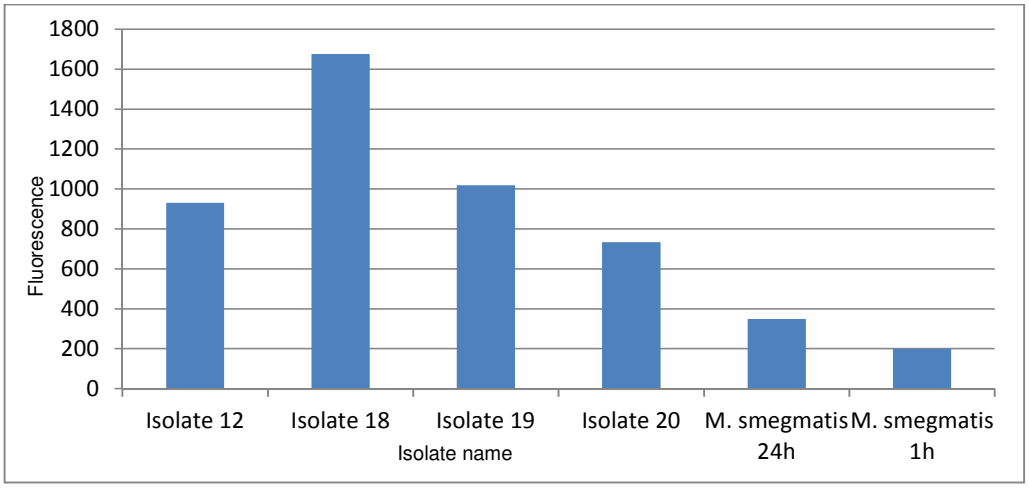


Figure 3.5.6. Relative fluorescence following infection of *M. smegmatis* with mycobacteriophage Tm4::pYubSSmRFP (Isolates 12, 18, 19 and 20). *M. smegmatis* cultures of 10ml each were used for infection followed by 24h incubation along with 2 negative controls consisting of uninfected *M. smegmatis* cultured for 24h. An amount of 300 µl of each sample was loaded onto a fluorescence plate reader and used to quantify fluorescence (as described in Section 2.5.3). Isolate 18 showed about 5 times the background expression levels of *M. smegmatis* cultured for 24h and more than 8 times the expression levels of an *M. smegmatis* culture of 1h. Note that this data represents a single experiment and needs to be interpreted with caution.

The ability to detect *M. smegmatis* cells, following infection with mycobacteriophage Tm4::pYubSSmRFP, by means of affinity chromatography and fluorescence measurement was also tested using several isolates (as shown in Figure 3.5.7). Figure 3.5.7 indicated that the fluorescence levels of His-tag purified eluates of *M. smegmatis* cultures infected with Tm4:pYubSSmRFP was up to 7 times higher than that from uninfected *M. smegmatis.* This shows that the new diagnostic avenue by means of chromatography and fluorescence detection shows promise and generates results which are clearly discernible from negative controls.

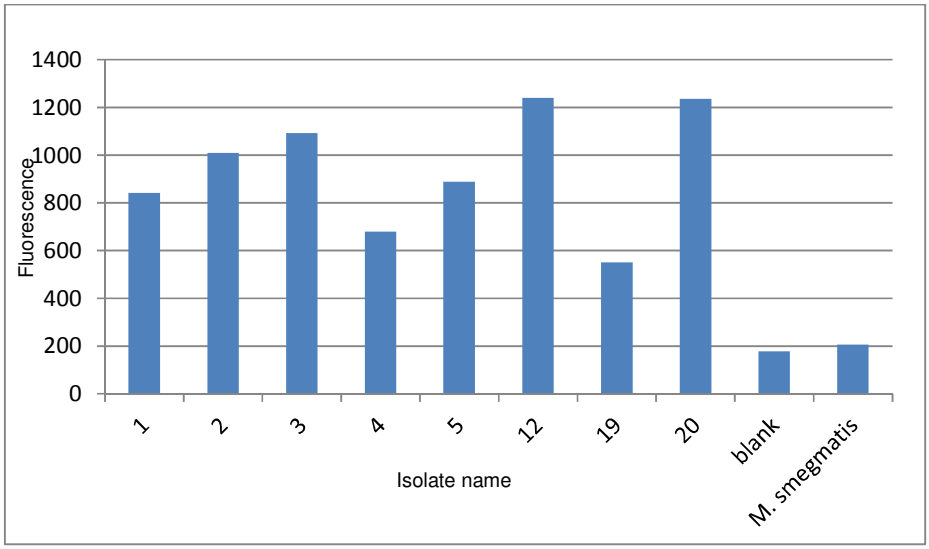


Figure 3.5.7. Fluorescence levels from His-tag purified eluates performed on *M. smegmatis* cells infected with isolates of Tm4::pYubSSmRFP (Isolate 1, 2, 3, 4, 5, 12, 19 and 20). A his-tag purification performed on a water sample as well as on uninfected *M. smegmatis* cells was included as negative controls. Note that this data represents a single experiment and needs to be interpreted with caution.

A protocol was developed for testing Tm4::pYubSSmRFP in *M. tb* H37RV (as described Appendix D.1). A high titer (10^9 pfu/ml) of Tm4::pYubSSmRFP was used to test for expression of fluorescence in *M. tb* following 6h infection and incubation. The samples were tested for fluorescence development using fluorescence microscopy as shown in Figure 3.5.7. The samples had been processed and stored for 4 weeks prior fluorescence microscopy which indicates that fluorescence levels are stable for extensive periods of time, despite the harsh paraformaldehyde treatment step. Furthermore, 3 different tests with paraformaldehyde treatment of the *M. tb* mycobacteriophage-infected cells, showed total killing of all cells in the sample which was confirmed by culture testing on MGIT for up to 4 weeks. This shows that the samples may be processed outside of a biohazard facility following paraformaldehyde treatment. Unfortunately the relative fluorescence of *M. tb* cells infected with Tm4::pYubSSmRFP compared to uninfected cells was not determined due to time constraints.

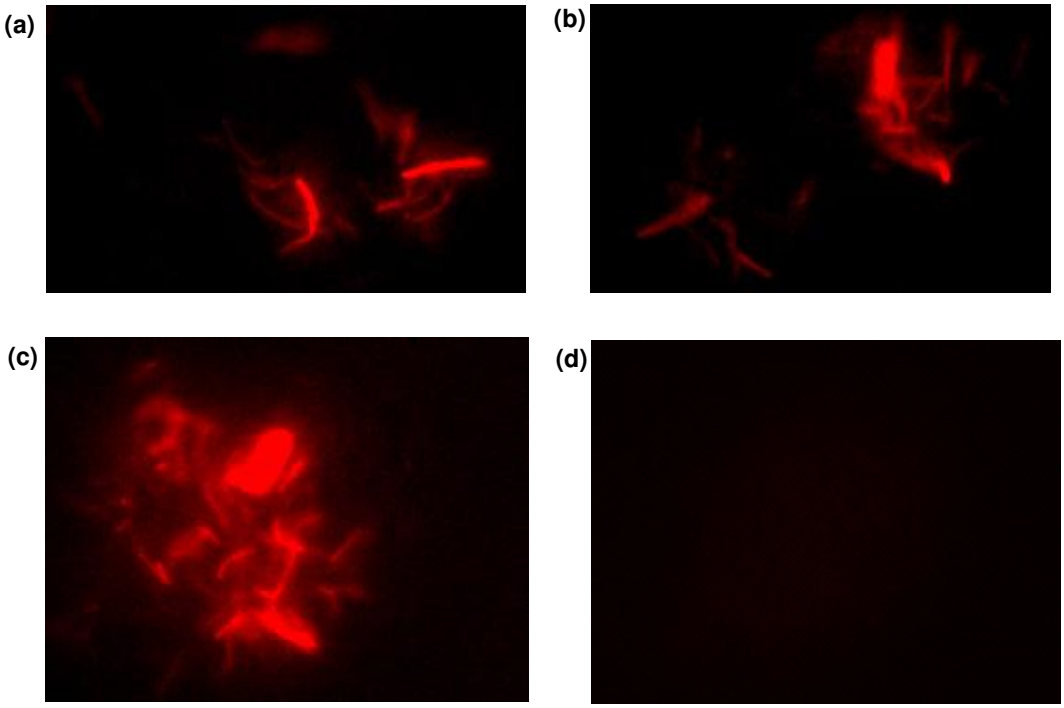


Figure 3.5.8. Fluorescence microscopy of *M. tb* cells following 6h infection with Tm4::pYubSSmRFP. **(a), (b) and (c)** *M. tb* infected with three different isolates of Tm4::pYubSSmRFP**.** Fluorescence can be seen following infection and 6h incubation, 90 min paraformaldehyde treatment and 4 weeks of storage at 4℃ prior to microscopy. **(d)** Negative control consisting of uninfected *M. tb* cells (a large clump) showing no fluorescence or auto-fluorescence.

Chapter 4 Discussion

The continuous spread of *M. tb* is the source of major mortality and morbidity world-wide (World Health Organization, 2009a). One third of the world has been estimated to be infected with this pathogen (Corbett *et al.*, 2003, World Health Organization, 2009a). Furthermore, the emergence of drug resistant strains is a new cause for concern, with MDR- and XDR-TB spreading globally and having been reported in as many as 58 countries around the world (World Health Organization, 2010). Drug treatment of TB is problematic since the treatment regimens spans over extended periods of time of between 6 and 8 months for drug sensitive TB and 18 to 24 months for MDR-TB (World Health Organization, 2009a). Patients are also liable to relapse following successful treatment, which leads to an increased risk of drug resistance (World Health Organization, 2009b). The current lack of effective anti-TB drugs presents a large problem in the control of the *M. tb* epidemic. Diagnostics are thus required to effectively detect and treat infected individuals before extensive transmission can take place. However, current routine diagnostics are not fulfilling this role effectively. Culture is the current gold standard TB diagnostic which is time consuming and requires biohazard facilities which are not ideal for field use. Smear microscopy is another widely-used TB diagnostic but it is insensitive. Additionally, current novel rapid TB diagnostics are out of reach for resource poor settings due to infrastructure, cost and skills requirements. Bacteriophage-based technology offers several advantages which could lead to simple, rapid and field-useable diagnostic technologies for bacterial detection. Mycobacteriophages have previously been investigated for use in *M. tb* diagnostics. However, these diagnostics have impractical requirements for field use, such as expensive reporter signal detection equipment, biohazard facilities and skilled staff. In this study, the construction of an affinity-tagged fluorescent reporter mycobacteriophage is described, which was engineered to act as a rapid TB diagnostic, offering an improvement on previous mycobacteriophage-based TB diagnostics. The construction of this reporter mycobacteriophage consisted of several steps. First, several mycobacterial promoters were screened for their suitability to drive expression of reporter genes, and the most optimal promoters were selected for further analyses. Secondly, various combinations of promoters, reporter genes, affinity tags and expression vectors were compared to establish which construct presented the most optimal and stable expression. The most suitable plasmid construct was then selected and the promoter, reporter gene and affinity tag from this construct was transferred to a

cosmid in preparation for transferral to a suitable mycobacteriophage. Thirdly, in order to construct a novel reporter mycobacteriophage, several mycobacteriophages were investigated for suitability. Reporter cosmids were cloned into the most suitable mycobacteriophage, which lead to the production of a novel reporter mycobacteriophage capable of detecting *M. tb.*

4.1 Promoter screening

Several promoters were investigated for use in the initial reporter plasmid constructs to drive expression of reporter genes, including the Hsp60, p19K, and pSmyc promoters. Additionally, 10 intergenic regions from *M. tb* H37Rv ESAT-6 gene cluster 5 were evaluated for the presence of promoters *in silico* as well as *in vivo*. Two promoter prediction software programs, "NNPP" and "BPPROM" were used to analyze the 10 intergenic regions as described in Section 2.6.2. The results of these programs did not fully correspond, with "NNPP" predicting more potential promoters within the 10 intergenic regions than "BPPROM". The promoter prediction software detected several of the promoters identified in the *in vitro* results; however two of the strongest promoters ("Int 2" and "Int 5"), as detected by the β-galactosidase assay, were only detected by one of the two programs, respectively. The software, however, could only act as an additional tool to help include promoter regions for screening and does not provide suitable evidence to exclude a region. The 10 intergenic regions were cloned into promoter probe vector pJem15 and used for promoter activity screening by transforming *M. smegmatis* and by plating onto solid media containing X-gal. Blue / green colonies indicated β-galactosidase activity and thus promoter activity. Figure 3.1.4 showed that "Int1", "Int2", "Int3", "Int5", "Int7", "Int8" and "Int10" have detectable promoter activity using the plating method. A β-galactosidase assay was performed and revealed the relative promoter strength for the intergenic regions as shown in Figure 3.1.5. This figure shows that "Int1", "Int2", "Int5" and "Int8" are strong promoter elements relative to the Hsp60 promoter. These results were in accordance with the β-galactosidase activity observed on the solid media containing X-gal, with the exception of "Int10", which is a relatively weak promoter according to the β-galactosidase assays. Promoter "Int1" was specifically chosen for use in the subsequently constructed reporter constructs since it has strong promoter strength, yet slightly weaker than the Hsp60 promoter. The reason for aiming to select a promoter weaker than Hsp60 was to avoid mutations and deletions which have previously been shown

to occur in very strong promoters such as Hsp60 and mycobacteriophage L5 pLeft promoters (Al Zarouni & Dale, 2002, Bardarov *et al*., 2003).

4.2 Reporter plasmid constructs

Mycobacterial plasmid expression vectors were initially used in order to test the various promoter, reporter gene and affinity tag combinations in *M. smegmatis*, of which the best reporter construct would be cloned into a suitable cosmid and subsequently transferred to a suitable mycobacteriophage. Table 18 (Section 2.6.3) shows a list of the reporter plasmids constructed during this study and the various combinations of promoters, reporter genes and affinity tags within these. These constructs are discussed below and grouped according to the specific reporter genes utilized (first *DsRed,* thereafter *mRFP1* and lastly *LacZ* constructs).

4.2.1 pSD21Hsp60DsRedNHis

To construct pSD21Hsp60DsRedNHis, the Hsp60 promoter and N-Term His-tagged *DsRed* gene were cloned into expression vector pSD21 (as described in Section 2.6.3.1). The results are shown in Section 3.2.1. Expression of *DsRed* from this construct in *M. smegmatis* (as well as in *E. coli*) was exceptionally high in certain instances, with cell pellets and His-tag purified eluates being visibly red to the naked human eye (as displayed in Figure 3.2.1.4a and b, respectively). However, the construct displayed unstable expression profiles after subculturing of the visibly red cultures containing construct pSD21Hsp60DsRedNHis. A several-fold decrease in visual red signal was observed in cell pellets, as well as in His-Tag purified eluates, despite similar culture conditions to the parent culture. This is likely due to the excessively strong promoter activity of the Hsp60 promoter, which leads to mutations and / or deletions in the promoter itself (Al Zarouni & Dale, 2002). A recent study also reported unreliable expression of *DsRed* in *M. smegmatis* using the Hsp60 promoter and suggested that frame-shift mutations could also be the cause (Carroll,P. 2010). Although the majority of cell pellets (grown from PCR positive colonies) displayed little visual red signal to the human eye, several of the cultures had a culture supernatant with visible red colour indicating possible cellular export of *DsRed*. However, as export of *DsRed* has not been reported in the literature, and due to the size of the tetramer, this is less likely. A more probable explanation for the visibly red supernatant could be due to cellular lysis of cells

expressing *DsRed*, releasing *DsRed* into the supernatant. In this study we observed that cultures (containing pSD21Hsp60DsRedNHis) which had a visibly red cell pellet following centrifugation often did not have a red supernatant. Conversely, it was also found that cultures (containing pSD21Hsp60DsRedNHis) with a red supernatant often did not have a red cell pellet. This mutual exclusion seems to indicate that DsRed is only released in certain cultures, notwithstanding the fact that culturing conditions were exactly the same. It is not clear whether this is due to active secretion or due to lysis of the cells. Considering the previously reported unstable expression in constructs that make use of the Hsp60 promoter, it is possible that this is also the case for the unstable expression from pSD21Hsp60DsRedNHis (Al Zarouni & Dale, 2002). In certain instances, expression of *DsRed* was not observed for the pellet or supernatant at all, despite positive PCR colony screening results for the pSD21Hsp60DsRedNHis construct. This further supports that expression from this promoter construct is unstable. Despite the variable visual results, fluorescence microscopy of samples with a red supernatant or red cell pellet revealed the presence of individual fluorescing cells (as displayed in Figure 3.2.1.5). The unreliable expression of construct pSD21Hsp60DsRedNHis makes it an unsuitable construct for the purpose of this study and further use of it was discontinued.

4.2.2 p19KProHsp60DsRedNHis

To construct p19KProHsp60DsRedNHis, the Hsp60 promoter and N-term His-tagged *DsRed* gene was cloned into vector p19KPro (as described in Section 2.6.3.2). The results for this construct are shown in Section 3.2.2. The expression of *DsRed* from *M. smegmatis* cultures containing construct p19KProHsp60DsRedNHis was similar to the expression observed for some of the cultures containing construct pSD21Hsp60DsRedNHis (discussed in Section 4.2.1). The similarity was that culture supernatants (from *M. smegmatis* cultures containing p19KProHsp60DsRedNHis) were frequently (almost consistently) red with cell pellets which were not visibly red. It is unclear whether this could be due to cellular lysis or due to export of the *DsRed* protein. However, as stated in Section 4.2.1, this has not been previously reported for *DsRed* and is less likely due to the large size of the *DsRed* tetramer. Despite the lack of a visibly red cell pellet, it was possible to detect single cells by means of fluorescence microscopy as displayed in Figure 3.2.2. Since none of the cell pellets were visibly red, it appears as though p19KProHsp60DsRedNHis could offer a worse expression level of *DsRed* than

pSD21Hsp60DsRedNHis. However, fluorescence microscopy showed that single fluorescent cells could be detected for most of the cultures, thus suggesting an increased stability in expression compared to pSD21Hsp60DsRedNHis. Furthermore, the frequently red supernatants also suggest increased expression of *DsRed* compared to *DsRed* expression from pSD21Hsp60DsRedNHis. A factor to take into account, when analysing the expression results from p19KProHsp60DsRedNHis, is the possible role of the p19k promoter which remains intact following construction of p19KProHsp60DsRedNHis as displayed in Figure 2.2. The Hsp60 promoter and *DsRed* gene was cloned into a single cutting site of p19Kpro. Thus, theoretically in half of the constructs the Hsp60 promoter and *DsRed* gene should be in the same orientation as the p19K promoter. The p19K promoter could thus potentially act synergistically with the Hsp60 promoter to yield increased promoter strength, increasing expression of *DsRed*. Additionally, the p19K promoter could perhaps grant promoter activity leading to *DsRed* expression in constructs with mutations or deletions in the Hsp60 promoter (as discussed in Section 4.2.1) which might explain the increased stability in expression over pSD21Hsp60DsRedNHis. However, it could also be argued that the possible additional promoter strength granted by the p19K promoter could further increase the occurrence of mutations and deletions in the Hsp60 promoter due to the increased combined promoter strength. It is unclear as to the exact reason for the less than ideal expression levels from construct p19KProHsp60DsRedNHis. However, due to the superior expression levels of reporter genes from other constructs made in this study, it was decided to discontinue further use of this construct for the purpose of this study.

4.2.3 pSD21Hsp60DsRedCHis

To construct pSD21Hsp60DsRedCHis, the Hsp60 promoter and C-term His tagged *DsRed* gene was cloned into vector pSD21 (as described in Section 2.6.3.3). The results are shown in Section 3.2.3. *M. smegmatis* cultures containing pSD21Hsp60DsRedCHis displayed no detectable expression of *DsRed* by means of fluorescence microscopy. The reason for the lack of *DsRed* expression is not clear. It would have been expected that pSD21Hsp60DsRedCHis would have similar expression to pSD21Hsp60DsRedNHis (Section 4.2.1) especially since the culture conditions used were exactly the same. As discussed in Section 4.2.1 and 4.2.2, use of the Hsp60 promoter lead to some cultures with no detectable *DsRed* expression which could also be the case for pSD21Hsp60DsRedCHis.

Interestingly, when the Hsp60 promoter, together with the C-terminal His-tagged *DsRed* gene, was excised from pSD21Hsp60DsRedCHis and cloned into vector pACE (which had its existing pACE promoter removed), the expression of *DsRed* was restored (as discussed in Section 4.2.4). Since transferral of the exact same promoter and affinity tagged reporter gene construct to another expression vector restored *DsRed* expression, it suggests that vector pSD21 contains some element which prevented optimal expression of *DsRed*. The lack of *DsRed* expression from construct pSD21Hsp60DsRedCHis made this construct unsuitable for further use in this study and its use was discontinued.

4.2.4 pACEHsp60DsRedCHis

To construct pACEHsp60DsRedCHis, the Hsp60 promoter and C-term His tagged *DsRed* gene was cloned into vector pACE (as described in Section 2.6.3.4). The results are shown in Section 3.2.4. The expression of *DsRed* was consistently high between cultures of *M. smegmatis* containing pACEHsp60DsRedCHis and single cells could be detected by fluorescence microscopy (as displayed in Figure 3.2.4.2). It is unclear why construct pACEHsp60DsRedCHis, despite its dependence on the Hsp60 promoter for *DsRed* expression, had more stable expression that previous constructs which utilized the Hsp60 promoter (as discussed in Sections 4.2.1, 4.2.2 and 4.2.3). One concern regarding the construct pACEHsp60DsRedCHis was the possibility that due to its situation at the C-terminus of *DsRed,* the affinity tag could be non-functional. It was previously suggested that C-terminal protein fusions to the *DsRed* tetramer could be non-functional (Yarbrough *et al*., 2001). However, the C-termini of *DsRed* monomers are partially exposed to solvent (Yarbrough *et al*., 2001), making it possible for small protein fusions, such as affinity tags, to remain functional as long as they are exposed to solvent. In this study, His-tag purification performed on the WCL of *M. smegmatis* containing pACEHsp60DsRedCHis resulted in an eluate which displayed fluorescence levels several-fold higher than the His-tag purified eluate of a control containing the same vector without a His-tag, indicating functional C-terminal His-tags. Furthermore, *DsRed* is an obligate tetramer and only fluoresces optimally when it this form (Yarbrough *et al*., 2001). It is thus not possible that the fluorescence observed was due to monomers or due to poorly fluorescing dimers (Yarbrough *et al*., 2001), which suggests that the His-tags fused to the tetramer did not affect tetramerization of DsRed.

The results for pACEHsp60DsRedCHis indicate that it is a suitable construct to consider for transferral into a cosmid vector. However, use of the Hsp60 promoter was considered less than ideal, as previously described, and thus use of pACEHsp60DsRedCHis was discontinued in favour of other superior constructs developed in this study.

4.2.5 pSD21Int1DsRedNHis

In order to address the poor and variable performance of the Hsp60 promoter, this promtoer was replaced with the Int1 promoter identified in the promoter analysis (discussed in Section 4.1). The expression from this construct was expected to correlate to the promoter analysis results for the Int1 promoter from the promoter analysis results as described in Section 4.1.1. However, since this promoter has not previously been described in literature its performance in expressing fluorescent proteins was unknown. To construct pSD21Int1DsRedNHis, the Int1 promoter and N-term His-tagged *DsRed* gene was cloned into vector pSD21 (as described in Section 2.6.3.5). The results are shown in Section 3.2.5. Interestingly, despite the good expression from this promoter in the promoter probe vector, the expression levels of *DsRed* were poor in *M. smegmatis* cultures containing pSD21Int1DsRedNHis. The reason for this is uncertain as construct pSD21Int1DsRedNHis is the same as construct pSD21Hsp60DsRedNHis except for the difference in promoter. The results from the comparative promoter analysis showed that the Int1 promoter was somewhat weaker than the Hsp60 promoter (shown in Figure 3.1.5, Section 3.1). Furthermore, use of the Int1 promoter should theoretically provide a more stable and reliable expression of *DsRed* as discussed in Section 4.1. This was in fact shown to be the case in another construct. Construct pACEInt1DsRedCHis (discussed in Section 4.2.6) showed high levels of *DsRed* expression when utilizing the Int1 promoter (see Section 4.2.6). This once again indicates potential problems with the use of expression vector pSD21. Later analyses also showed that expression vector pSD21 gave problematic expression in constructs pSD21Hsp60DsRedNHis (Section 4.2.1), pSD21Hsp60DsRedCHis (Section 4.2.3) and pSD21Hsp60mRFPNHis (Section 4.2.8). As discussed in Section 4.2.3, transfer of the Hsp60 promoter and His-tagged *DsRed* gene from pSD21Hsp60DsRedCHis (which had poor DsRed expression, Section 4.2.3) to expression vector pACE (to create pACEHsp60DsRedCHis, Section 4.2.4) restored expression of DsRed. All of these results thus suggest that the use of vector pSD21

potentially leads to problems in expression of these reporter genes. Given this, the Int1 promoter was also tested in other constructs (Sections 4.2.6, 4.2.10 and 4.2.15) and showed better expression activity. Thus, construct pSD21Int1DsRedNHis was not further utilized in this study.

4.2.6 pACEInt1DsRedCHis

To construct pACEInt1DsRedCHis, the Int1 promoter and C-term His-tagged *DsRed* gene was cloned into vector pACE (as described in Section 2.6.3.6). The results are shown in Section 3.2.6. Expression levels of DsRed from *M. smegmatis* cultures containing pACEInt1DsRedCHis were high and single cells could be detected using fluorescence microscopy as in Figure 3.2.6.2. His-tag purification performed on the WCL of *M. smegmatis* cells containing construct pACEInt1DsRedCHis lead to an eluate with fluorescence levels several-fold higher than a negative control. These results corresponded to the results obtained with construct pACEHsp60DsRedCHis (Section 4.2.4) which show that a 6 amino-acid C-terminal fusion to the DsRed tetramer, such as a His-tag, remains functional and is thus exposed to the solvent. These results also show that the Int1 promoter is a strong promoter which drives expression of *DsRed* to a level which enables single-cell detection by means of fluorescence microscopy. Due to the strong promoter activity of Int1 in construct pACEInt1DsRedCHis, it is thus likely that the poor expression from construct pSD21Int1DsRedNHis (discussed in Section 4.2.5) is attributable to the use of the pSD21 vector. The expression of *DsRed* from construct pACEInt1DsRedCHis indicated that it would be suitable for further use in downstream cloning and transfer into mycobacteriophage. However, pACEInt1DsRedCHis was not utilized further in this study due to the superior performance of other constructs (discussed later in this chapter).

4.2.7 pSD21Hsp60mRFPNHis

To construct pSD21Hsp60mRFPNHis, the Hsp60 promoter and N-term His-tagged mRFP1 gene was cloned into vector pSD21 (as described in Section 2.6.3.7). The results are shown in Section 3.2.7. A low level of fluorescence was detected among some of the *M. smegmatis* cultures containing construct pSD21Hsp60mRFPNHis. However, the expression of *mRFP1* from this construct matched the unstable expression of *DsRed* from pSD21Hsp60DsRedNHis (as discussed in Section 4.2.1). Other constructs from this study, which also utilized the Hsp60 promoter, also displayed poor

expression of their reporter genes (see Sections 4.2.1, 4.2.2, 4.2.3 and 4.2.5). This supports the previous observation in this study that the Hsp60 promoter results in unstable reporter gene expression. However, constructs in pSD21 also showed poor performance regardless of which promoter was used (see Section 4.2.1, 4.2.3, 4.2.5, with the exception of Section 4.2.4), so that the vector could also contribute to the poor performance. It is thus possible that constructs which utilize both the Hsp60 promoter and pSD21 are at highest risk to have poor expression. In fact, all constructs which utilized both the Hsp60 promoter in combination with vector pSD21 had expression ranging from poor to none (see discussion of these constructs in Sections 4.2.1, 4.2.5 as well as this section). Due to the poor *mRFP1* expression observed from pSD21Hsp60mRFPNHis, its further use in thus study was discontinued.

4.2.8 p19KProp19KmRFPNHis

To construct p19kProp19kmRFPNHis, an N-term His-tagged *mRFP1* gene was cloned into vector p19KPro, placing the *mRFP1* gene under control of the p19K promoter (Section 2.6.3.8). Expression was expected to be stable due to favourable results from previous studies using this promoter (Dave,J.A. 2002). The results are shown in Section 3.2.8. Expression of *mRFP1* from *M. smegmatis* cultures containing construct p19kProp19kmRFPNHis was consistently stable among cultures and sub-cultures containing p19kProp19kmRFPNHis. The cultures did not, however, lead to a visibly red cell pellet or eluates following His-tag purification. Despite the lack of a visibly red cell pellet, single cells were easily detected using fluorescence microscopy, as displayed in Figure 3.2.8.2. It thus appears as though the p19Kpro is not a suitably strong promoter for further use in this study. Despite the expression levels of *mRFP1* being consistent among cultures, the expression levels were not as high as other constructs from this study. Thus the use of p19kProp19kmRFPNHis was discontinued.

4.2.9 pSD21Int1mRFPNHis

To construct pSD21Int1mRFPNHis, the Int1 promoter and N-term His tagged *mRFP1* gene was cloned into vector pSD21 (as described in Section 2.6.3.9). The results are shown in Section 3.2.10. Expression levels of *mRFP1* were stable in cultures of *M. smegmatis* containing vector pSD21Int1mRFPNHis and single cells were easily detected using fluorescence microscopy (as

displayed in Figure 3.2.9). In this study, constructs that utilized the Int1 promoter lead to superior (higher and more stable) expression compared to constructs that utilized the Hsp60 promoter (with the exception of pSD21Int1DsRedNHis as discussed in Section 4.2.5). The Int1 promoter is thus a good candidate promoter for use in a reporter construct due to its consistent levels of expression and stability. However, due to the superior performance of other constructs, use of pSD21Int1mRFPNHis was discontinued.

4.2.10 pDMN1pSmycmRFPNHis

To construct pDMN1pSmycMRFPNHis, the N-term His tagged *mRFP1* gene was cloned into pDMN1 (as described in Section 2.6.3.10). This promoter was previously shown to drive strong and stable expression of fluorescent proteins in *M. smegmatis* (Carroll,P. 2010). The results from this study are shown in Section 3.2.10. pDMN1pSmycMRFPNHis displayed high levels of *mRFP1* expression in all cultures and sub-cultures of *M. smegmatis* cells containing the construct. Single fluorescing cells were identifiable in all cultures and sub-cultures containing this construct. This indicated stable and constitutive expression from the pSmyc promoter contained in this construct (in contrast to earlier constructs which utilized the Hsp60 promoter). The superior expression of *mRFP1* observed from this construct makes the promoter and reporter gene combination an appropriate construct for transferral to a cosmid and subsequently into a mycobacteriophage. Additionally, cultures of pDMN1pSmycMRFPNHis also gave a visibly red eluate following His-tag purification performed on the WCL, proving that this construct would be useful for concentration of reporter signal. All these factors made pDMN1pSmycMRFPNHis the most suitable reporter construct for transferral into mycobacteriophage and it was thus selected to be cloned into a cosmid and subsequently into mycobacteriophages.

4.2.11 pJem15Hsp60LacZNHis

To construct pJem15Hsp60LacZNHis, the Hsp60 promoter and N-term His-tagged *LacZ* gene was cloned into pJem15 (as described in Section 2.6.3.11). The results are shown in Section 3.2.11. The expression of *LacZ* the gene, and functionality of the His-tag fusion, was shown by first performing a His-tag purification on the WCL of cells containing the construct, followed by addition of X-gal which

resulted in a colorimetric reaction (as shown in figure 3.2.11d). Construct pJem15Hsp60LacZNHis proved to be a suitable construct for further use in this study, however, its use was discontinued due to favourable results from other constructs.

4.2.12 pJem15Int1LacZNHis

To construct pJem15Int1LacZNHis, the Int1 promoter and C-term His-tagged *LacZ* gene was cloned into pJem15 (as described in Section 2.6.3.12). The results are shown in Section 3.2.12. A His-tag purification performed on the WCL of *M. smegmatis* cells containing construct pJem15Int1LacZNHis produced an eluate which did not lead to a colorimetric signal after the addition of X-gal as with construct pJem15Hsp60LacZNHis (discussed in Section 4.2.11). It is possible that the partial digest performed on pJem15 lead to removal of DNA from the *LacZ* gene which inactivated the enzyme. The construct was thus not suitable for further consideration in this study and was not further utilized.

4.3 Cosmid constructs

The optimal and most stable expression of a reporter gene was observed from construct pDMNpSmycmRFPNHis (Section 4.2.10), consisting of the pSmyc promoter and N-term His-tagged *mRFP1* gene cloned into expression vector pDMN1. This promoter and reporter gene combination was selected to be sub-cloned into a suitable cosmid in order to allow Lambda packaging together with mycobacteriophage DNA to create a novel reporter mycobacteriophage. This brings two limitations into play, the head capsid size of bacteriophage lambda and the mycobacteriophage head capsid size of the selected mycobacteriophage limit the amount of DNA that can be stored. The theoretical maximum size that the head capsid of bacteriophage lambda can accommodate has been determined to be between 38 and 52 kb (Chauthaiwale,V.M. 1992). This means that the ligation product between mycobacteriophage DNA and cosmid DNA cannot exceed this amount in order to allow lambda packaging. In turn, the mycobacteriophage head capsid can only accommodate 52,797 bp for mycobacteriophage Tm4 and 49 136 bp for mycobacteriophage D29 (Pedulla,M.L. 2003). The integrating cosmid pYub412 (Appendix B.11) was used for the majority of cloning attempts. A non-integrating version of this cosmid was also created. Furthermore, a new cosmid was constructed,

based on expression vector pDMN1 (Appendix B.8). Table 19 (Section 2.6.4) shows a list of the cosmid constructs created in this study.

4.3.1 pYub412mRFPPvuI

Integrating reporter cosmid pYub412mRFPPvuI consisted of the *mRFP1* gene driven by the pSmyc promoter, cloned into the *Pvu*I RE site of pYub412 (as described in Section 2.6.4.1). The results are shown in Section 3.3.1. Construct pYub412mRFPPvuI failed to produce viable transformants following lambda packaging into *E. coli.* It is unclear why this construct failed to produce viable transformants. The cloning disrupted the ampicillin resistance gene, but since the *Pvu*I RE site is situated only about halfway into the ampicillin resistance gene, it would be unlikely to disrupt any essential genes. Additionally, disruption of the ampicillin resistance gene on its own could not be the explanation, as it would leave the role of selectable marker to the intact hygromycin resistance gene. The only plausible explanation for the lack of viable transformants (other than incorrect RE map information) could be that the *E. coli* origin of replication makes use of promoter read-through from the ampicillin resistance gene. It is possible that this origin of replication is disrupted by insertion of the reporter construct into the *Pvu*I RE site. This assumption is substantiated by the fact that the origin of replication is situated directly adjacent to the ampicillin resistance gene. This construct was thus found to be unsuitable for the purpose of this study and additional RE sites of pYub412 were investigated for use in constructing a more suitable reporter cosmid.

4.3.2 pYub412mRFPSapI

Integrating reporter cosmid pYub412mRFPPvuI was constructed by cloning of the N-terminal His-tagged *mRFP1* gene driven by the pSmyc promoter into the *Sap*I RE site of pYub412 (as described in Section 2.6.4.2). The results are shown in Section 3.3.2. Construct pYub412mRFPSapI failed to produce viable transformants following electroporation into *E. coli.* A possible reason for the lack of viable transformants following ligation of the reporter construct into the *Sap*I site of pYub412 might be ligation failure. This would result from using incompatible DNA overhangs during the ligation step. In order to clone an insert into a *Sap*I RE site it is necessary to have the exact sequence information of the *Sap*I RE site since the entire DNA overhangs are not dependent on the *Sap*I RE recognition site

and are determined by the specific DNA target site. However, since the overhangs of the digested promoter and reporter gene insert were complementary to the SapI digested overhangs of pYub412, according to the sequence information available on pYub412, it was unclear why the DNA failed to ligate. Furthermore, since the *Sap*I RE site is situated in a non-essential region in the pYub412 cosmid, it is unlikely that the insert disrupted any essential genes. Due to the failure of this construct to produce viable transformants following ligation, further use of it was discontinued.

4.3.3 pYub412mRFPNdeIBglII

Cosmid vector pYub412 was modified from an integrating cosmid to an episomal consmid by restriction digesting with NdeI and BglII which removed the AttP site as well as a part of the integrase gene. The episomal reporter cosmid pYub412mRFPPvuI was constructed by cloning the N-terminal His-tagged *mRFP1* gene driven by the pSmyc promoter into the *Nde*I and *Bgl*II RE sites (as described in Section 2.6.4.3). The results are shown in Section 3.3.3. Construct pYub412mRFPNdeIBglII failed to produce viable transformants following electroporation into *E. coli.* It is not clear why no viable colonies were observed following electroporation of the construct into *E. coli.* The region excised from pYub412, and replaced by the pSmyc promoter and *mRFP1* gene, contained non-essential DNA according to the vector map (Appendix B.12). Nevertheless, other RE sites were available and utilized in this study in order to construct a functional reporter cosmid (as discussed later in this section). Thus pYub412mRFPNdeIBglII was not further utilized in this study.

4.3.4 pDMNLCosmRFP

Granted that pYub412mRFPPvuI, pYub412mRFPSapI and pYub412mRFPNdeIBglII (discussed in Section 4.3.1, 4.3.2, and 4.3.3 respectively) were found to be unsuitable for the purposes of this study. Construction of a novel cosmid based on the selected reporter plasmid (pDMNpSmycmRFPNHis) was attempted. Episomal reporter cosmid pDMNLCosmRFP consisted of the construct pDMNpSmycmRFPNHis (see Section 4.2.10) with a (minimally size reduced) lambda cos site cloned into it (as described in Section 2.6.4.4). The results are shown in Section 3.3.4. The construct was successfully cloned, however, failed to lambda package together with mycobacteriophage DNA. The reason why the construct failed to produce viable colonies following ligation to mycobacteriophage

DNA and lambda packaging was initially unclear. This was especially the case since the recombinant reporter cosmid possessed all the features required for lambda packaging (an *E. coli* origin of replication, selectable marker and lambda cos site). Failure of similarly-sized cosmids to package along with mycobacteriophage DNA in this study later revealed that the most likely reason for packaging failure of this construct was due to size constraints of the lambda head capsid. It was shown that the cosmid pYubmRFP (Section 4.3.5) also did not successfully package, however, a size reduced version of it (pYubSSmRFP, Section 4.3.6) could successfully be lambda packaged. It was not possible to further reduce the size of this cosmid extensively, so unfortunately the functionality of the minimally size reduced lambda cos site used in pDMNLCosmRFP could thus not be tested in this study. Due to the failure of the novel reporter cosmid to be packaged along with mycobacteriophage DNA into *E. coli*, the pDMNLCosmRFP cosmid was not further utilized in this study.

4.3.5 pYubmRFP

As with construct pYub412mRFPNdeIBglII (Section 4.3.3), cosmid vector pYub412 was again modified from an integrating cosmid to an episomal cosmid by restriction digestion, this time with *Sal*I and *Eco*RV, which removed a larger region including the hygromycin resistance gene, the *AttP* site as well as the whole *integrase* gene. Episomal reporter cosmid pYub412mRFPPvuI was constructed by cloning the N-terminal His-tagged *mRFP1* gene driven by the pSmyc promoter into the *Sal*I and *Eco*RV RE sites (as described in Section 2.6.4.5). The results are shown in Section 3.3.5. Due to the removal of the large region, and the insertion of only a small construct, the resulting cosmid was considerably size reduced compared to pYub412. It was envisaged that this could result in a cosmid small enough to be accompanied together with mycobacteriophage DNA in phage lamba heads during lambda packaging into *E. coli*. Despite the removal of the non-essential genes, the cosmid could, however, still not be lambda packaged together with mycobacteriophage DNA due to size constraints. It seemed that the construct still needed to be much smaller to allow packaging. According to Figure 3.4.3.3a (Section 3.4.3), which displays the excision of the existing luciferase reporter cosmid from phAE142, the existing luciferase reporter cosmid was in fact considerably smaller than pYubmRFP (~4500bp compared to 5919bp). Thus, our reporter cosmid needed to be smaller than around 4500bp in order to ensure that the size constraints of bacteriophage lambda do not prevent packaging. For this

purpose, a further size reduced version of pYubmRFP was constructed as discussed in the following section (Section 4.3.6).

4.3.6 pYubSSmRFP

The size reduced cosmid pYub412mRFPPvuI was constructed by further removal of a 2032bp region of non-essential DNA, leaving the construct with only one remaining functional lamba cos site (as described in Section 2.6.4.6). The results are shown in Section 3.3.6. Although a double cos site improves lambda packaging efficiency, only one site is necessary, and the priority was to create a cosmid of the right physical size to allow lambda packaging. Thus, the reduced packaging efficiency due to using only one cos site was an acceptable trade off. However, this cosmid did have one drawback in that expression of *mRFP1* from the cosmid construct in *M. smegmatis* could not be tested. The reason for this was that the only remaining selectable marker in pYubSSmRRP was the ampicillin resistance marker, to which *M. smegmatis* is resistant. This meant that *M. smegmatis* cells containing the construct could not be selectively propagated in liquid or solid media, since both transformed and non-transformed cells would replicate equally well. However, favorable results were previously observed for the reporter plasmid containing the same DNA sequence for the promoter and reporter gene (pDMNpSmycmRFPNHis, Section 4.2.10). The reporter cosmid pYubSSmrfp was used for cloning into mycobacteriophage as described in Section 4.4.

4.4 Reporter mycobacteriophages

4.4.1 Selection of a suitable mycobacteriophage for cloning

The choice of mycobacteriophage plays a large role in the dynamics and performance of a reporter assay based on mycobacteriophage detection technology. Lysogenic mycobacteriophages will integrate into the host cells and will lead to gradual buildup of reporter signal, enabling single cell detection. Lytic mycobacteriophages, however, will lead to the rapid expression of reporter protein, but also host lysis and phage replication, rapidly leading to progeny phage buildup and new cycles of infection. Host lysis is the most suitable scenario for affinity-tagged mRFP1 expression prior to purification of reporter protein. Twenty-one novel South African mycobacteriophages isolated from soil samples, as well as mycobacteriophages D29 and Tm4 were considered for cloning to create a

reporter mycobacteriophage. Host range analyses indicated that the novel mycobacteriophages were not capable of infecting and replicating in *M. tb* and were found to be limited to *M. smegmatis* and possibly other unidentified soil mycobacteria. These were thus not found to be suitable for the purpose of this study. According to the literature there are currently 26 mycobacteriophages (the entire cluster K as well as mycobacteriophage L5 and D29) which are known to infect fast and slow growing mycobacteria and currently 223 genomes mycobacteriophages in genbank. This gives the rough estimate that about 1 in ten mycobacteriophages can infect fast and slow growing mycobacteria. It is thus unfortunate that the 21 mycobacteriophages screened in this study could only infect *M. smegmatis.* Their host range could, however, include other mycobacteria which have not been screened for in this study. Both D29 and Tm4 were found to be suitable since they are both lytic mycobacteriophages capable of infecting *M. tb.* Furthermore, their use as reporter mycobacteriophages has previously been demonstrated (Dusthackeer *et al.*, 2008, Pearson *et al.*, 1996). The dynamics of reporter protein expression is further influenced by the choice between an integrative or an episomally replicating cosmid for cloning into the mycobacteriophage. However, in this study only episomal cosmids could successfully be utilized due to size constraints. Incidentally, episomal reporter cosmids would be preferable since it would lead to rapid build-up of progeny mycobacteriophage and exponential signal amplification. In contrast, integrative reporter mycobacteriophage would lead to strong expression, but lower levels of lysis, and thus less extracellular reporter signal suitable for affinity purification-based signal detection.

In order to construct a recombinant mycobacteriophage, several approaches can be followed. If insertion site sequence information is available and if size constrains allow, recombination offers a relatively easy method of creating a recombinant mycobacteriophage. However, recombination was not utilized in this study since D29 DNA was wild-type and required a size reduction in order to accommodate additional DNA, making recombination unsuitable. Also, recombination could not be used for Tm4 since information on the insertion site of the existing luciferase cosmid (which had to be excised to disable luciferase expression as well as to allow space for the new cosmid) was not available. Self-ligation of the cosmid and mycobacteriophage followed by electroporation into *E. coli* or *M. smegmatis* offers a low efficiency alternative (compared to the lambda packaging method). A third

method for construction of recombinant mycobacteriophage is excision of non-essential mycobacteriophage DNA, followed by ligation to a suitable reporter cosmid, and thereafter the use of lambda packaging to create a reporter phasmid. Lambda packaging requires that the DNA which is targeted for packaging contain a lambda cos site and must be of suitable size (not too small or too large in order to be accommodated in the lambda phage heads). In addition, an *E. coli* origin of replication and a selectable marker needs to be present on the target DNA. However, ligation into the mycobacteriophage genome would naturally have to be into a non-essential region as well as into a region flanked by the mycobacteriophage cos sites to avoid cleavage excision after transfer into a mycobacterial host following activation of the mycobacteriophage lytic machinery. Additionally, the total size of the cosmid and mycobacteriophage DNA would also have to be of a suitable size to be accommodated in the mycobacteriophage heads (in addition to the lambda phage heads).

4.4.2 D29 reporter mycobacteriophage

Mycobacteriophage D29 DNA was obtained as wild-type DNA which would not be able to accommodate additional DNA in the D29 head capsid. A restriction enzyme size reduction strategy was adopted which makes use of partial RE digest and cloning of large (yet size reduced) DNA fragments into reporter cosmid pYubmRFP followed by lambda packaging. This method removed non-essential DNA from the D29 genome and replaced it with a reporter cosmid selected in this study. The cloning method is shown in Section 2.6.5.2 and the results are shown in Section 4.4.2. Working with linear double stranded mycobacteriophage DNA proved to be difficult, in particular obtaining large quantities of partially digested D29 DNA. Several attempts were made to construct a viable mycobacteriophage by ligating partially digested D29 DNA to digested pYubmRFP. Several hundreds of transformant colonies, resulting from lambda packaging of the ligations into *E. coli*, were PCR screened and DNA was extracted from colonies testing positive. However, transformation of the extracted DNA into *M. smegmatis* did not result in the production of a recombinant mycobacteriophage. Although the DNA packaged was large (as detected by cosmid DNA extraction and gel electrophoresis) the essential genes for infectivity and the lytic cycle must have been lost during the partial RE digest. To avoid the size constraints of phage lambda packaging, we attempted to electroporate the cosmid and partially-digested D29 DNA ligation directly into *M. smegmatis,* but this proved to be equally time consuming and unsuccessful. In order to achieve success with the RE size reduction method, clearly inordinate amounts of colonies would have to be further screened, which could potentially be simplified by resorting to Southern blotting to detect positive colonies. However, since several hundred colonies yielded no viable reporter mycobacteriophages by PCR, it was unlikely that a different screening method would provide a solution to the lack of viable recombinant mycobacteriophages. Granted the amount of time required to screen additional hundreds of colonies and / or plaques by either PCR or Southern blotting, other cloning methods proved to be superior to obtain a novel reporter mycobacteriophage, as discussed in Section 4.4.3.

4.4.3 Tm4 mRFP mycobacteriophage

Mycobacteriophage Tm4 was obtained in the form of phAE142, a luciferase reporter mycobacteriophage utilizing the L5 promoter to drive firefly luciferase from an unknown cosmid. phAE142 contains RE sites which could be utilized to excise the luciferase-containing cosmid and allows the possibility to replace it with another reporter cosmid. This method offers, in principle, a simplified cloning strategy over the D29 RE size reduction method (discussed in Section 4.4.1). Initial RE digest attempts to excise the luciferase cosmid appeared to excise a faint band of DNA. However, ligation of a reporter cosmid to the presumably digested DNA failed to produce viable transformants (Section 3.3). Additionally, attempts to directly electroporate the ligation between the cosmid and the (presumably) digested mycobacteriophage DNA failed to produce viable mycobacteriophage (Section 3.3). It was later revealed that mutations or deletions were most likely present in luciferase cosmid. According to literature, the luciferase cosmid has been reported to have mutations or deletions when phAE142 is propagated in wild-type *M. smegmatis*, and requires a special strain of *M. smegmatis* which expresses an L5 promoter repressor (Bardarov *et al*., 2003). However, the special strain of *M. smegmatis* was unavailable.

Thus, an alternative methodology was devised to obtain adequate amounts of phAE142 without the presence of mutations or deletions. Instead of propagating the DNA as mycobacteriophage, which subjects the DNA to selective pressures leading to mutations or deletions, the linear mycobacteriophage DNA was instead self-ligated and lambda packaged into *E. coli*. This allowed handling of the mycobacteriophage DNA in the form of circular phasmid DNA in *E. coli,* which was of benefit, as the L5 promoter is not active in the *E. coli* host. As such, a phasmid DNA extraction was performed on the DNA which yielded purified phAE142 DNA without, to our knowledge, mutations or deletions. This method preserved the RE sites in the unknown luciferase cosmid DNA sequence which subsequently allowed the successful excision of the luciferase cosmid and the release of the *Pac*I-digested Tm4 genome (as shown in Figure 3.4.3.3a). The minimally size reduced reporter cosmid pYubSSmRFP (as discussed in Section 4.3.6) was cloned into the *Pac*I RE site of *Pac*I-digested mycobacteriophage Tm4 and was successfully lambda packaged. Colonies testing PCR positive were cultured and a phasmid DNA extraction was performed on the cultures. The electroporation of the

purified phasmid DNA successfully lead to plaque formation, after which plaque purification was performed to obtain a large amount of recombinant Tm4::pYubSSmRFP stock. The novel reporter mycobacteriophage Tm4::pYubSSmRFP was then tested in both *M. smegmatis* and *M. tb* as described in Section 4.5.

4.5 Evaluation of reporter mycobacteriophage Tm4::pYubSSmRFP

Novel reporter mycobacteriophage Tm4::pYubSSmRFP consisted of the episomal reporter cosmid pYubSSmRFP (as discussed in Section 4.3.6) cloned into the *Pac*I RE site of mycobacteriophage Tm4. Mycobacteriophage Tm4::pYubSSmRFP was initially tested in *M. smegmatis* in order to evaluate its performance. The preliminary evaluation made use of a 96-well format fluorescence plate reader as well as fluorescence microscopy to test three aspects of the reporter mycobacteriophage important for its function and suitability as a reporter mycobacteriophage. These aspects were (1) variation in *mRFP1* expression between isolates, (2) the optimal titer, and (3) change in fluorescence levels as a function of time. The results are shown in Section 3.5.

In order to determine if there was any variation in *mRFP1* expression between isolates of Tm4::pYubSSmRFP, isolates were diluted to the same titer and used to infect *M. smegmatis.* Figure 3.5.1 displays the fluorescence levels observed, which indicated that some level of variation exists between different isolates. However, this variation was not significant since the expression from each isolate was considerably higher than that of uninfected *M. smegmatis* cells. Furthermore, good expression of *mRFP1* was observed using fluorescence microscopy which leads to the detection of individual cells of *M. smegmatis* as shown in Figure 3.5.5. Possible explanations for the level of variation could possibly due to mutations in the genomes of the isolates or due to unanticipated variance in the conditions of infection. It is also possible that some level of variation may be attributed to variance in the growth stages of the *M. smegmatis* cells. Sequencing of the pSmyc promoter and *mRFP1* reporter gene did not show any mutations or deletions in the 20 isolates which lead to two conclusions. First, if mutations or deletions are responsible for the observed expressional variance, then these mutations must reside elsewhere in the Tm4 genome. Secondly, the lack of mutations and deletions suggest that Tm4:pYubSSmRFP is more stable than phAE142 (Bardarov *et al.*, 2003) during

propagation in wild-type *M. smegmatis* which simplifies large scale production of the reporter mycobacteriophage Tm4::pYubSSmRFP for wide-spread use in diagnostics. Other than mutations or deletions, the remaining source for variation is due to variations during the infection of each *M. smegmatis* sample either by variations in the titer ranges of the mycobacteriophage isolates or due to variations in the amount of *M. smegmatis* used for infection or a combination of these aspects. Variations in the titer ranges could arise due to the inherent level of inaccuracy of titer ranges due to the downstream amplification of pipetting errors. Furthermore, considering that the *M. smegmatis* cultures used for infection were cultured in the absence of Tween-80 (leading to clumping of cells), it is possible that some level of variation occurred between samples during pipetting uneven amounts of cells. This is apparent since Figure 3.5.2 (Section 3.5) shows that fluorescence levels from infected cells are strongly associated with the amount of *M. smegmatis* cells used. It is thus likely that a combination of these two sources of variation is at play to yield the fluorescence variations in Figure 3.5.1.

In order to determine the optimal titer of Tm4::pYubSSmRFP to yield maximum expression of *mRFP1,* alarge dilution range was tested, ranging from 10^9pfu/ml to 1 pfu/ml as shown in Figure 3.5.3. The optimal fluorescence was observed from the undiluted samples corresponding to a concentration of 10^9 pfu/ml. Interestingly, one of the samples had a higher fluorescence at a concentration of 10^8 pfu/ml compared to 10^9 pfu/ml. This could be due to an excessively high MOI leading to LO occurring.

In order to determine how the fluorescence levels changed as a function of time, infection of *M. smegmatis* cultures with mycobacteriophage Tm4::pYubSSmRFP were performed over various time frames as shown in Figure 3.5.4. Figure 3.5.4 shows that an increase in fluorescence levels can be detected as early as 3h with a maximum fluorescence at more than 46h. It was also possible to detect single fluorescing cells by means of fluorescence microscopy by as early as 5h following infection of *M. smegmatis* (as shown in Figure 3.5.5). This roughly corresponded to the maturation time required for *mRFP1* of about 6h (Campbell *et al*., 2002). According to Figure 3.5.4, the highest fluorescence is between 8 and 46h. However, these results indicate that an incubation time of between 6-8h yields the fastest incubation time for an easily discernible result compared to the negative controls. Four isolates

of Tm4::pYubSSmRFP were selected and used to infect *M. smegmatis* cells using the suitably optimal incubation time of 24h (as shown in Figure 3.5.4) and the optimal titer of 10^8pfu/ml to 10^9pfu/ml (as shown in Figure 3.5.3). The results are shown in Figure 3.5.6, which indicates that the isolate at a concentration 10^9pfu/ml had significantly higher fluorescence values that those at 10^8pfu/ml. Thus 10^9pfu/ml is the optimal titer for maximum fluorescence. Additionally, isolate 18 showed fluorescence levels which were more than 8 times that of uninfected *M. smegmatis* cells which were cultured for 1h, and about 5 times higher than *M. smegmatis* cells which were allowed to grow for 24h. This shows that Tm4::pYubSSmRFP infected cells would show a considerably higher fluorescence level compared to contaminant bacteria with background fluorescence levels equal to that of a 24h *M. smegmatis* culture. The ability to detect the presence of Tm4::pYubSSmRFP infected *M. smegmatis* cells by means of affinity chromatography was also investigated (as described in Section 2.7.3). Figure 3.5.7 shows the resultant fluorescence levels following fluorescence reading of the his-tag purified eluates which show that the eluates have up to 7 times higher fluorescence levels than the eluate of uninfected *M. smegmatis* which provides a diagnostic avenue with a high level of discrimination.

The performance of the Tm4::pYubSSmRFP reporter mycobacteriophage was also tested in *M. tb* using the protocol listed in Appendix C.1. Using a MOI of 10^9 pfu/ml and overnight infection and incubation, single *M. tb* cells were able to be detected by means of fluorescence microscopy as shown in Figure 3.5.8. This indicates that the reporter cosmid pYubSSmRFP was successfully cloned into the Tm4 genome and that the reporter mycobacteriophage Tm4::pYubSSmRFP was able to infect *M. tb* and express the reporter gene *mRFP1* under control of the pSmyc promoter. These results also show that Tm4::pYubmRFP offers single cell detection of *M. tb* after only 6h incubation with the reporter mycobacteriophage. Furthermore, 3 different tests with formaldehyde treatment of the *M. tb* mycobacteriophage-infected cells, for 90 min each, showed that no viable cells remained in the samples. This was confirmed by culture testing on MGIT for up to 4 weeks after the 90 min treatment. Additionally, despite 4 weeks of storage at 4°C of the formaldehyde treated samples, individual cells could still be detected by means of fluorescence microscopy (as displayed in Figure 3.5.8). This shows that the analysis of the sample may be performed outside of a biohazard facility, following the 90 min

paraformaldehyde treatment, due to the very efficient bactericidal effect of the paraformaldehyde, which increases its usefulness to settings without specialized biohazard facilities. Furthermore, the four week results showed that the samples do not have to be processed immediately following formaldehyde treatment, which is an improvement on luciferase assays in which the reporter signal degrades as soon as the substrate and ATP is consumed. As culture testing is not necessary in this protocol, it could decrease the total diagnostic time for the assay to an estimated 8h. However, it is possible to increase the sensitivity of the assay by a brief 24h selective culture step by incubating the sample together with the 21 mycobacteriophages isolated in this study as they have been shown to not include *M. tb* in their host range (as described in Appendix D). The selective culture and detection of *M. tb* protocol is described in Section C.2). The assay offers the distinct advantage of signal concentration which is an improvement on existing mycobacteriophage-based TB diagnostics. His-tag purification of the samples following infection with Tm4::pYubSSmRFP provides a rapid and simple diagnostic avenue. In summary, the TB diagnostic based on Tm4::pYubSSmRFP provides a simpler diagnostic method to existing mycobacteriophage based assays without the need for P3 facilities, which makes the assay more suitable for field use.

4.6 Conclusion

Construction of the novel reporter mycobacteriophage Tm4::pYubSSmRFP led to the construction of several intermediates consisting of varying promoter, reporter gene, affinity tag and expression vector combinations. Initial constructs were evaluated using plasmid expression vectors. The combination of promoter and affinity tagged reporter protein which lead to the highest and most stable expression of the reporter gene was subsequently cloned into a cosmid to allow lambda packaging together with mycobacteriophage DNA. Several reporter cosmids were in turn cloned into mycobacteriophage Tm4 and analyzed for functionality. This lead to the successful production of novel reporter mycobacteriophage Tm4::pYubSSmRFP. The reporter mycobacteriophage Tm4::pYubSSmRFP was able to detect both *M. smegmatis* and *M. tuberculosis* using a multi-plate fluorescence reader as well as by detecting single cells using fluorescence microscopy. The optimal MOI was determined to be 10^9 pfu/ml which lead to detectable fluorescence levels after 3h with optimal expression between 24 to 46h. Incubation for a period of between 6 and 8h appeared to give the fastest results with a definitive

result compared to the negative controls. This corresponded to the maturation time required for *mRFP1* of about 6h (Campbell *et al.*, 2002). Fluorescence was stable following formaldehyde treatment and 4 weeks storage at 4°C. This is an extensive time window for detection compared to luciferase assays which only produce a detectable signal in the presence of ATP and substrate. This extensive time-frame eases logistics for the assay and adds to the versatility of the diagnostic. Additionally, analysis of the sample may be performed outside of a biohazard facility after fixing of the cells due to the bactericidal effect of the paraformaldehyde treatment step. The assay further offers the distinct advantage of signal concentration through the histidine affinity tag which is an improvement on existing mycobacteriophage-based TB diagnostics.

4.7 Future directions

In this study we developed a novel reporter mycobacteriophage that could detect single cells of *M. tb* by means of fluorescence microscopy, and also present a novel concept whereby the signal can be concentrated by means of affinity chromatography. Future studies should investigate the sensitivity and specificity presented by the novel reporter mycobacteriophage Tm4::pYubSSmRFP performed on clinical samples by means of fluorescence detection following affinity chromatography. Improvement on novel reporter mycbacteriophage Tm4::pYubSSmRFP could provide an even more suitable reporter mycobacteriophage for *M. tb* detection. Future studies to improve the characteristics of Tm4::pYubSSMRFP should investigate and compare the use of various combinations of *M. tb* specific promoters, reporter genes, affinity tags, mycobacteriophages as well as means of detection.

Use of mycobacteriophages which are specific for *M. tb* could offer a reporter mycobacteriophage with a specificity of 100%. However, construction of such a reporter mycobacteriophage, utilizing the methods described in this study, would require propagation of the mycobacteriophage in *M. tb* which presents an extensive biohazard risk. Use of a mycobacteriophage with a slightly wider host range could potentially decrease the biohazard risk, such as mycobacteriophage DS6A which is specific to the *M. tb* complex (REDMOND & CATER, 1960). Another means to derive a reporter mycobacteriophage with a high specificity for *M. tb* is to utilize *M. tb* specific promoters which are not active in other mycobacteria. It is also possible to increase the sensitivity of reporter

mycobacteriophage assays by investigating the use of other reporter genes. Enzymatic reactions in particular offer a very high amount of reporter signal production for a relatively low amount of reporter protein (enzyme), for example β-galactosidase enzymes. Use of a reporter bacteriophage which makes use of ice-nucleation genes as reporter could also provide a useful diagnostic method. Use of ice-nucleation genes as reporters offers a single-tube reaction which is simple and highly sensitive with the main drawback of requiring accurate temperature control devices. Finally, use of affinity-tag purification offers a natural solution to the problem that contaminant bacteria could express similar reporter genes through a affinity chromatography purification step. The use of biotin as affinity-tag, in particular, should be investigated in future studies due to its strong binding to streptavidin.

Appendix A - Solutions

A.1 Antibiotic stock solutions

	Antibiotic name	Concentration	Amount per ml of culture media	Conditions
A.1.1	Ampicillin	50mg/ml	1µl/ml	Made up in water, filter sterilised*, stored at -20°C
A.1.2	Kanamycin	50mg/ml	1µl/ml	Made up in water, filter sterilised*, stored at 4°C
A.1.3	Hygromycin B	50mg/ml	2µl/ml	Made up in water, filter sterilised*, stored at 4°C
A.1.4	Tetracyclin	50mg/ml	10µl/ml	Made up in methanol, filter sterilised*, stored at -20°C

* using a Sterillin 0.22µm PVDF filter unit

A.2 Culture Media

A.2.1 7H9

Dissolve 21g of Middlebrook 7H9 media in 900ml H_2O, add 3ml glycerol and autoclave for 20 min at 121°C. Once cooled to about 45°C, add 100ml Middlebrook Oleic-Acid-Albumin-Dextrose-Catalase (OADC, Appendix A.2.8).

A.2.2 7H10 plates

Dissolve 19g of Middlebrook 7H10 media in 900ml H_2O, add 5ml glycerol and autoclave for 20 min at 121°C. Once cooled to about 40°C, add 100ml Middlebrook Oleic-Acid-Albumin-Dextrose-Catalase (OADC, Appendix A.2.8) and the appropriate antibiotic(s). Slowly mix the media and pour onto plates. Allow the plates to set and immediately use or stored upside down at 4°C for up to 4 weeks.

A.2.3 7H11 plates

Dissolve 21g of Middlebrook 7H11 media in 900ml H_2O, add 3ml glycerol and autoclave for 20 min at 121°C. Once cooled to about 45°C, add 100 ml Middlebrook Oleic-Acid-Albumin-Dextrose-Catalase (OADC, Appendix A.2.8) and the appropriate antibiotic(s). Slowly mix the media and pour onto plates. Allowed the plates to set and immediately use or store upside down at 4°C for up to 4 weeks.

A.2.4 Top Agar

Dissolve 4.7g of Middlebrook 7H9 broth and 7g Bacto agar (Difco) in 1L H_2O and autoclave for 20 min at 121°C. Once cooled to about 45°C, add $CaCl_2$ to a concentration of 1mM and use immediately.

A.2.5 Kircheners medium

Dissolve 3g Na_2HPO, 4g KH_2PO_4, 1.07g $MgSO_4.7H_2O$, 2.5g Trisodiumcitrate and 5g Asparagine in 980ml distilled H_2O, add 20ml Glycerol and autoclave 20 min at 121°C.

A.2.6 Lysogeny Broth (LB)

Dissolve 10g Bacto-tryptone, 5g Yeast Extract and 5g NaCl in distilled H_2O, fill to 1L and autoclave for 20 min at 121°C. Once cooled to room temperature, add the appropriate antibiotics prior to use.

A.2.7 Lysogeny Broth (LB) agar plates

Dissolve 10g Bacto-tryptone, 5g Yeast Extract, 5g NaCl and 12g Bacto-agar in distilled H_2O, fill to 1L and autoclave for 20 min at 121°C. Once cooled to about 45°C, add the appropriate antibiotics, slowly mix the media and pour onto plates. Allow the plates to set and use immediately use or store upside down at 4°C for up to 4 weeks.

A.2.8 Oleic Acid-albumin-Dextrose Catalase (OADC)

Middlebrook OADC enrichment for mycobacterial growth media, supplied as a 100ml liquid (Merck).

A.2.9 Super Optimal Broth (SOB) medium

Dissolve 2g Bacto-tryptone, 0.5g yeast extract, 0.496g NaCl and 0.186g KCl in 900ml H_2O, adust the pH to pH7 and fill to 1L. Aliquote the media into 10ml autoclavable tubes and autoclave for 20 min at 121°C

A.2.10 Super Optimal broth with Catabolite repressor (SOC) medium

Add 200µl filter-sterilized glucose and 100µl filter-sterilized $MgCl_2$ to 10ml of SOB.

A.3 Buffers and solutions

A.3.1 Acid alcohol (3%)

Add 30ml 1M HCL to 970ml 95% ethanol

A.3.2 Acrylamide-bisacrylamide (30%)

Dissolve 30g Acrylamide and 0.8g Bis-acrylamide in 100ml H_2O, filter the solution through a Whatman filter paper (no.1) and store at 4℃ in the absence of light.

A.3.3 Ammonium persulphate (APS) (10%w/v)

Dissolve 1g $(NH_4)_2S_2O_8$ in H_2O and fill to 10ml, use immediately or store for up to one week at 4℃.

A.3.4 Ammonium Sulfate (Saturated)

Dissolve 80g ammonium sulfate in 100ml H_2O by mixing the solution for 5 hours. Siphon off the saturated solution from the undissolved crystals and store at room temerature.

A.3.5 Blocking buffer

Dissolve 20g Fat free milk powder in 200ml of TBS-T wash buffer (Appendix A.3.43) together with 2g BSA and 4ml 5M Azide ($NaN_{3)}$. Use immediately or store the solution at 4^{o}C.

A.3.6 Bromophenol Blue (0.75%)

Dissolve 75mg Bromophenol Blue in 10ml H_2O.

A.3.7 BSA blocking buffer (1%)

Dissolve 1g BSA in 100ml PBS-T buffer (Appendix A.3.27).

A.3.8 Carbol Fuchsin (ZN Staining)

Dissolve 10g basic fuchsin, 50g phenol in H_2O, add 100ml absolute ethanol and fill to solution to 1L. Filter the solution using a Whatman paper (no.1) before use.

A.3.9 Coomassie blue staining solution

Dissolve 2.5g Coomassie blue (Merck) in 450ml methanol and add 100ml acetic acid. Filter the solution using Whatman paper (no.1) before use.

A.3.10 Destaining solution

Add 50ml Glycerol, 187.5ml Glacial acetic acid and 125ml Methanol to 637.5ml distilled H_2O.

A.3.11 EDTA (0.5M)

Dissolve 186.12g EDTA in 800ml H_2O, adjust pH to pH8.0, fill to 1L and autoclave for 20 min at 121°C.

A.3.12 Ethanol (70%)

Add 30ml distilled and autoclaved sterile H_2O to 70ml 100% ethanol (merk) and store at -20°C

A.3.13 Ethanol (95%)

Add 5 ml distilled and autoclaved H_2O to 95ml 100% ethanol (merk) and store at -20°C.

A.3.14 Ethidium Bromide stock solution

Dissolve 10mg ethidium bromide in 1ml TE buffer (Appendix A.3.43)

A.3.15 Glycerol (10%)

Add 100ml Glycerol to 900ml H_2O and autoclave for 20 min at 121°C.

A.3.16 Isopropyl-β-D-thiogalactoside (IPTG, 0.1M)

Dissolve 238mg IPTG in 10ml water and filter sterilize the solution.

A.3.17 Klenow filling buffer (10X)

Make up 33µM dNTPs, 10mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$ and 1mM Dithiothreitol in sterile autoclaved H_2O and adjust the pH to pH7.9.

A.3.18 Maltose (1%)

Dissolve 1g Maltose in 100ml distilled and autoclaved H_2O and filter sterilize the solution using a 0.22µm filter.

A.3.19 Methylene Blue

Dissolve 0.3g methylene blue in 100ml distilled and autoclaved H_2O.

A.3.20 Mg solution (100X)

Add 1ml 1M $MgCl_2$ (Appendix A.3.21), 3.1674ml β-mercaptoethanol (sigma) to 5.8326ml distilled and autoclaved H_2O.

A.3.21 $MgCl_2$ (1M)

Supplied as a 1M solution (sigma).

A.3.22 $MgSO_4$ (1M)

Dissolve 12.03g $MgSO_4$ in 100ml water and filter purifyie the solution using a 0.22µm filter.

A.3.23 Mycobacteriophage (MP) buffer

Add 25ml 1M Tris (pH 7.6, Appendix A.2.48), 4.35g NaCl, 0.47g $MgCl_2$ and 0.11g $CaCl_2$ to 475ml H_2O and filter sterilize the solution using a 0.22µm filter.

A.3.24 ONPG (o-nitrophenyl-b-D-galactopyraniside, 1X)

Dissolve 4mg ONPG in 1ml 0.1M phosphate buffer (Appendix A.3.32).

A.3.25 Paraformaldehyde (4%)

Dissolve 4g Paraformaldehyde in 100ml distilled and autoclaved H_2O and stored the solution in a foil-covered container in the absence of light.

A.3.26 PBS (phosphate buffered saline) pH 7.4

Dissolve 8g NaCl, 0.2g of KCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4 in 900ml H_2O, fill to 1L and autoclave for 20min at 121°C.

A.3.27 Phage dilution buffer

Make up 10mM Tris-HCL, 100mM NaCl and 10mM $MgCl_2$ in 1L H_2O and autoclave 20 min at 121°C.

A.3.28 Phenol (buffer equilibrated)

Add Sigma buffer equilibration mixture to phenol as per provider instruction (sigma), incubate the solution at room temperature for 2-4 hours and store in a foil-coverd container in the absence of light at 4°C.

A.3.29 Phenol:Chloroform

Add an equal amount of Phenol (Appendix A.3.27) to chloroform in a glass container, store in a foil-covered container at 4°C.

A.3.30 Phenol:Chloroform:Isoamyl alcohol (PCI, 25:24:1)

Add 250ml Phenol (Appendix A.3.29), 240ml chloroform and 10ml isoamyl alcohol to a glass container covered with foil and store at 4°C.

A.3.31 Phosphate buffer (0.1M, pH 7)

Dissolve 1.61g $Na_2HPO_4.7H_2O$ and 0.55g $NaH_2PO_4.H_2O$ in 80ml distilled H_2O, adjust the pH to pH7 and fill the solution to 100ml.

A.3.32 Ponceau Red dye

Dissolve 2g Ponceau S, 30g trichloracetic acid and 30g sulfosalicylic acid in 100ml H_2O.

A.3.33 Proteinase K

Supplied as 100mg powder (promega). Make up to 2µg/ml using sterile autoclaved H_2O, use immediately or store as frozen aliquots at -20°C.

A.3.34 Proteinase K buffer (10X)

Add 25ml 1M Tris-HCl (pH 7.6, Appendix A.3.46) and 25ml 0.5M EDTA (pH 8.0, Appendix A.3.11) to 200ml sterile autoclaved H_2O and autoclave to solution for 20 min at 121°C.

A.3.35 Sample Reducing buffer (2X)

Add 3.4ml 1M Tris-HCl (pH6.8, Appendix A.3.47), 2ml glycerol, 3ml SDS (20% m/v), 0.5ml Bromophenol Blue (0.75%), 0.2ml EDTA (0.5M, Appendix A.3.11) and 1ml β-mercatoethanol to 10ml tube and store at 4°C.

A.3.36 SDS (Sodium dodecyl sulfate, 10% m/v)

Dissolve 10g SDS in 900ml distilled and autoclaved H_2O, adjust the pH to pH7.2 and fill to 1L.

A.3.37 SDS-PAGE Running buffer

Dissolve 6g Tris and 28.8g glycine in 1.5L H_2O, add 20ml SDS (10%) and fill to 2L with H_2O.

A.3.38 Sodium Acetate (3M)

Dissolve 40.8g Sodium Acetate (merck) in 100ml H_2O.

A.3.39 Sodium Borate Buffer (SB)

Dissove 19.1g di-Sodium tetraborate decahydrate (merk) in 500ml distilled H_2O to yied a 10X solution. Add 100ml of the 10X stock to 900ml distilled H_2O to yield a 1X working solution.

A.3.40 Sodium Carbonate (1M)

Dissolve 1,06g Sodium Carbonate (merck) in 10ml distilled H_2O.

A.3.41 TBS-T (Tris Buffered Saline Tween) buffer

Add 20ml Tris (1M, ph7.6 Appendix A.3.48), 16.01g $NaCl_2$ and 2ml Tween 20 to 1.5L H_2O, make up the solution to 2L.

A.3.42 Transfer buffer

Dissolve 3.03 g of Tris (25 mM), 14.4 g Glycine (192 mM) and 200 ml Methanol (20%) in 700ml H_2O and make up the solution to 1L. Store at 4°C.

A.3.43 Tris-EDTA buffer (TE)

Make up 10mM Tris and 1mM EDTA in 1L H_2O, adjust pH to pH7.5 and autoclave for 20 min at 121°C.

A.3.44 Tris-Acetic-acid-EDTA (TAE buffer)

Dissolve 242g Tris base in 750 mL H_2O, add 57.1 ml glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0), (Appendix A.3.12) and adjust the volume to 1L.

A.3.45 1M Tris-HCl pH 6.8,

Dissolve 121.1g Tris in 900 ml H_2O, adjust the pH to pH6.8 and make up the solution to 1L.

A.3.46 1M Tris-HCl pH 7.6,

Dissolve 121.1g Tris in 900 ml H_2O, adjust the pH to pH7.6 and make up the solution to 1L.

A.3.47 1.5 M Tris-HCl; pH 8.8

Dissolve 90.75g Tris in 400ml H_2O, adjusted the pH to to pH 8.8 and make up the solution to 500ml.

A.3.48 Tween (20%)

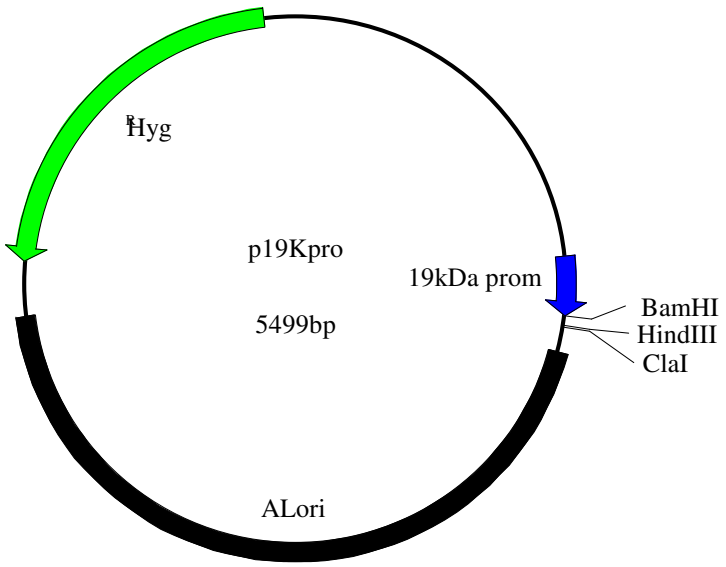
Add 20ml Tween 80 to 80ml H_2O and filter sterilize.

A.3.49 X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

Dissolve 250mg X-gal (Sigma) in 10ml dimethylsulfoxide (DMSO).

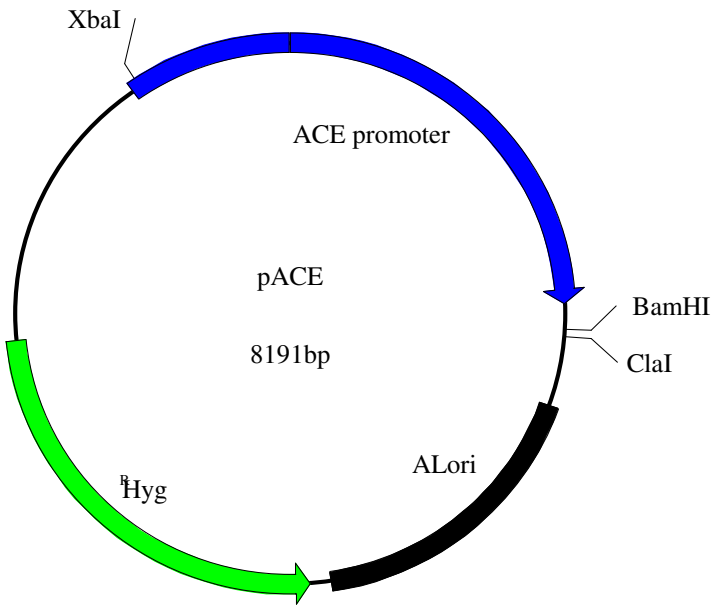
Appendix B - Vector maps

B.1 p19Kpro



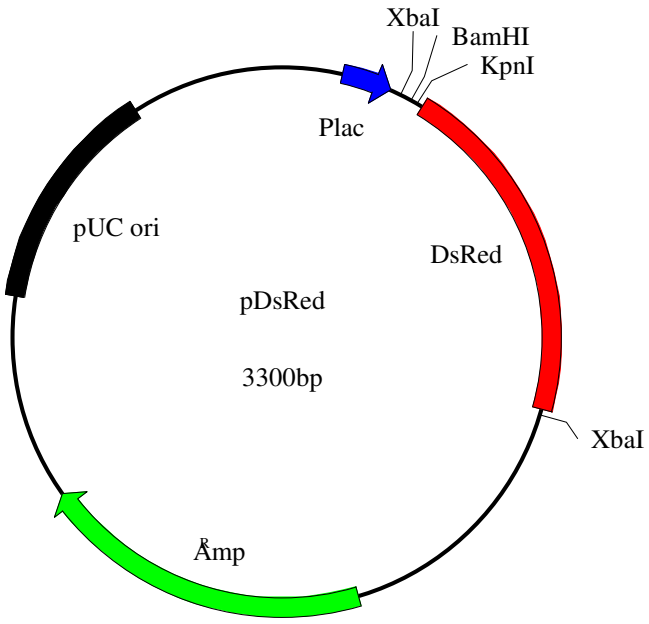
P19Kpro is a mycobacterial expression vector with hygromycin resistance. It contains a promoter region from *M. tuberculosis* H37Rv chromosomal DNA which is upstream of the 1 kDa antigen gene, (Ashbridge *et al.*, 1989). The promoter drives expression in mycobacterial hosts such as *M. tb* and *M. smegmatis*. Additionally it contains the the ALori origin of replication which is derived from *M. fortuitum* pAL5000 plasmid and is functional in both *E. coli* and mycobacteria, enabling cloning steps in *E. coli* host. The p19Kpro plasmid was constructed by Dr. Koen de Smet (Imperial College Medical School at St Mary`s, London, UK) and used with permission from Dr. Douglas Young (Imperial College Medical School at St Mary`s, London, UK).

B.2 pACE



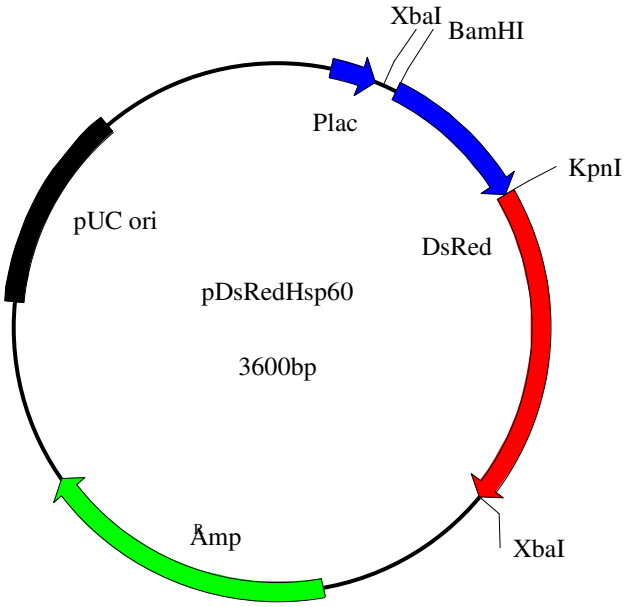
pACE is a mycobacterial expression vector with hygromycin resistance. The vector contains a 3kb region upstream of the acetamidase gene from *M. smegmatis*, (Mahenthiralingam *et al.*, 1993). The vector allows concentration dependant acetamide-inducible expression from the ACE promoter situated in the 3kb region. The vector contains the ALori origin of replication enabling replication in *E. coli* and mycobacteria. The pACE plasmid was constructed by Dr. Koen de Smet (Imperial College Medical School at St Mary`s, London, UK) and used with permission from Dr. Douglas Young (Imperial College Medical School at St Mary`s, London, UK).

B.3 pDsRed



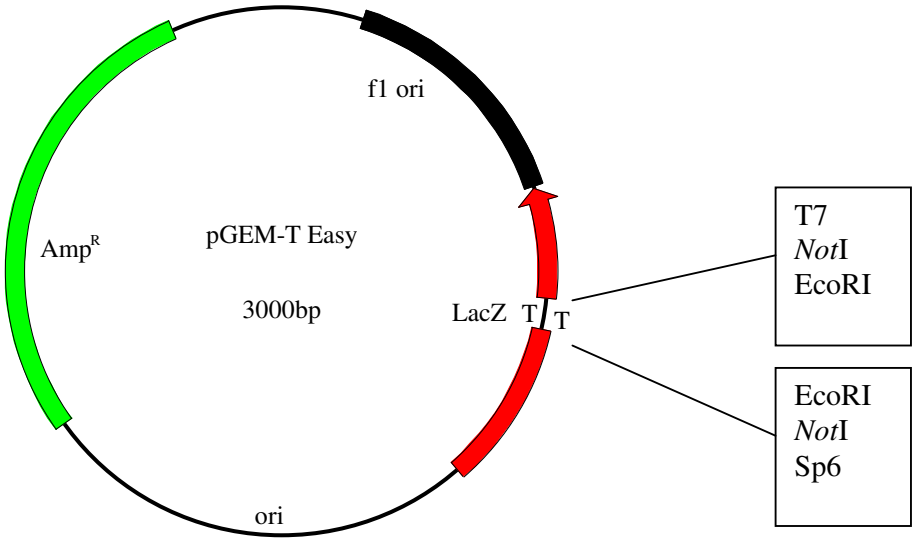
pDsRed (clonetech) is a prokaryotic expression vector which encodes the red fluorescent protein DsRed. DsRed, isolated from coral of the discosoma genus, has an excitation maximum of 558nm and emission maximum of 583nm. The *DsRed* gene in pDsRed is cloned in frame with the P_{lac} promoter allowing expression in *E. coli*. The vector contains a high copy number origin of replication as well as a gene for ampicillin resistance.

B.4 pHsp60promDsRed



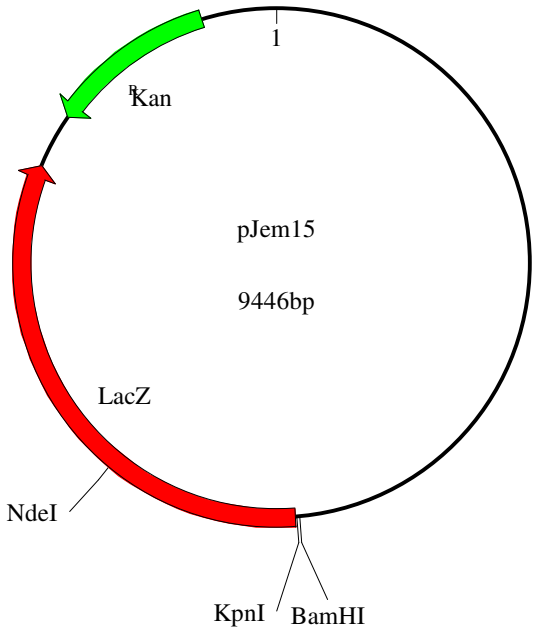
pDsRedHsp60 contains the Hsp60 promoter cloned into vector pDsRed (Appendix B.3) using the restriction enzyme sites *Bam*HI and *Kpn*I. The gene for the fluorescent protein, DsRed, is cloned in frame with the Hsp60 promoter enabling expression in *E. coli.*

B.5 pGem-T-Easy



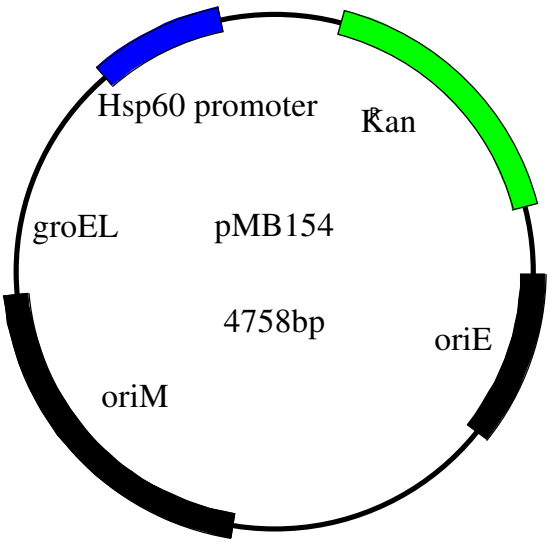
The Promega pGEM-T-Easy vector is a linear (shown circular) T-cloning vector for cloning PCR products with overhanging deoxyadenine base pairs as generated by DNA-polymerases without proofreading activity. The vector contains overhangs of 3` thiamine (T) which bind to the deoxyadenine overhangs on the PCR product which to form a circular construct. The plasmid contains T7 and SP6 RNA polymerase promoters on two multiple cloning sites which flank the PCR product cloning site. The insertion site is within the coding region of β-galactosidase, insertional inactivation of β-galactosidase allows colour screening of recombinant clones on plates containing X-gal (Appendix B.3.49). The vector also contains an *E. coli* high copy number origin of replication and an ampicillin resistance gene (Amp^R).

B.6 pJem15



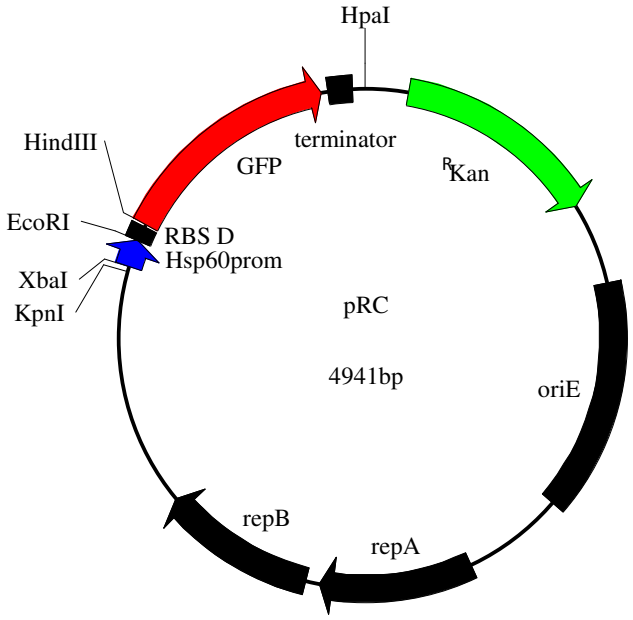
pJem15 is a mycobacterial promoter probe plasmid vector used for relative quantification of promoter activity. The vector has a multiple cloning site upstream of the lacZ gene which allows promoter activity to be determined by detection of LacZ expression by β-galactosidase activity assay. The vector contains the kanamycin resistance marker (Kan^R) as well as an origin of replication enabling cloning in *E. coli* host. The pJem15 promoter probe vector was obtained from and used with permission from Dr. Brigitte Gicquel (Institute Pasteur).

B.7 pMB154Hsp60



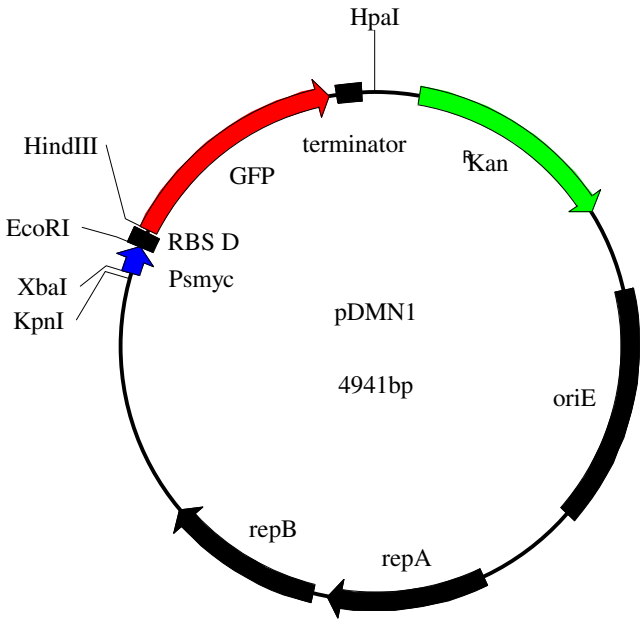
pMB154 is an *E. coli* - mycobacteria shuttle vector with kannamycin resistance. pMB154 used in this study was constructed previously to contain the Hsp60 promoter in frame with the first 3 amino acids from *M. tb H37RV Esat6* gene cluster 5 (N.C Gey van Pittius, unpublished results). The vector contains origins of replication for both mycobacteria and *E. coli.*

B.8 pRC



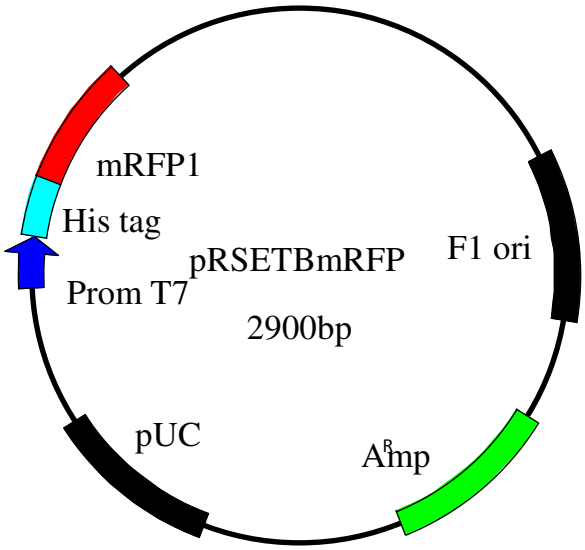
pRC is a mycobacterial expression vector containing GFP (green fluorescent protein) cloned downstream of the mycobacterial Hsp60 promoter and a ribosome binding site. Additionally the vector makes use of a terminator sequence downstream of the GFP gene. The vector contains genes for kannamycin resistance as well as *E. coli* and mycobacterial origins of replication enabling cloning in *E. coli* host. The vector was a kind gift, and used under permission, from Dr. Ros Chapman (University of Cape Town, infectious diseases).

B.9 pDMN1



pDMN1 is a mycobacterial expression vector containing GFP (green fluorescent protein) cloned downstream of the strong constitutive mycobacterial promoter P_{smyc} which has been previously described, (Mailaender *et al.*, 2004), (Kaps *et al.*, 2001). The GFP gene is flanked by a ribosomal binding site as well as a terminator sequence. The vector contains genes for kannamycin resistance as well as origin of replication sequences for mycobacteria and *E. coil* enabling cloning on *E. coli* host. The vector was a kind gift, and used under permission, from Dr. Ros Chapman (University of Cape Town, infectious diseases).

B.10 pRSETBmRFP



pRSETBmRFP is a prokaryotic expression vector which utilizes the inducible T7 promoter to express histidine tagged mRFP1 (monomeric red fluorescent protein). Utilizing bacterial strain BL21(DE3)pLysS to encode T7 RNA polymerase strong expression can be achieved. The vector contains a gene for ampicillin resistance as well an *E. coli* origin of replication. The vector is used with permission from Roger Y. Tsien (Howard Hughes Medical Institute, USA).

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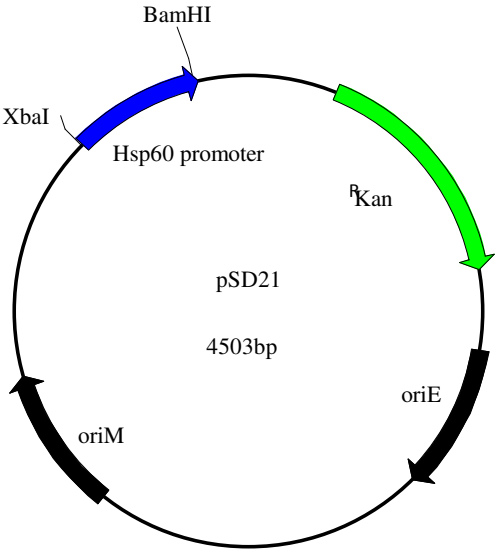
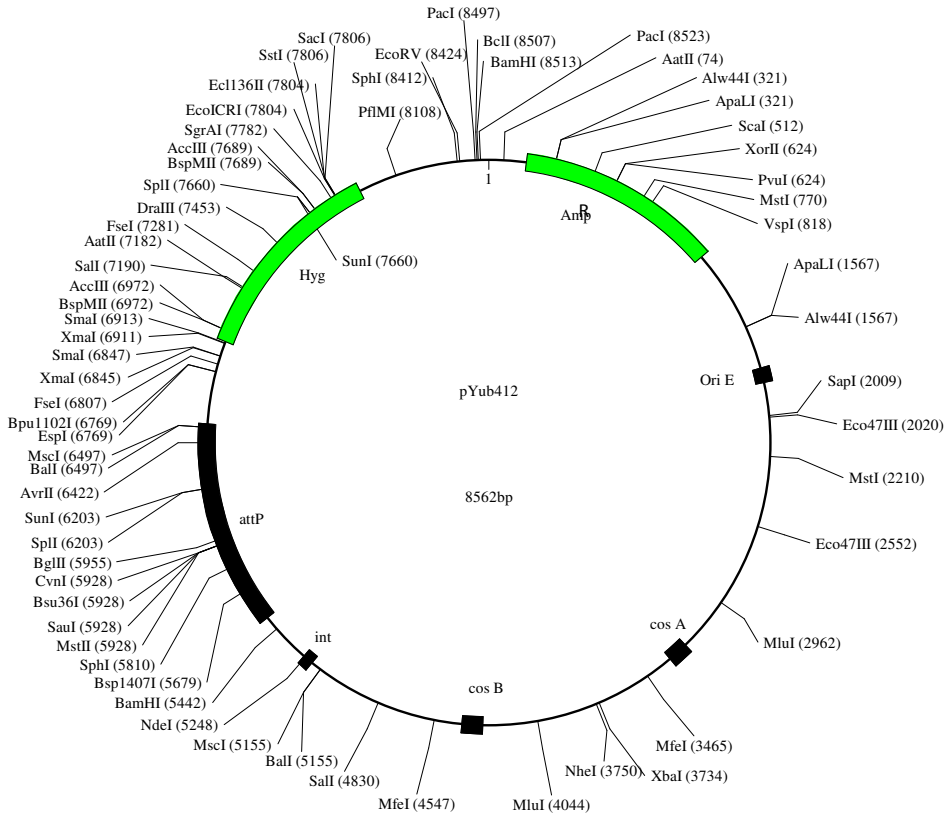
B.11 pSD21



pSD21 is a mycobacterial expression vector that contains the mycobacterial hsp60 promoter. The vector has a kanamycin resistance marker, mycobacterial as well as *E. coli* origins of replication (oriM and oriE respectively).

B.12 pYub412



pYub412 is an *Pac*I-excisable integrative cosmid cloning vector. The vector contains both ampicillin and hygromycin resistance. The cosmid also contains the mycobacteriophage L5 attachement site (attP), the L5 integrase gene (int) which allows stable integration into the mycobacteriophage L5 attachment site (attB) of the mycobacterial chromosome, (Lee *et al.*, 1991). As such the vector is a suicide vector in mycobacteria and can only replicate in *E. coli*

Appendix C – TB Diagnostic protocols

C.1 TB diagnostic protocol including culture testing

Protocol for detecting *M. tuberculosis* with the reporter mycobacteriophage Tm4::pYubSSmRFP

1. Culture *M. tuberculosis* sample in 7H9 OADC + Tween

2. Aliquot 1 ml of culture to 2 tubes

3. Centrifuge the tubes and resuspend the pellets in in 7H9-OADC without Tween

4. Aliquot 200µl from each tube into a new tube

5. Add 100µl of a 10^9 pfu/ml stock of Tm4::pYubSSmRFP to all tubes, except the control tube.

6. Incubate the tube for 6h at 37℃.

7. Add 300µl of paraformaldehyde (4% in PBS)

10. Leave at room temperature for 90 min to ensure killing of the bacteria.

11. Centrifuge, resuspend the pellet in 400µl PBS

12. Centrifuge, resuspend the pellet in 30µl PBS.

13. Spread 15µl onto a labeled glass microscope slide, cover with a coverslip and store at 4℃ until step 14 shows that no viable cells remain in the sample.

14. Use 15ul of the cells to inoculate a fresh MGIT culture and culture for 4 weeks, if no viable cells are detected, proceed to step 15.

15. Examine slides with fluorescence microscope, 100x objective with oil immersion.

C.2 Optimized TB diagnostic protocol

1. Decontaminate sputum sample with NaOH and subsequently neutralize with HCL.

2. Incubate the *M. tuberculosis* sample in 7H9 0ADC + Tween together with a cocktail of 21 novel South African mycobacteriophages for 24h.

3. Centrifuge the sample and resuspend the pellet in 100µl 7H9-0AD without Tween.

4. Infect the samples with 100µl of a 10^9 PFU Tm4::pYubSSmRFP mycobacteriophage stock and incubate for 6 hours 37℃.

5. Add 300µl paraformaldehyde (4% in Phosphate Buffered Saline) and incubate for 90 min.

6. Centrifuge the sample and resuspend the pellet in 400µl PBS.

8. Centrifuge the sample and resuspend the pellet in 50µl PBS.

9. Detect fluorescence by a fluorescence plate reader, or by detecting a red signal on a Nickel coated membrane.

Figure C.1 is a visual illustration of the optimized diagnostic.

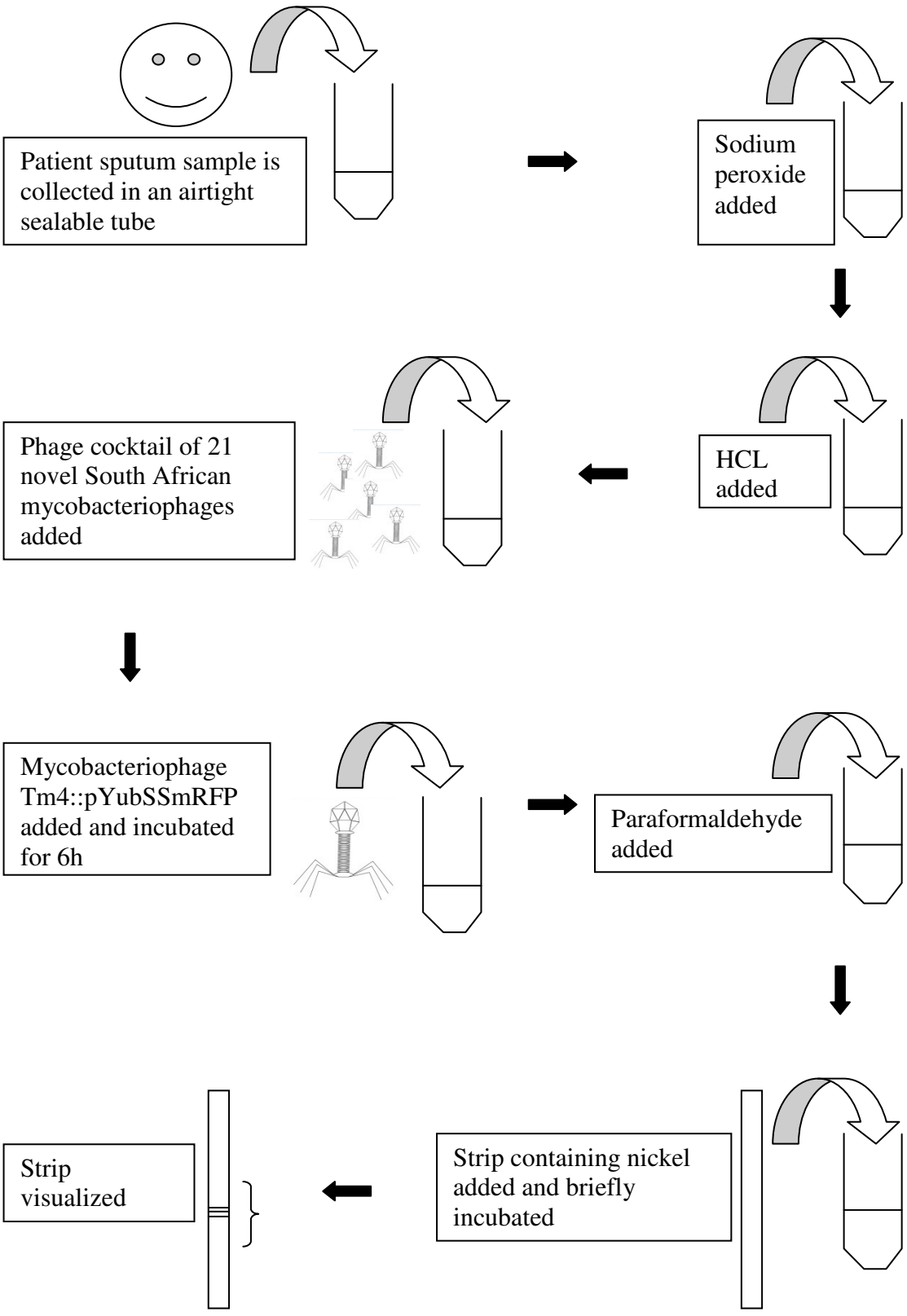


Figure C.1. Diagram illustrating the diagnostic procedure for detecting *M. tb* with mycobacteriophage Tm4::pYubSSmRFP.

Appendix D – ADDITIONAL WORK DONE

The isolation, characterization and comparative genomics of novel South African mycobacteriophages from soil samples

D.1 Introduction

Mycobacteria, especially Mycobacterium tuberculosis the causative agent of tuberculosis, pose a serious health threat worldwide. Mycobacteriophages are mycobacterium specific phages that are naturally occurring and have been isolated from various niches such as soil or even human sources. Various mycobacteriophages have been isolated, characterized, sequenced and investigated on a genetic level, however none originate from South Africa, a country with an extremely high TB burden. Mycobacteriophages have proven useful as molecular tools to manipulate mycobacteria, such as through generating knockouts, site specific integration, recombineering, improved transformation, novel transposon delivery vehicles and opportunity to investigate novel means of combating the organism. This includes drug development as well as rapid and cheap diagnostics. Isolation, characterization and comparative genomic analysis of novel mycobacteriophages offer further tools to address and investigate pathogenic mycobacteria.

D.2 Aim

The aim of this study is to isolate novel mycobacteriophages originating from South African soil samples, to characterize them by electron microscopy, to determine their host range, to extract and sequence their genomic DNA and to perform comparative genomics on their genomes.

D.3 Methods

D.3.1 Purification and DNA extraction.

Soil samples were collected from various regions in South Africa and tested for the presence of mycobacteriophages using indicator strain *M.smegmatis* as described in Section 2.4.1. Resulting plaques were then purified to create large stocks as described in Section 2.4.2. The mycobacteriophages where then purified from the large stocks by utracentrifugation (as described in Section 2.4.3) after which the resulting mycobacteriophage band was used for dialysis (as described in Section 2.4.4). The purified mycobacteriophage samples were then investigated by means of electron microscopy (as described in Section 2.4.4) and their genomic DNA was extracted (as described in Section 2.4.6). Each mycobacteriophage tested for its ability to infect *M. tb* by means of a spot test (as described in Section 2.4.1). Additionally, the mycobacteriophages were used for whole sequencing, and genome annotation and comparative genomcis.

D.3.2 Sequencing and genome annotation

Novel South African mycobacteriophages that were isolated from soil and used for DNA extraction (Section 2.4) were sent for whole genome sequencing at the Texas A&M University using an Illumina with *de novo* assembly in collaboration with Prof. Thomas Ioerger. Several bioinformatics tools and parameters were used to annotate the novel South African mycobacteriophages, as described below.

An initial genome sequence homology search was done using the NCBI blast website: (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome) and the closest matching mycobacteriophage data was stored for each novel genome. Positional annotation was performed using the annotation software "DNA Master" (available on the website: http://en.bio-soft.net/dna/dnamaster.html). DNA Master was used with the following parameters: The default translation table was set to "Bacteria and Plant Plastid", the codons ATG, GTG as well as TTG was added as start codons. The "auto-annotate" feature was used for preliminary positional annotation using NCBI

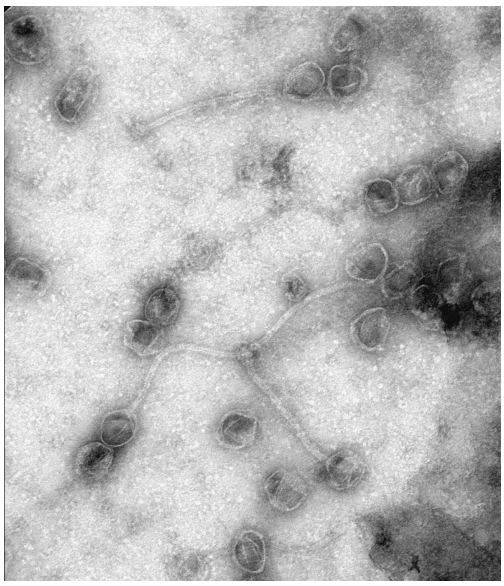
blast searches. The settings used for the “auto-annotate” feature were as follows: Aragorn was used to document tRNA`s, open reading frames were analysed with both Glimmer and GeneMark (GeneMark

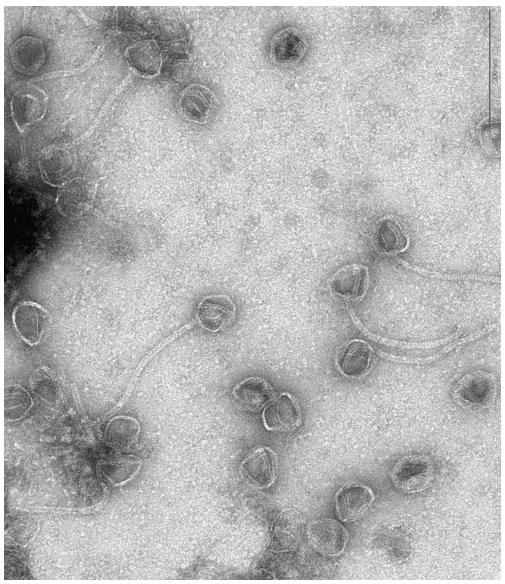
calls were favoured), “examine and learn gene features” was selected, “Perform BLAST search on nr database” was selected with the restrictions of saving hits with E-values less than 10^{-3}, however 1 hit was saved regardless of E-value and the number of hits was limited to 50. Open reading frames were predicted using Glimmer, which is embedded into the DNA Master suite. GeneMark was used to identify putative open reading frames ((http://opal.biology.gatech.edu/GeneMark/genemark_prok_gms_plus.cgi). The host selection parameter was set to mycobacteria. tRNAScan SE (Version 1.21) was used to predict potential tRNA genes (http://lowelab.ucsc.edu/tRNAscan-SE/). Aragorn (Version 1.2.28.c) was also used for tRNA prediction as well as for tmRNA (the software is accessible from the DNA Master suite). Frame Shift Finder was used to detect programmed translational frameshifts (http://chainmail.bio.pitt.edu/~junxu/webshift.html). *M. tuberculosis* was used as the coding potential model and RBS model, and the other settings were left as default.

D.4 Results

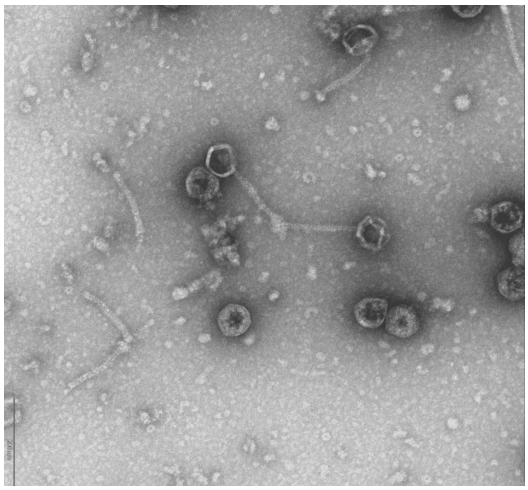
21 mycobacteriophages were isolated from various regions in South Africa. Plaque morphology showed a wide degree of variation. Transmission electron microscopy performed on the first two mycobacteriophages revealed them to be siphoviridae with typical head and tail properties. Figure D.1 shows the TEM images for the first two mycobacteriophages isolated. The host range of the 21 mycobacteriophages excluded *M. bovis* BCG and *M.tb* according to host range tests performed by spot testing. Field inversion gel electrophoresis indicates the genomes of the novel mycobacteriophages ranged between 50kb and 120kb which is within the range of previously isolated mycobacteriophags. Whole genome sequencing and annotation of the first South African mycobacteriophage, to be sequenced at the time, was successfully completed with sequencing of the others underway. The first fully sequenced South African mycobacteriophage was named mycobacteriophage Luiperd. It has a genome of 54885bp (~55KB), GC content of 65.4% and is in cluster I. Luiperd shows high levels of similarity to mycobacteriophage Che9c (with regions of >95% similarity) and with 47 (out of 83) open reading frames (ORFs) closely matching those of Che9c. 9 ORFs with no genbank matches were found, 4 of which contain conserved protein predictions according to BLASTp (3 belong to the XRE family transcriptional regulators and one contains NrdH-redoxin (NrdH)). 15 ORFs were found to be more closely related to bacterial genes than mycobacteriophage genes. tRNAScan revealed no tRNA`s in the genome. Luiperd also appears to have a divergent lysin B with only 42% BLASTP identities to a known mycobacteriophage lysin B (mycobacteriophage Giles). Table D.1 shows a list of the annotated open reading frames in Luiperd and Figure D.2 shows a visual illustration of the genome map of Luiperd.

(a) (b)





(c) (d)



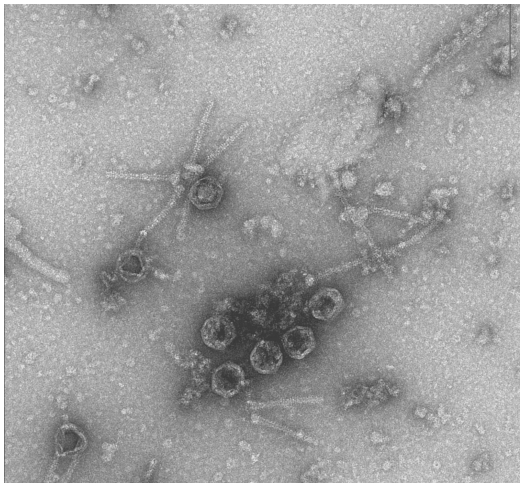


Figure D.1 TEM images of two novel mycobacteriophages isolated from soil samples. (a) and (b) TEM image of a novel mycobacteriophage of lytic nature**. (c) and (d)** TEM images of a novel mycobacteriophages of lysogenic nature.

Table D.1 Annotation of open reading frames in novel South African mycobacteriophage Luiperd.

Product	Closest Match	Predicted function based on DNA homology
gp1	Che9c gp1	
gp2	Che9c gp2	Terminase
gp3		
gp4	Che9c gp4	Portal
gp5	Che9c gp5	Prohead Protease
gp6	Che9c gp6	Capsid
gp7	Che9c gp7	
gp8	Che9c gp8	
gp9	Che9c gp9	
gp10	Che9c gp10	
gp11	Che9c gp11	
gp12	Che9c gp12	
gp13	Che9c gp13	
gp14	*M. smegmatis*	Hypothetical protein smegmatis 2145
gp15	Che9c gp15	Tape measure protein
gp16	Che9c gp16	
gp16a	Che9c gp16a	
gp17	Che9c gp17	
gp18	Brujita gp19	
gp19	Che9c gp18	
gp20	Che9c gp19	
gp21	Marinobacter aquaeolei	Fis family Transcriptional regulator?
gp22	Qyrzula gp31	
gp23	Che9c gp22	
gp24	Pipefish gp36	
gp25	Che9c gp24	

gp26	Che9c gp25	Lysin A? (Contains putative peptidoglycan binding domain)
gp27	Brujita gp30	Lysin B
gp28	Tm4 gp31	
gp29	Tm4 gp33	
gp30	predator gp39	
gp31	Actinomyces urogenitalis	Hypotherical protein Actinomyces urogenitalis DSM 15434
gp32	Actinomyces urogenitalis	DNA-methyltransferase?
gp33	Thermofilum pendens	methyltransferase type 11?
gp34	Sphaerobacter thermophilus	Hypothetical protein
gp35	Sphaerobacter thermophilus	transferase, transferring glycosyl groups?
gp36	Rhodococcus equi	gp43 family protein
gp37	Rhodococcus equi	Hypothetical protein
gp38	Che9c gp35	PPE superfamily, Antigenic varation for host?
gp39	Che9c gp36	
gp40	Che9c gp38	H37RV LpqJ
gp41	Che9c gp39	
gp42	Che9c gp41	phiLC3 phage Integrase
gp43	Che9c gp42	Actinobacteria protein superfamily DUF3263
gp44	Che9c gp43	
gp45		transcriptional regulator, XRE family
gp46		transcriptional regulator, XRE family
gp47	Streptomyces	DNA-damage-inducible protein D
gp48	Che9c gp50	transcription regulator, HTH MerR-SF superfamily
gp49	Che9c gp51	
gp50	Corynebacterium matruchotii	hypothetical protein CORMATOL_00734
gp51		LuxR family transcriptional regulator?
gp52	Giles gp41	
gp53		

gp54	Streptomyces lividans	transcriptional regulator, required for differentiation and sporulation.
gp55	Brujita gp44	
gp56	Che9c gp58	
gp57	Brujita gp48	YqaJ-like viral recombinase domain, Exonuclease
gp58	Brujita gp49	Recombination and repair recT superfamily
gp59		
gp60	Che9c gp62	
gp61		NrdH-redoxin (NrdH)
gp62	Salinibacter ruber	methyl-accepting chemotaxis protein
gp63	ctinomyces sp. oral taxon	glutaredoxin, NrdH-redoxin
gp64	Wee gp74	
gp65	Beggiatoa sp. PS	sensor signal transduction histidine kinase?
gp66	Che9c gp67	
gp67	Che9c gp68	RADICAL SAM superfamily
gp68	Brujita gp60	
gp69	Trypanosoma brucei gambiense	conserved hypothetical protein
gp70	Che9c gp71	
gp71	Che9c gp72	
gp72	Che9c gp73	
gp73	Che9c gp75	
gp74	Angelica gp3	
gp75		
gp76	Che9c gp77	
gp77	Che9c gp78	
gp78	Che9c gp79	
gp79	Che9c gp80	

gp80	Che9c gp81	
gp81	Che9c gp82	
gp82	Che9c gp83	
gp83	Che9c gp84	

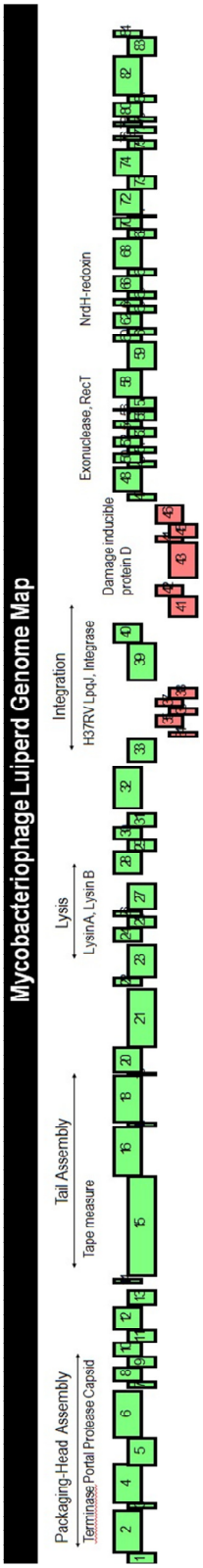


Figure D.2 Visual illustration of the open reading frames in mycobacteriophage Luiperd.

D.5 Discussion

Twenty-one novel South African mycobacteriophages were isolated from soil samples. Transmission electron microscopy of two of the mycobacteriophages revealed that are siphoviridae with head and tail lengths similar to previously described mycobacteriophages. Field inversion gel electrophoresis showed them to have genome sizes within the range of previously published mycobacteriphages. Each has been submitted for whole genome sequencing, one of which has been fully annotated, named mycobacteriophage Luiperd, the first South African mycobacteriophage to be sequenced. Comparative genomics showed that Luiperd has a high degree of similarity to mycobacteriophage Che9c, originating from Chenai, India. Luiperd, however, was isolated from a historically barren and unpopulated area in the Karoo landscape in the Western cape of South Africa. The proximity of the two regions suggest that it is improbable that there was trafficking of soil, mycobacteria or mycobacteriophages between the regions, which makes the degree of genome similarity surprising. However, geographical distance is not a good indicator of genome diversity since samples taken in close proximity have been known to differ to a large extent. A high level of conservation in structurally encoding genes (head, tail, capsid, tape-measure etc.) between Che9c and Luiperd was observed and a total of 47 out of 83 ORFs closely match those of Che9c. The lack of divergence in structural genes is a typical occurrence. Luiperd also contains ORFs that contain novel genes not in the Genbank database as well as several genes not common to mycobacteria or mycobacteriophages which suggests a high rate of divergence in DNA encoding for non-structural components (see Table D.1 and figure D.3 for a list of ORFs found in Luiperd). Luiperd has given us initial insight to South African mycobacteriophage genome diversity; further comparative genomics will provide deeper insight as to their diversity. This could potentially populate Genbank with several novel mycobacteriophage genes since the majority of genbank mycobacteriophages originate for Japan, India and USA. Investigation of the unique attributes in the novel genomes of mycobacteriophages could offer further insight and novel tools for the understanding and manipulation of mycobacterial genetics as well as potential new drug treatments and diagnostics for mycobacterial infections.

Appendix E – Sequence data

E.1 Intergenic regions in ESAT-6 gene cluster region 5

The following shows the sequence data for the 10 intergenic regions from the *M. tb* H37Rv gene cluster region 5, the primer binding locations are shown in bold.

Int1: Rv1779c-80

>M. tuberculosis H37Rv genome: 421 bp - region from 2014379 bp to 2014799 bp

cgctcgccgagttcgcgccagtgtccgccgccgagccaggtaagcagcccctcgattccggacacggccgagcgc**tgctcagcatattcgtgg**
gcgcacatgagactgaaaacacctcctgctggtcaagcctggcaggcccccgcccgacacaccgaatcgaagcggccccttgtggtgttgttcac
aactgcgcgagagatgacgcagatcacgtcgcggctgcccagccgaatcctcagcgagttcaatgtcaaaattaccgcggcgcgagcggatca
gcggccattatggcag**gtgacgtgagacggtataca**cctatgcaaaatcacgactacgttacctacgaagagttcggccgcagattcttcgaggt
agcagttaccccggaccgcgtcgccgccgcgtttgccgacatcgc

Int2: Rv1781-82

>M. tuberculosis H37Rv genome: 465 bp - region from 2017376 bp to 2017840 bp

gcctcggagaccagcacctgccggccagtccagtccgtgtactcggtggcaatgccgaatcg**tcgggcaagttcgaccag**cgaaggcgcgag
ctcggtcatgtcgcccatcttgcgtccggcacccgtgtgcgggcgagcgcaggaatctgagccttccgtcagcacagcacggttggctaccgaaca
ccactacgttgcaggtcaacgaggtagactgcggagcggacagttccacaggcggactcggtcattcgccgctaccatgcccagtgaagacacg
acgaatccttgggggatccgcgcagtggcaaatacccaggtcaatgtccaggtgttctgagcagaccgga**aggtgatctagcgtggc**tgaaga
gagccgcgggcagcgggggtcggggtatggccttgggttgtccacgcggacccaggtaaccggttatcagttcctggcgcgtcgaaccgc

Int3: Rv1783 – 84

>M. tuberculosis H37Rv genome: 671 bp - region from 2020119 bp to 2020734 bp

catttcggtgactcgcggcggcacgacgcggcgggtaacgcgcggatggtctaca**cctcggttcgtgagtttgc**cgcagagcaagccgaattat
tcgcgggccgtggttctttcacgcctcgacacgcgagttcgtcggcgcagaccccgaccccgcacaccgtgatcatcgccgacgtcgacgatccgc
aatgggagtacgtgatcagcgccgagggtgtcgacggggtgacgttcttcgacctgaccggctcttcgatgtggactgacatcccggagcggaagc
tgcagttcgacaagaccggcgtgatcgaggcgctgccccgcgaccgcgacacctggatggtgatcgacgacaaggcttggttcttcgctctcaccg
accaagtcagcatcgccgaggcagaagagttcgcgcagaagctggcgcagtggcggctggctgaggcctatgaagagatcggccagcgggtt
gcccacattggtgcccgagacatctagtcctactacgggattgacgatcctggcaacatcgacttcgactcgctgtgggc**tagccggaccgacac**
catgggacggtcgcgattgcgggcgccgttcggtaatcgctccgacaacggcgagctgctgttcttggatatgaaatcgctcgacgaaggcggcg
acggccc

Int4: Rv1786-87 (PPE25)

>M. tuberculosis H37Rv genome: 471 bp - region from 2024931 bp to 2025401 bp

cagagcacgaggtgcggcccgaggacatgcagctgaccagagacggtgtggccgcttgcccc**gaaatggcgctcatcct**cgaggaggacga
cgcggactgacgattccgggtcataccacaaaattaacgctggccaaacgatcgtttacgaggaatgaatatttggcgtcatcggcgctggaggcc
ggtattgcaatctaatgtgttttctatgcaacagttgcgcagcgacgccgttatcgactagcggtgctatattcggcgccttttcgatgccgagcgcgcgt
ctcgttggccacgtttggtggcaatgctcatcagggctcatccggatcgccaacgcgatcgtgtgtg**gagagggaggactggttg**gacttcggggc
gttaccgccggagatcaattcgggccgtatgtattgcggtccggggtcggggccgatgctggctgcggccgcggcctgggacggggt

Int5: Rv1787-88 (PPE25/PE18)

>M. tuberculosis H37Rv genome: 280 bp - region from 2026298 bp to 2026577 bp

Gactgccgctagggtcgcggatcactcggcgtagcggcgcctttgcccaccgatatgggttccgt**cacagtgtggttgcccg**cccgccatcggcc
ggataacgccatgacctcagctcggcagaaatgacaatgctcccaaaggcgtgagcacccgaagacaac**taagcaggagatcgcatg**tcgtt
tgtgactacccaaccagaagcactggcggcggcggccggcagtctgcagggaatcggctccgcattgaacgcccagaatgcggctgcggcgac

Int6: Rv1789-90 (PPE26/PPE27)

>M. tuberculosis H37Rv genome: 655 bp - region from 2027871 bp to 2028525 bp

tgttgggcggcctgccgctagctggtgcgggcggggccggcgcgggtccacgctacggattccgtcccaccgtc**atggctcgcccacccttc**gc
cggatagtcgctgccgcaacgtattaacgcgccggcctcggctggtgtggtccgctgcgggtggcaattggtcggcgccgagatctcggtgggttatt
tgcggtgggatttttttcccgaagccgggttcagcaccggatttcctaacggtcccgcgactcaacggcaccgcgccgtcagcaagttccggtggtgtt
gatcgcggtatccatgcaggtggtgatggcgcggcgagactggtcgtgtgcgctgaagcacagggtacttggcggttgtggctcccgggatgtagct
ggccgcccaacgtcccgcagcgtcggggtcagcggcggagcagcacggcgatttagcctcacaaccgagcagctagctcgcgtttcccagcgg
ctcaatccccgtcgagccattgaaaggcacctcagatgtcgtttgcgactccgcaaccggag**aaagggttcggaatggac**ttcggggcgttaccg
ccggagatcaattcgggccgtatgtattgcggtccggggtcggggccgatgctggctgcggccgcggcctgggacggggt

Int7: Rv1790-91 (PPE27/PE19)

>M. tuberculosis H37Rv genome: 628 bp - region from 2029377 bp to 2030004 bp

gaggcataccgctggcgagagcggggcggcgtacgggcgccttcgctcaccgatacgggt
tccgccacagcgtgattacccg**gtctccgtcggcgggata**gctttcgatccggtctgcgcggccgccggaaatgctgcagatagcgatcgaccg
cgccggtcggtaaacgccgcacacggcactatcaatgcgcacggcgggcgttgatgccaaattgaccgtcccgacggggctttatctgcggcaag
atttcatccccagcccggtcggtgggccgataaatacgctggtcagcgcgactcttccggctgaattcgatgctctgggcgcccgctcgacgccgag
tatctcgagtgggccgcaaacccggtcaaacgctgttactgtggcgttaccacaggtgaatttgcggtgccaactggtgaacacttgcgaacgggtg
gcatcgaaatcaacttgttgcgttgcagtgatctactctcttgcagagagccgttgctgggattaattggg**agaggaagacagcatgtc**gttcgtgac
cacacagccggaagccctggcagctgcggcggcgaacctacagggtattggcacgacaatgaacgcccagaacgcggccgcggctgc

Int8: Rv1791-92 (PE19/esxM)

>M. tuberculosis H37Rv genome: 345 bp - region from 2030103 bp to 2030447 bp

cccaggccgcggccattcacgaaatgttcgtgaacacgctggtggccagttctggctcatacgcggccacc**gaggcggccaacgcag**ccgctg
ccggctgaacgggctcgcacgaacctgctgaaggagaggggaacatccggagttctcgggtcaggggttgcgccagcgcccagccgattcag
ctatcggcgtccataacagcagacgatctaggcattcagtac**taaggagacaggcaacatg**gcctcacgttttatgacggatccgcatgcgatgc
gggacatggcgggccgttttgaggtgcacgcccagacggtggaggacgaggctcgccggatgtg

Int9: Rv1793-94 (esxN/1794)

>M. tuberculosis H37Rv genome: 289 bp - region from 2030878 bp to 2031166 bp

aggtgatctacgagcaggccaacgcccacgggcagaaggtgcaggctgccggcaacaacatggcgcaaaccg**acagcgccgtcggctcc**
agctgggcctaaaactgaacttcagtcgcggcagcacaccaaccagccggtgtgctgctgtgtcctgcagttaactagcactcgacc**gctgaggt**
agcgatggatcaacagagtacccgcaccgacatcaccgtcaacgtcgacggcttctggatgcttcaggcgctactggatatccgccacgttgcgc
ctgagttacg

Int10: Rv1794-95

>M. tuberculosis H37Rv genome: 473 bp - region from 2031868 bp to 2032340 bp

tggctatctgcccggctaccccgcagttggtgcaagtaggagtgaagaccgttttggatacactgccctacggcg**agtggaaaacacacagca**g
agtatgacgccagggcgtgaaacccgaagtacaacaacaaatttgagcatcagatacaacccagatacgtacagggcaaattgctctagaatcg
actgcaatactgcaaggcaaggtcaaccacaacgatttggtcgcgaggcaaggcaaatgaaatcggagttagtcgagccgcagctcccggtgg
gctaccgcgcctcggtgcctacaccgacggagctccccgcgccactgaagccacggtgtaacacgtttgccatggc**agggggtacaggacgat**
gaccgcagtagctgacgcacctcaggctgacattgagggtgtggcatcgccccaggctgtcgtcgtgggcgtcatggccggcgaaggcgtccag
atcgg

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