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Development of a multiplex molecular method for identification of extensively drug resistant *Mycobacterium tuberculosis* by padlock probes

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ABSTRACT

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (MTB), is generally treated by a regimen of antibiotics. Resistance to the first-line-antibiotics (isoniazid and rifampicin) is called multi-drug-resistant TB (MDR-TB) while resistance to at least isoniazid and rifampicin, to any fluoroquinolones, and to any of the three injectables (kanamycin, amikacin and capreomycin) is called extensively drug-resistant TB. Diagnosis of drug-resistant TB by conventional phenotypic methods require weeks to months. For rapid diagnosis of drug resistant TB, we have tested circularizing oligonucleotides called padlock probes that could detect the mutations associated with the anti-TB drugs resistance. Mutations in *rpoB, katG, gyrA, rrs* genes and promoter region of *mabA-inhA* confer resistance to anti-TB drugs. Here, we have constructed padlock probes which circularize after detecting each of the above mentioned wild type and mutated genes. The circularized padlock probes were amplified via two rounds of rolling circle amplification method and detected using single molecule detection method. In this study with the combination of padlock probes, rolling circle amplification and single molecule detection we could detect synthetic wild type and mutated DNA with a sensitivity of 1 attomole (6.02 x 10^5 molecules).

ABBREVATIONS

DNA	Deoxyribonucleic acid
TB	Tuberculosis
MTB	*Mycobacterium tuberculosis*
MDR-TB	Multidrug-resistant TB
XDR -TB	Extensively drug-resistant TB
RIF	Rifampicin
INH	Isoniazid
PZA	Pyrazinamide
EMB	Ethambutol
FQs	Fluoroquinolones
KAN	Kanamycin
AMI	Amikacin
CAP	Capreomycin
PCR	Polymerase chain reaction
RCA	Rolling circle amplification
RCP	Rolling circle product
C2CA	Circle to circle amplification
CO	Capture oligonucleotide
RO	Restriction oligonucleotide
RE	Restriction enzyme
DO	Detection oligonucleotide
SMD	Single molecule detection
BSA	Bovine serum albumin
PC	Positive control
NC	Negative control

DEFINITION OF TERMS

Sensitivity is used to describe the lowest concentration or amount of analyte that a given method can measure.

Specificity is used to describe the ability of a method to distinguish target molecules from any other molecules.

Multiplexing is the ability of an assay to measure multiple analytes in parallel in one reaction.

Multidrug-resistant TB (MDR-TB) is resistance to INH and RIF, with or without resistance to other first line drugs.

Extensively drug-resistant TB (XDR-TB) is resistance to at least INH and RIF, to any FQ, and to any of the three second-line injectables (AMI, CAP, and KAN).

INTRODUCTION

Tuberculosis, the global epidemic

"The biggest disease today is not leprosy or tuberculosis, but rather the feeling of being unwanted." -Mother Teresa. Humanistically Mother Teresa is right in her saying but technically it needs a small correction, i.e. 'Tuberculosis is the biggest disease', biggest in terms of its incidence. Tuberculosis (TB) is the third leading cause of human deaths, and annually eight to ten million new TB cases occur as per World Health Organization (WHO) estimation[1]. Millions of children are becoming orphans every year as a result of parental deaths (approx. 2 million annually) caused by TB[1]. According to the sixteenth global report on tuberculosis published by WHO, in the year 2010, there were 8.8 million incident cases of TB, and 1.45 million deaths[2]. TB was once thought to be a disease of the past, particularly in the developed countries but now it has made a dramatic comeback for various reasons and is developing to a global epidemic[3].

Mycobacterium tuberculosis, the causative agent of TB

Mycobacterium tuberculosis (MTB), an aerosol borne pathogenic bacterium that can infect any part of the body, most commonly the lungs is the causative agent of TB. Mycolic acids in the cell wall, slow growth rate, resistance to weak disinfectants and the ability to survive in dry state for weeks are some of its unique characteristics[4]. The MTB genome comprises 4,411,529 base pairs, contains around 4,000 genes, and has a very high guanine + cytosine content[5]. Infection with MTB where the bacteria are alive but not currently causing any disease symptoms is called latent TB infection while the MTB infection with disease symptoms is called active TB infection[6]. Approximately 10% of the patients with latent TB infection will develop active TB infection in later stages of their life for various reasons affecting the immune system[2] like the onset of a disease (such as AIDS), disease treatments (like chemotherapy for cancer), malnutrition or aging .

Treatment of TB, a long regimen of antibiotics

Once the latent TB infection turns active, the host needs to be treated with some bacteriostatic and bactericidal drugs. Antibiotics like rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), ethambutol (EMB), fluoroquinolones (FQs), kanamycin (KAN), amikacin (AMI), capreomycin (CAP) etc are used in TB treatment[7]. Each of these antibiotics interferes with the cellular mechanisms of the MTB in a specific fashion and either suppresses its growth or kills it[7]. The standard short course treatment of TB involves daily therapy with the first line drugs: INH, RIF, and PZA for two months and then with INH plus one other primary drug (EMB or RIF) for the next four months. In WHO's fourth edition of 'Treatment of tuberculosis', it is recommended to prefer RIF over EMB during the four months course of the treatment to reduce the number of relapses and failures[8]. MTB strains resistant to the first line therapy are treated with the second line drugs such as FQs, KAN, AMI, CAP etc[7]. These drugs should be used for longer duration than the first line drugs and so the chances for patients to miss doses or failing to complete the treatment regimen are high. Second line drugs

are more expensive, less effective than the first line drugs and they have more toxic side-effects as well[9].

Drug resistance, a major problem in TB treatment

The drug resistance profile of the MTB strains has a great impact on the TB treatment regimen. Recent US data show that about 13% of all new cases are resistant to at least one first-line drug, and 1.6% are resistant to both INH and RIF[7]. Resistance to INH and RIF, with or without resistance to other first line drugs is called multidrug-resistant TB (MDR-TB)[10]. Resistance to at least INH and RIF, to any FQ, and to any of the three second-line injectables (AMI, CAP, and KAN) is called extensively drug-resistant TB (XDR-TB)[10]. The primary mechanism of drug resistance in MTB is the acquisition of point mutations in its genome while many other bacteria use mechanisms like horizontal gene transfer (genes on plasmids) to acquire drug resistance[11]. Some of the mutations that confer drug resistance in MTB are described below (shown in Table 1).

RIF binds to the beta subunit of ribonucleic acid (RNA) polymerase and inhibits its transcriptional activity. The beta subunit of RNA polymerase is encoded by *rpoB* gene. Mutations in *rpoB* gene affect the amino acid residues in the enzyme coded by it leading to RIF-resistance. The most commonly mutated codons in rpoB gene are 516, 526, 531 and 533[12].

The molecular mechanism of INH activity is complex and not well understood. However, it is postulated that INH inhibits the biosynthesis of mycolic acids. Catalase-peroxidase, an enzyme encoded by *katG* gene converts INH to a biologically active form. Mutations in *katG* gene alter the structure of enzyme resulting in a decreased conversion of INH to its biologically active form. Amino acid replacement at position 315 (Ser→Thr) of catalase-peroxidase is found in most of the INH-resistant strains. The *inhA* locus (*mabA-inhA* operon) that codes for enzymes required for fatty acid biosynthesis is also associated with INH-resistance. Mutations in the upstream of *mabA-inhA* operon (especially at position 15) prevent the inhibition of fatty acid synthesis by INH[12].

FQs (ciprofloxacin and ofloxacin) target the bacterial DNA gyrase that catalyzes negative supercoiling of DNA. High-level resistance to FQs is associated with amino acid replacements in the A-subunit of the DNA gyrase encoded by *gyrA* gene. Mutations in *gyrA* prevent the inhibition of supercoiling by FQs. Codons 90 and 94 in *gyrA* are frequently mutated in FQ-resistant strains[12].

The second line drugs KAN, AMI and CAP interfere with the protein synthesis by inhibiting the normal function of ribosomes. The *rrs* gene that codes for 16S rRNA (subunit of ribosome) is observed to have single nucleotide variations that lead to KAN-, AMI- and CAP-resistance. High-level resistance to these drugs is often associated with mutations at positions 1400 and 1401 in *rrs* gene[7].

Table 1. The most common mutations in the genes that make MTB resistant against antibiotics.

Antibiotic	Gene	Mutation[a]
Rifampin	*rpoB*	D516V (GAC/GTC)
	rpoB	D516Y (GAC/TAC)
	rpoB	H526Y (CAC/TAC)
	rpoB	H526D (CAC/GAC)
	rpoB	S531L (TCG/TTG)
	rpoB	S531W (TCG/TGG)
	rpoB	L533P (CTG/CCG)
Isoniazid	*katG*	S315T(AGC/ACC)
	inhA[b]	-15 (C/T)
Fluoroquinolones	*gyrA*	A90V (GCG/GTG)
	gyrA	D94G (GAC/GGC)
Amikacin		
Kanamycin	*rrs*	1401 (A/G)
Capreomycin		

[a]: Wild type amino acid, codon number, mutated amino acid (wild type codon/mutated codon)
[b]: mabA-*inhA* promoter

Diagnostic method for drug resistant TB, the challenge ahead

The infection status of TB is diagnosed using methods like skin test, chest X-ray, computerised tomography (CT) scan or smear microscopy[13]. These methods are not suitable for typing of TB drug resistance. Culture (i.e., growing MTB on different antibiotic containing medium tubes) is the gold standard for diagnosis of drug-resistant TB. However, one major limitation here is the long turnaround time (weeks to months) due to the slow growth of the bacteria. A few diagnostic methods use amplification techniques like polymerase chain reaction (PCR) to detect the nucleic acids that are specific to MTB while a few detect the mutations that render MTB strains resistant to antibiotics [14]. Though mutations can be detected with high sensitivity and specificity using molecular techniques based on PCR, they cannot be performed in a highly multiplex format. Padlock probes, explained below are potentially better detectors of mutations (single nucleotide polymorphisms) in a multiplex format[15].

Padlock probes, detectors of single nucleotide variants

A padlock probe is a single stranded DNA molecule (70-110 nucleotides long) comprising two target-specific sequences (15-20 nucleotides long) at both ends connected by a linker segment (40-70 nucleotides long) containing sequences for amplification and identification. The probe is designed to bring the two target specific ends (binding arms) into juxtaposition upon hybridization to the specific target sequence. This conformation allows a DNA ligase to catalyze the phophodiester bond formation between the both ends making the probe circularized[16] (Figure1A). A mismatch of the 3'-end nucleotide of the padlock probe

inhibits the circularization of the probe by DNA ligase (see Figure 1B). This high specificity of the padlock probes towards their target enables them to distinguish target molecules that closely resemble one another[16].

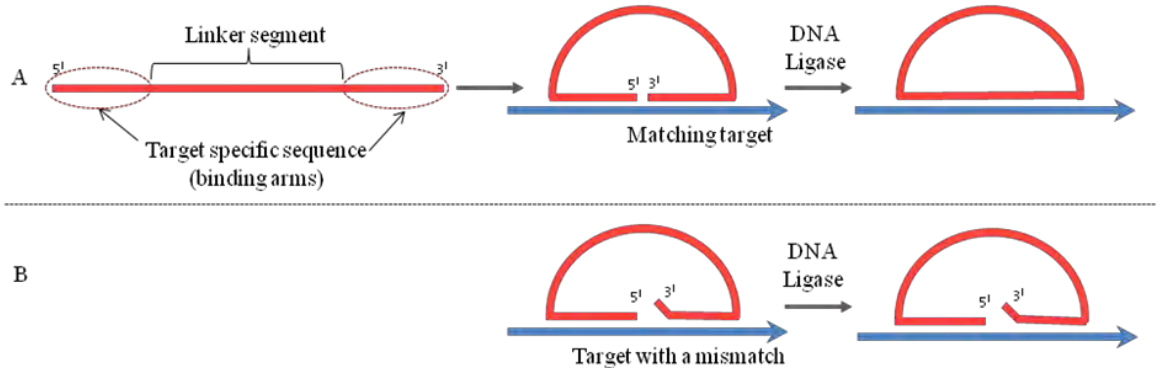

Figure 1. Target dependent padlock probe circularization. A. Perfect complementarity between the binding arms of the padlock probe (red) and the target (blue) brings the arms into juxtaposition after which they can be ligated and circularized by DNA ligase. Circularized padlock probes are wound around the target strand due to the helical nature of DNA (not shown in the figure). B. Mismatch(es) between the ends of binding arm(s) and the target does not bring the arms into juxtaposition and thereby DNA ligase cannot ligate and circularize the probe.

Amplification and detection of padlock probes

The target-dependent circularized padlock probes (the signal) have a diameter of approximately 20 nm which is too small to visualize under the microscope. Hence, the circularized padlock probe is further amplified by rolling circle amplification (RCA) (Figure 2A). During the RCA, the circular padlock probe acts as an endless template. As a result, the padlock probe can be amplified into a long single-stranded concatemer DNA molecule containing approximately 1000 complement copies of the circularized padlock probe after 1 hour (Figure 2B)[17], called rolling circle products (RCPs). The RCPs are digested into monomers using the restriction oligonucleotides (RO) that hybridize once per monomer in a RCP and the restriction enzyme (RE) that recognizes their hybridization (Figure 2C). The monomerized RCPs are further re-ligated into circles by DNA ligase using the RO as a template. Primed by the RO, the circularized monomers are in turn amplified in another round of RCA. This process is called circle to circle amplification (C2CA)[17](Figure 2C). C2CA results in long single stranded DNA molecules (RCPs) that spontaneously collapse into coils with a diameter of approximately 1µm and are concatemers of the padlock probe sequence[18] (shown in Figure 2B).

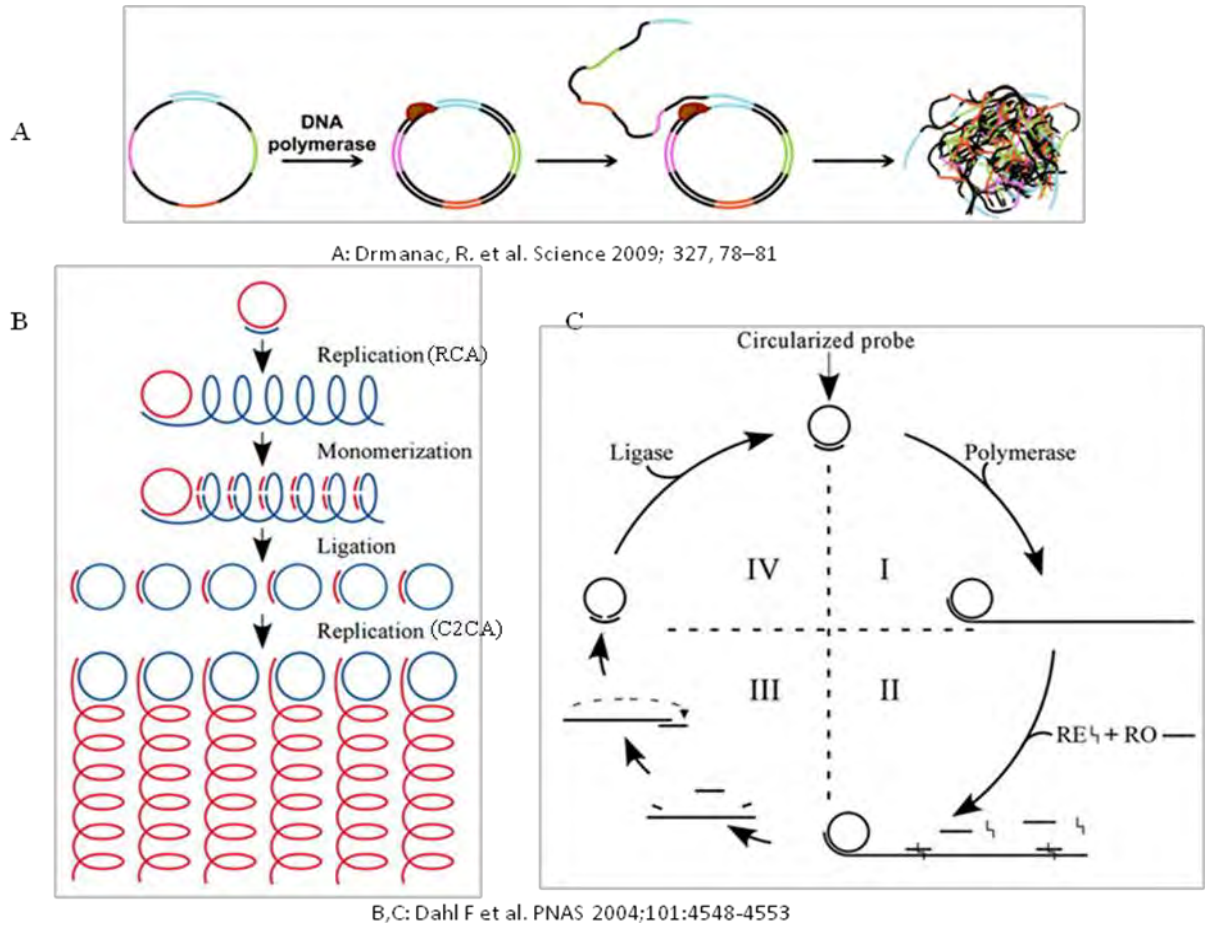

Figure 2. A. Rolling circle replication (RCA). The polymerase uses circular DNA as an endless template and synthesizes a complementary strand beginning at the bound primer (blue). The rotation induced by polymerase unwinds the synthesized strand from the target, and produces many copies of the original circle, linked together as a single molecule (a concatemer)[19–21]. B. Circle to circle amplification (C2CA). The padlock probes of (+) polarity (red) are circularized in a template (blue)-dependent fashion. The circularized padlock probes are extended into long products (RCPs) composed of repeated copies of the circle of (⁻) polarity (blue). The products are restriction digested to monomers and then circularized. These circles then serve as templates for the next RCA, primed by an RO^{+} (red). As a result a long single stranded DNA concatemer with repeats of circularized padlock probe sequence is synthesized[17]. C. Comprehensive explanation of one cycle of C2CA procedure. In step I, the target dependent circularized padlock probe is amplified by a DNA polymerase in RCA mechanism. As a result, a long single stranded concatemeric DNA (RCP) is synthesized. In step II, the polymerase is heat-inactivated, and an RO that hybridizes once per monomer of the RCP is added. RE recognizes a specific sequence in the RO hybridized sites and makes a cut resulting in monomers. In step III, the fragments of RO flanking at the ends of the monomers are dissociated by raising the temperature. As the temperature is lowered, remaining RO (undigested) hybridize to the monomers. In step IV, RO guide the re-ligation of monomers into DNA circles that can be amplified once again in RCA.

Fluorescent molecule–tagged probes (detection oligonucleotides) that are complementary to a portion in the RCPs are used in detecting the amplified signal (Figure 3B). The randomly coiled RCPs after fluorescent labelling appear as clusters of fluorophores which under fluorescence microscope are seen as bright objects with a diameter of approximately 1 µm. Using single-molecule detection method (SMD), individual DNA molecules are detected and quantified by pumping the solution of fluorescently labelled RCPs

through a thermoplastic micro-channel mounted in a standard confocal fluorescence microscope operating in line-scan mode (Figure 3C). The readout from the microscopic scan is further converted to a binary output file, which allows counting and classification of individual objects (RCPs)[18] (Figure 3D). A high count of objects in the solution means that the padlock probe is efficient in recognizing the target.

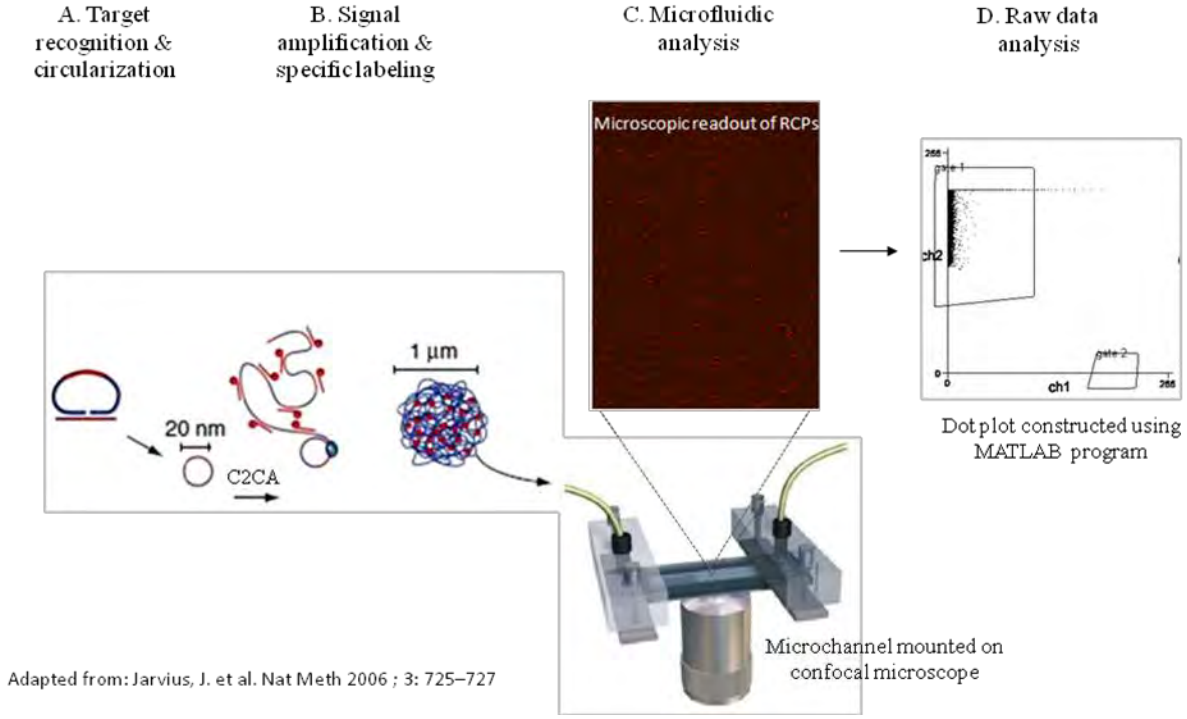

Figure 3. Working mechanism of single-molecule detection (SMD), "Blob counting". The target dependent circularized padlock probe (A) is amplified into RCPs using the C2CA mechanism (B); as a result, the diameter of padlock probe increases from 20 nm to approximately 1 µm. The 1µm diameter RCPs in solution are fluorescently labelled (B) after which they appear like bright objects (red dots in C) when observed under a standard fluorescence microscope. The raw data from the microfluidic analysis are further translated into a binary output using a MATLAB program from which the count of RCPs can be obtained.

The number of RCPs is estimated using the following equation[18]:

$$\textbf{Number of RCPs} = \frac{A \times n \times V_A \times N_a}{1000 \times V_T}$$

Where

Amplification factor $A \cong \left[\left(\frac{1000}{60}\right)^{x-1} \times \left(\prod_{i=1}^{x-1} t_i\right)\right]$ where x is the number of RCAs preformed and t_i is the amplification time in i^{th} RCA

n is the number of moles of padlock probe circularized

V_A is the volume of sample analyzed for detection

V_T is the total volume of sample

N_a is an Avogadro's constant

Solid phase RCA

RCA is often performed on a solid phase (magnetic beads) to simplify the subsequent removal of unreacted padlock probes. In a solid phase RCA, the target is captured using a biotinylated oligonucleotide, called capture oligonucleotide (CO). CO hybridizes at the 5' end of the target without interfering with the padlock probe's hybridization site. When a target specific padlock probe is added to the [CO-template] complex, the padlock probe circularizes on it forming the [CO-target-padlock probe] complex. This complex is in turn coupled to streptavidin coated magnetic beads via biotin-streptavidin affinity interaction. The free 3' end of the target is then digested using the 3'-5' exonuclease activity of the phi 29 DNA polymerase. Once the polymerase encounters the double stranded padlock probe-target, it stops digesting and starts extending the target using the circularized padlock probe as an endless template. As a result, a concatemer of DNA (RCP) hangs from the [magnetic bead-CO-target-padlock probe] complex. The RCP is further restriction digested as described above. As a result, the monomers are released into the solution from the [magnetic bead-CO-target-padlock probe] complex (Figure 4). Once RCPs are monomerized the [magnetic bead-CO-target-padlock probe] complex is removed from the solution using a magnet. The monomers are re-ligated into circles and C2CA is performed as described above and its products are detected using SMD method[26].

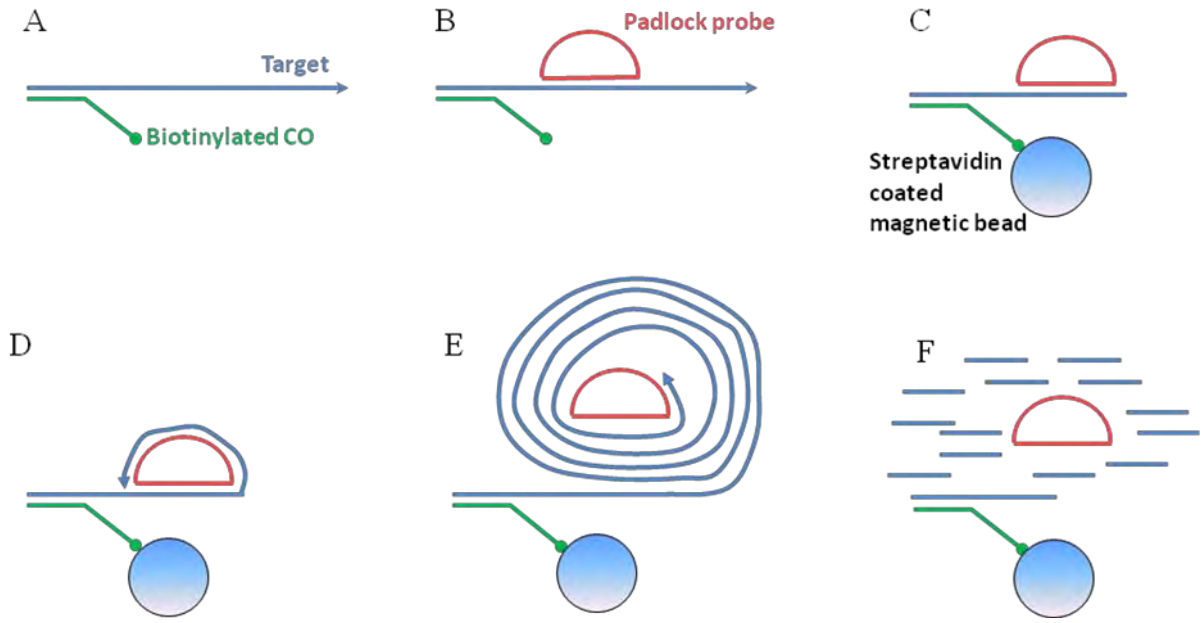

Figure 4. Solid phase RCA. Target is captured using biotinylated CO (A). The padlock probe circularizes upon binding to its respective target and forms a [CO-target-padlock probe] complex (B). This complex in coupled to streptavidin coated magnetic beads and forms a [magnetic bead-CO-target-padlock probe] complex. The phi 29 DNA polymerase (not shown in the above figure) digests the long, free 3' end of the target which is then extended using the padlock probe as an endless template. In RCA mechanism a long concatemeric DNA is synthesized (C, D, E). The concatemeric DNA is restriction digested into monomers and dissociated from the [magnetic bead-CO-target-padlock probe] complex (F).

Aim of the study

The main objective of this study was to develop padlock probes that could efficiently detect the mutations associated with XDR-TB. Padlock probes were designed to detect the common mutations in *rpoB, inhA, katG, gyA,*and *rrs* genes, as well as their corresponding wild type sequences. The efficiency of the designed padlock probes was evaluated using C2CA and SMD approach. Subsequently, the specificity of the padlock probes towards their respective targets was tested.

MATERIALS AND METHODS

Templates

Single stranded synthetic DNA molecules with the sequence of wild type genes (*rpoB, katG, gyrA, rrs*) and respective mutated genes were used as templates (targets) to test the padlock probes. Sequences were obtained from the *M. tuberculosis* H37Rv genome (GenBank accession no. NC_000962; NCBI bank). One amol (=6.02 x 10^5 molecules) of a template was used for testing padlock probes. Templates used are listed in Table 3.

Padlock probes and oligonucleotides

Design of padlock probes:

All the padlock probes had been designed in a way that the last nucleotide in its 3' end binds to the mutated or mutation susceptible nucleotide in the template. Padlock probes were designed for wild type and mutated type sequences of *rpoB* codons 516, 526, 531 and 533; upstream of *inhA* promoter at position -15; *katG* codon 315; *gyrA* codons 90 and 94; and *rrs* codon 1401. Padlock probes for *rpoB* codons 526, 531 and 533 were designed to hybridize on the minus strand (reverse strand) as it was hypothesized that downstream padlock probes would block RCA for upstream probes hybridized to the target. Various versions of padlock probes were designed to each target (Table 2). An in silico model, 'Mfold' was used to predict the secondary structures of the padlock probes[22].

All padlock probes were chemically 5' phosophorylated by mixing 1 µl padlock probe (100 µM) with 1x PNK-buffer A, 1 mM ATP, and 10 U T4 Polynucleotide Kinase to a volume of 100 µl and incubating at 37ºC for 30 min followed by T4PK inactivation at 65ºC for 20 min. Padlock probes and oligonucleotides used are presented in Tables 3, 4 and 5.

Table 2. List of different versions of padlock probes designed for detecting wild type and mutated codons in MTB genes.

Gene	Codon	Wt[a] seq	Mut[b] seq	Versions of the padlock probe	
				Wt	Mut
rpoB	516	GAC	GTC	*rpoB* 516 wt1, wt2	*rpoB* 516 GTC
rpoB			TAC		*rpoB* 516 TAC v[c]1 - v10
rpoB	526	CAC	TAC	*rpoB* 526 wt RS[d]	*rpoB* 526 TAC RS v1 - v9
rpoB			GAC		*rpoB* 526 GAC RS v1 - v7
rpoB	531	TCG	TTG	*rpoB* 531 wt RS v1 - v3	*rpoB* 531 TTG RS v1 - v3
rpoB			TGG		*rpoB* 531 TGG RS v1 - v12
rpoB	533	CTG	CCG	*rpoB* 533 wt RS v1, v2	*rpoB* 533 CCG RS v1 - v6
katG	315	AGC	ACC	*katG* 315 wt v1, v2	*katG* 315 ACC
inhA	-15	C	T	*inhA* -15 wt v1, v2	*inhA* -15T
gyrA	90	GCG	GTG	*gyrA* 90 wt	*gyrA* 90 GTG
gyrA	94	GAC	GGC	*gyrA* 94 wt v1, v2	*gyrA* 94 GGC
rrs	1401	A	G	*rrs* 1401 wt	*rrs* 1401 G

[a]: Wild type [b]: Mutated type [c]: Version [d]: Reverse strand

Ligation and amplification

C2CA

Padlock probes were circularized in a template-dependent fashion by preparing 20 µl of ligation mixture that has 10 µl of 0.1pM (1amol) synthetic MTB template, 1 x Ampligase buffer, 250 mU/µl Ampligase (Epicenter), 100 nM of 5' phosphorylated padlock probe, 0.2 µg/µl bovine serum albumin (BSA), and 50 nM of the biotinylated capture oligonucleotide (CO) followed by incubation at 60°C for 5 min. Using 50 µg MyOne Dynabeads T1 (Invitrogen) that could bind to the biotin on CO, the non-circularized padlock probes were washed away with 1xWtw buffer (10 mM TRIS-HCl pH 7.5, 5 mM EDTA, 0.1% Tween-20, 0.1 M NaCl). By adding an amplification mixture of 20 µl (composed of 125 µM dNTP, 0.2 µg/µl of BSA, 1x Phi29 buffer, 100 mU/µl Phi 29 DNA polymerase (Thermo Fisher Scientific)) to the Dynabead-CO-template-padlock probe complexes, RCA of the templates using circularized padlock probe as endless mother strand was performed for 20 min at 37ºC followed by inactivation at 65ºC for 1 min. The amplified templates (RCA products) were monomerized by adding restriction oligo (RO) L11060 (120 nM) complementary to the replication sequence in the RCA products together with 40 mU/µl of *Alu*I in 1 x Phi 29 buffer. Monomerization was done by incubation at 37ºC for 1 min followed by inactivation at 65ºC for 1 min. After monomerization the beads were discarded, the monomerized RCA products were circularized in RO-dependent fashion and amplified once again (C2CA) by adding 25 µl of amplification mix that was prepared using 14 mU/µl T4 DNA ligase, 1 x Phi 29 buffer, 0.2 µg/µl BSA, 0.67 mM ATP, 100 µM dNTP, 60 mU/µl Phi 29 DNA polymerase and incubated at 37ºC for 20 min followed by inactivation at 65ºC for 1 min.

Quantification of RCA products

The resultant RCA products (RCPs) after C2CA were fluorescently labelled by adding a labelling mix (50 µl) containing 5 nM detection oligo (DO) L10921 complementary to a sequence in RCP, 20 mM EDTA, 20 mM tris-HCl, 10% Tween-20 and 1 M NaCl and incubated for 2 min at 70ºC followed by 15 min at 55ºC. DOs hybridize all over the RCPs making them visible as a cluster of flurophores sized about 1 µm. The labelled RCPs were pumped through a micro-channel mounted in a standard confocal fluorescence microscope operating in a line-scan mode, and individual DNA molecules were detected and quantified. About 2.5 µl of labelled RCPs was analyzed. In the current study, RCP counts higher than 15,000 were considered to be good signal amplification.

Table 3: List of templates and oligonucleotides used.

ID	Name	Sequence (5'-3')	Kind of oligo	Modification 5'	Modification 3'
L10919	TB rpoB wt SW	ATCACACCGCAGACGTTGATCAACATCCGGCCGGTGGTCGCCGCGATCAAGGAGTTCTTCGGCACCAGCCAGCTGAGCCAATTCATGGACCAGAACAACC CGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTGGGGCCCGGCGGTCTGTCACGT	Template	-	-
L11488	TB rpoB 516 GTC	ATCACACCGCAGACGTTGATCAACATCCGGCCGGTGGTCGTCTCTCCCAGCTGAGCCAATTCATGGTCCAGAACAACCCGCTG	Template	-	-
L11487	TB rpoB 516 TAC	ATCACACCGCAGACGTTGATCAACATCCGGCCGGTGGTCGTCTCTCCCAGCTGAGCCAATTCATGTACCAGAACAACCCGCTG	Template	-	-
L11800	TB rpoB SW RS wt	GGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACCTCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTT GTGGGTCAACCCCGACAGCGGGTTGTT	Template	-	-
L11801	TB rpoB SW RS mut	GGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACCTCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCAACAGTCGGCGCTT GTGGGTCAACCCCGACAGCGGGTTGTT	Template	-	-
L11881	rpoB SW RS 526 TAC	GGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACCTCCAGCCCGGCACGCTCACGTGCCCCAGCGCCGACAGTCGGCGCTTGTAGGTCAACCCCG ACAGCGGGTT	Template	-	-
L11882	rpoB SW RS 526 GAC	GGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACCTCCAGCCCGGCACGCTCACGTGCCCCAGCGCCGACAGTCGGCGCTTGTCGGTCAACCCCGA CAGCGGGTT	Template	-	-
L11880	rpoB SW RS 531 TGG	GGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACCTCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCCACAGTCGGCGCTTGTGGGTCAA	Template	-	-
L11879	rpoB SW RS 533 CCG	GGCACATCCGGCCGTAGTGCGACGGGTGCACGTCGCGGACCTCCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCGGCGCCGACAGTCGGCGCTTGTGGGTC	Template	-	-
L11857	mabA-inhA SW wt	GTAACCCCAGTGCGAAAGTTCCCGCCGGAAATCGCAGCCACGTTACGCTCGTGGACATACCGATTTCGGCCCGGCCGCGGCGAGACGATAGGTTGTCGGG GTGACTGCCACAGCCACTGAAGGGGCCAAACCCCCATTCGTATCCCGTTCAGTC	Template	-	-
L11877	gyrA SW wt	TTCCGCCCGGACCGCAGCCACGCCAAGTCGGCCCGGTCGGTTGCCGAGACCATGGGCAACTACCACCCGCACGGCGACGCGTCGATCTACGACAGCCTGGTGC GCATGGCCCAGCCCTG	Template	-	-
L12559	inhA -15 T target	GTAACCCCAGTGCGAAAGTTCCCGCCGGAAATCGCAGCCACGTTACGCTCGTGGACATACCGATTTCGGCCCGGCCGCGGCGAGATGATAGGTTGTCGGGGTGA CTGCCACAGCC	Template	-	-
L12560	katG 315 ACC target	GAACCCGAGGCTGCTCCGCTGGAGCAGATGGGCTTGGGCTGGAAGAGCTCGTATGGCACCGGAACCGGTAAGGACGCGATCACCACCGGCATCGAGGTCGTATGGAC	Template	-	-
L12561	gyrA 90 GTG target	TTCCGCCCGGACCGCAGCCACGCCAAGTCGGCCCGGTCGGTTGCCGAGACCATGGGCAACTACCACCCGCACGGCGACGTGTCGATCTACGACAGCCTGGTGCGCA	Template	-	-
L11859	katG SW wt	GAACCCGAGGCTGCTCCGCTGGAGCAGATGGGCTTGGGCTGGAAGAGCTCGTATGGCACCGGAACCGGTAAGGACGCGATCACCAGCGGCATCGAGGTCG TATGGACGAACACCCCGACGAAATGGGACAACAGTTTCCTCGAGATCCTGTACG	Template	-	-
L12720	mabA-inhA SW wt v2	GTAACCCCAGTGCGAAAGTTCCCGCCGGAAATCGCAGCCACGTTACGCTCCCCGGCCGCGGCGAGACGATAGGTTGTCGGGGTG	Template	-	-
L12721	katG SW wt v2	GAACCCGAGGCTGCTCCGCTGGAGCAGATGGGCTTGGGCTGGAAGAGCTCGTATGGTAAGGACGCGATCACCAGCGGCATCGAGGTCGTATG	Template	-	-
L12722	gyrA SW wt v2	TTCCGCCCGGACCGCAGCCACGCCAAGTCGGCCCGGTCGGTTGCCGAGACACCCGCACGGCGACGCGTCGATCTACGACAGCCTGGTGCGCATG	Template	-	-
L12562	gyrA 94 GGC target	TTCCGCCCGGACCGCAGCCACGCCAAGTCGGCCCGGTCGGTTGCCGAGACCATGGGCAACTACCACCCGCACGGCGACGCGTCGATCTACGGCAGCCTGGTGCGC ATGGCCCA	Template	-	-
L12544	rrs 1401 A-G template	TGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCGCGTCATGAAAGTCGGTAACA	Template	-	-
L11792	TB SW rrs 1401 wt	TGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACA CCCGAAGCCAGTGGCCTAACCCTCGGGAGGGAGCTGTCGAAA	Template	-	-
L10918	TB rpoB CO	CTCTCTCTCTCTCTCTCTCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGAT	Capture oligo	5' Biotin	-
L11783	TB rpoB CO RS	CTCTCTCTCTCTCTCTCTGTCCGCGACGTGCACCCGTCGCACTACGGCCGGATGTGCC	Capture oligo	5' Biotin	3' Biotin
L11858	mabA-inhA CO	CTCTCTCTCTCTCTCTCTTGGCTGCGATTTCCGGCGGGAACTTTCGCACTGGGGTTAC	Capture oligo	5' Biotin	-
L11878	gyrA CO	CTCTCTCTCTCTCTCTCTCCGACCGGGCCGACTTGGCGTGGCTGCGGTCCGGGCGGAA	Adapter	5' Biotin	-
L11860	katG A CO	CTCTCTCTCTCTCTCTCTTTCCAGCCCAAGCCCATCTGCTCCAGCGGAGCAGCCTCGGGTTC	Capture oligo	5' Biotin	-
L11790	TB CO rrs 1401	CTCTCTCTCTCTCTCTCTCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCA	Capture oligo	5' Biotin	-
L11060	BNL_AluI_RO	GTGTATGCAGCTCCTCAGTA	Restriction oligo	-	-

Table 4: List of padlock probes used.

ID	Name	Sequence (5'-3')	Kind of oligo	Modification 5'	Modification 3'
L11764	rpoB 516 GTC	CCATGAATTCGCTCAGCTGCGATTCGAGAACGTGACTGCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGACAGCGGGTTGTTCTGGA	Padlock probe	-	-
L11765	rpoB 531 wt RS	GGCGCTGGGGCCCGGAGTGTCCTCCGAGCTGAGATGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCCACAAGCGCCGACTGTC	Padlock probe	-	-
L11766	rpoB 531 wt RS_1	GGCGCTGGGACCCGGAGTGTCCTCCGAGCTGAGATGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCCACAAACGCCGACTGTC	Padlock probe	-	-
L11767	rpoB 531 TTG RS	GGCGCTGGGGCCCGGCGAGTTCCCGTGCGTTAGATCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCCCACAAGCGCCGACTGTT	Padlock probe	-	-
L11768	rpoB 531 TTG RS_1	GGCGCTGGGGCCCGACGAGTTCCCGTGCGTTAGATCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCCCACAAGAGCCGACTGTT	Padlock probe	-	-
L11769	rpoB 533 wt RS	GGGGCCCGGCGGTCTGTAGATTACGGACTGTGCTTTGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGCGCCGACTGTCGGCGCT	Padlock probe	-	-
L11772	rpoB 526 wt RS	ACAAGCGCCGACTGTCGGAGATCGCAGGGTATCGCATCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCGCTGTCGGGGTTGACCC	Padlock probe	-	-
L11784	TB_rrs 1401 wt	GACGGGCGGTGTGTACAAGCACCACACTGGAGCGGTAATGTGTATGCAGCTCCTCAGTAATAGTGTCTTAAGTTACCGACTTTCATGACGT	Padlock probe	-	-
L11785	TB rrs 1401 A-G	GACGGGCAGTGTGTACAAGGCTATGCCTTCCGCGTGATTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGTTACCGACTTTCATGACGC	Padlock probe	-	-
P5263	katG 315 wt	TGGTGATCGCGTCCTTACCACAGGTCATCGAACTCTCAGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACATACGACCTCGATGCCGC	Padlock probe	-	-
P5264	katG 315 wt_1	TGGTGATCGCGTCCTTACCCCACTGGCTACCGATGAGTAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACATACGACCTCGATGCCGC	Padlock probe	-	-
P5265	inhA -15 wt	TCTCGCCGCGGCCGGGGCGATGTTCGTAACCCTTAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACACCCCGACAACCTATCG	Padlock probe	-	-
P5266	inhA -15 wt_1	TCTCGCCGCGGCCGGAGTAGCTCTAGCCGGTGACAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACACCCCGACAACCTATCG	Padlock probe	-	-
P5268	rpoB 531 wt RS_2	GGCGCTAGGGCCCGGGCTGTTCCGTAAGTGTGATCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCCACAAACGCCGACTGTC	Padlock probe	-	-
P5273	gyrA 94 1st wt	GTAGATCGACGCGTCGCACGCTAATGACGGCAGTGCAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACATGCGCACCAGGCTGTC	Padlock probe	-	-
P5274	gyrA 94 2nd wt	CGTAGATCGACGCGTCGCCGTCGGTTCAGGTCATATGTGTATGCAGCTCCTCAGTAATAGTGTCTTACATGCGCACCAGGCTGT	Padlock probe	-	-
P5285	rpoB 516 1st wt	CATGAATTGGCTCAGCTGGCGGCTCAGTGGTACGACATAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACACAGCGGGTTGTTCTGGTC	Padlock probe	-	-
P5286	rpoB 516 2nd wt	CCATGAATTGGCTCAGCTGAGGTCAGTTCACGAAGCTCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACACAGCGGGTTGTTCTGGT	Padlock probe	-	-
P5409	rpoB 516 TAC v1	CATGAATTGGCTCAGCTCGTTCCTAAAGCTGAGTCTGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACAGCGGGTTGTTCTGGTA	Padlock probe	-	-
P5410	rpoB 516 TAC v2	CATGAATTGGCTCAGCCGTTCCTAAAGCTGAGTCTGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACAGCGGGTTGTTCTGGTA	Padlock probe	-	-
P5411	rpoB 516 TAC v3	CATGAATTGGCTCAGCTACGTTCGTCAAGAGTCGCATGTGTATGCAGCTCCTCAGTAATAGTGTCTTACAGCGGGTTGTTCTGGTA	Padlock probe	-	-
P5412	rpoB 516 TAC v4	CATGAATTGGCTCAGCTACGTTCGTCAAGAGTCGCATGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCGGGTTGTTCTGGTA	Padlock probe	-	-
P5413	rpoB 516 TAC v5	CATGAATTGGCTCAGCTGCAGGGAATTGCCGACCATAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGGGTTGTTCTGGTA	Padlock probe	-	-
P5414	rpoB 516 TAC v6	CATGAATTGGCTCAGCTTAGATCAGTTGGACTCGATGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCGGGTTGTTCTGGTA	Padlock probe	-	-
P5415	rpoB 516 TAC v7	CATGAATTGGCTCAGCTCGTGCAAGTTACCGAGCTGAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACAGCGGGTTGTTCTGGTA	Padlock probe	-	-
P5416	rpoB 516 TAC v8	CATGAATTGGCTCAGCTTCGTGCCTTGTCATTCGGGAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGCGGGTTGTTCTGGTA	Padlock probe	-	-
P5417	rpoB 516 TAC v9	CATGAATTGGCTCAGCTTCGTGCCTTGTCATTCGGGAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACAGCGGGTTGTTCTGGTA	Padlock probe	-	-
P5418	rpoB 516 TAC v10	CATGAATTGGCTCAGCTCGTGCCTTGTCATTCGGGAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGCGGGTTGTTCTGGTA	Padlock probe	-	-
P5419	rpoB 526 GAC RS v1	ACAAGCGCCGACTGTAGTAGCCGTGACTATCGAGTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGTCGGGGTTGACCG	Padlock probe	-	-
P5420	rpoB 526 GAC RS v2	ACAAGCGCCGACTGTAGTAGCCGTGACTATCGAGTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTGTCGGGGTTGACCG	Padlock probe	-	-
P5421	rpoB 526 GAC RS v3 extra TTT	ACAAGCGCCGACTGTTTTAGTAGCCGTGACTATCGAGTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTTTTGTCGGGGTTGACCG	Padlock probe	-	-
P5422	rpoB 526 GAC RS v4	ACAAGCGCCGACTGTCAGAGTGTACCGACCTCGTAAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCTGTCGGGGTTGACCG	Padlock probe	-	-
P5423	rpoB 526 GAC RS v5	ACAAGCGCCGACTGTCATGTGACCCTGGATACCCGTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCTGTCGGGGTTGACCG	Padlock probe	-	-
P5424	rpoB 526 GAC RS v6	ACAAGCGCCGACTGTCTTTATGTGACCCTGGATACCCGTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTTTCTGTCGGGGTTGACCG	Padlock probe	-	-
P5425	rpoB 526 GAC RS v7	ACAAGCGCCGACTGTCGCGAGTGCTCCGTGCGAAATAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCTGTCGGGGTTGACCG	Padlock probe	-	-
P5426	rpoB 526 TAC RS v1	ACAAGCGCCGACTGTCGGCGGTTGATGTCATGTGTCGCACGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCCCGCTGTCGGGGTTGACCT	Padlock probe	-	-
P5427	rpoB 526 TAC RS v2	ACAAGCGCCGACTGTGTTGATGTCATGTGTCGCACGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTGTCGGGGTTGACCT	Padlock probe	-	-
P5428	rpoB 526 TAC RS v3	ACAAGCGCCGACTGTCATTAACTCGACTGCCGCGTGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCTGTCGGGGTTGACCT	Padlock probe	-	-
P5429	rpoB 526 TAC RS v4	ACAAGCGCCGACTGTCAACAACGATGAGACCGGGCTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCTGTCGGGGTTGACCT	Padlock probe	-	-
P5430	rpoB 526 TAC RS v5	ACAAGCGCCGACTGTAACAACGATGAGACCGGGCTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTGTCGGGGTTGACCT	Padlock probe	-	-

Table 5: List of padlock probes used.

ID	Name	Sequence (5'-3')	Kind of oligo	Modification 5'	Modification 3'
P5431	rpoB 526 TAC RS v6	ACAAGCGCCGACTGTATTGACCAAACTGCGGTGCGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTGTCGGGGTTGACCT	Padlock probe	-	-
P5432	rpoB 526 TAC RS v7	ACAAGCGCCGACTGTCCTCGACCGTTAGCAGCATGAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCTGTCGGGGTTGACCT	Padlock probe	-	-
P5433	rpoB 526 TAC RS v8	ACAAGCGCCGACTGTCGCCGAGATGTACCGCTATCGTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGCTGTCGGGGTTGACCT	Padlock probe	-	-
P5434	rpoB 526 TAC RS v9 mm	ACAAGCGCCGACTGTCGCCGAGATGTACCGCTATCGTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGCTGTCTGGGTTGACCT	Padlock probe	-	-
P5459	rpoB 531 TGG RS v1	GGCGCTGGGGCCCGTAGGACTTGCGTCTCTGTGCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACACAAGCGCCGACTGTG	Padlock probe	-	-
P5460	rpoB 531 TGG RS v2	GGCGCTGGGGCCCGGTAGGACTTGCGTCTCTGTGCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACACAAGCGCCGACTGTG	Padlock probe	-	-
P5461	rpoB 531 TGG RS v3	GGCGCTGGGGCCCGGATCCGGTCTCATCGCTGAATGTGTATGCAGCTCCTCAGTAATAGTGTCTTACACAAGCGCCGACTGTG	Padlock probe	-	-
P5462	rpoB 531 TGG RS v4	GGCGCTGGGGCCCATCCGGTCTCATCGCTGAATGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCAAGCGCCGACTGTG	Padlock probe	-	-
P5463	rpoB 531 TGG RS v5 extra TTT mismatch	GGCGCTGGGGCCCTTTATCCGGTCTCATCGCTGAATGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTTTCAAGCACCGACTGTG	Padlock probe	-	-
P5464	rpoB 531 TGG RS v6	GGCGCTGGGGCCCCTCACTAATCGTCTGCGGTGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCAAGCGCCGACTGTG	Padlock probe	-	-
P5465	rpoB 531 TGG RS v7	GGCGCTGGGGCCCGCTCACTAATCGTCTGCGGTGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCAAGCGCCGACTGTG	Padlock probe	-	-
P5466	rpoB 531 TGG RS v8 mismatch	GGCGCTGGGGCCCGGCACTAACTGGTCTGGGTCAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACACAAGCACCGACTGTG	Padlock probe	-	-
P5467	rpoB 531 TGG RS v9 extra TTT	GGCGCTGGGGCCCTTTGCACTAACTGGTCTGGGTCAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTTTACAAGCGCCGACTGTG	Padlock probe	-	-
P5468	rpoB 531 TGG RS v10	GGCGCTGGGGCCCGGCTGGAATGTGACCGTGCTCTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACACAAGCGCCGACTGTG	Padlock probe	-	-
P5469	rpoB 531 TGG RS v11	GGCGCTGGGGCCCGCTGGAATGTGACCGTGCTCTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCAAGCGCCGACTGTG	Padlock probe	-	-
P5470	rpoB 531 TGG RS v12 extra TTT	GGCGCTGGGGCCCGTTTCTGGAATGTGACCGTGCTCTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTTTCAAGCGCCGACTGTG	Padlock probe	-	-
P5471	rpoB 533 CCG RS v1 extra TTT	GGGGCCCGGCGGTCTTTGCGTGTGTGGACTCTCTCTAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTTTCCGACTGTCGGCGCC	Padlock probe	-	-
P5472	rpoB 533 CCG RS v2	GGGGCCCGGCGGTCTGCGTGTGTGGACTCTCTCTAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCCGACTGTCGGCGCC	Padlock probe	-	-
P5473	rpoB 533 CCG RS v3 mismatch	GGGGCCCGGCGGTCTGCGTGTGTGGACTCTCTCTAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCCGACAGTCGGCGCC	Padlock probe	-	-
P5474	rpoB 533 CCG RS v4	GGGGCCCGGCGGTCTATTTGACGAACGTATGCCGCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCCGACTGTCGGCGCC	Padlock probe	-	-
P5475	rpoB 533 CCG RS v5	GGGGCCCGGCGGTCTATTTGACGAACGTATGCCGCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGCCGACTGTCGGCGCC	Padlock probe	-	-
P5476	rpoB 533 CCG RS v6 mismatch	GGGGCCCGGCAGTATTTGACGAACGTATGCCGCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACCGACTGTCGGCGCC	Padlock probe	-	-
P5587	gyrA 94 GGC	CGTAGATCGACGCGTCGCTGCCTGCTGTAGACTCGTAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACATGCGCACCAGGSTGC	Padlock probe	-	-
P5588	gyrA 90 wt	CGTCGCCGTGCGGGTTACCGCGCTTGGGACATGATGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTGTCGTAGATCGACG	Padlock probe	-	-
P5589	gyrA 90 GTG	CGTCGCCGTGCGGGTGTGCCTCGTTCTCGTGTAGAGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTGTCGTAGATCGACA	Padlock probe	-	-
P5590	katG 315 ACC	TGGTGATCGCGTCCTTACCGAGTAGCCTTCCCGAGCATTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACATACGACCTCGATGCCGG	Padlock probe	-	-
P5591	inhA -15 T	TCTCGCCGCGGCCGGCAATCTAGTATCAGTGGCGCGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGTCACCCCGACAACCTATCA	Padlock probe	-	-

Evaluation of padlock probes

Various versions of padlock probes that were designed were evaluated on their respective synthetic MTB DNA using the specific capture probes and detection probes. List of the padlock probes that were evaluated and their respective targets, COs used are shown in Table 6.

Table 6. List of targets and COs used in evaluating the padlock probes.

Padlock probes	Targets	CO
rpoB 516 TAC v1 - v10	L11487	L10918
rpoB 526 TAC RS v1 - v9	L11881	L11783
rpoB 526 GAC RS v1 - v7	L11882	L11783
rpoB 531 TGG RS v1 - v12	L11880	L11783
rpoB 533 CCG RS v1 - v6	L11879	L11783
inhA -15 wt v1, v2	L11857	L11858
	L12720[a]	L11858
inhA -15T	L12559	L11858
katG 315 wt v1, v2	L11859	L11860
	L12721[a]	L11860
katG 315 ACC	L12560	L11860
gyrA 90 wt	L11877	L11878
gyrA 90 GTG	L12561	L11878
gyrA 94 wt v1, v2	L11877	L11878
	L12722[a]	L11878
gyrA 94 GGC	L12562	L11878
rrs 1401 G	L12544	L11790

[a]: Truncated version of the target above it.

Specificity tests

The specificity of the padlock probes was investigated by testing mutation specific padlock probes (hereafter denoted mutated type padlock probes) on wild type templates and vice versa. The specificity of the mutated type padlock probes was evaluated by preparing four different samples (A, B, PC and NC) which were amplified by C2CA and detected using SMD method. Sample 'A' is a mixture of wild type target and all respective mutation specific padlock probes; Sample 'B' is a mix of wild type target, its respective mutation specific padlock probes and a padlock probe that is specific to the wild type target in the mix. Sample 'PC', positive control, has only the wild type target and its respective padlock probe. The negative control (NC) has the padlock probe and water instead of target. The list of mutated type padlock probes evaluated and templates used is shown in Table 7.

Table 7. List of templates, CO and padlock probes used in specificity test for *rpoB* mutated type padlock probes.

Codon	Padlock probes	Target	CO
516	L11764[a]	L10919[b]	L10918
	P5417[a]		
	P5285[b]		
526 (RS)	P5427[a]	L11800[b]	L11783
	P5421[a]		
	P5281[a]		
	P5282[a]		
	P5277[a]		
	L11772[b]		
531 (RS)	L11768[a]	L11800[b]	L11783
	P5466[a]		
	P5268[b]		
533 (RS)	P5471[a]	L11800[b]	L11783
	L11769[b]		

[a]: Mutated type
[b]: Wild type

Sample A= Padlock probes[a] + Target[b]
Sample B= Padlock probes[a] + Padlock probe[b] + Target[b]
PC= Padlock probe[b] + Target[b]
NC = Padlock probe[b] + Water

The specificity of the padlock probes designed to detect the wild type codons (hereafter denoted wild type padlock probes) was evaluated by testing them on the possible mutated targets of a codon (Sample A). Wild type padlock probe on wild type target was used as a positive control (PC) and wild type padlock probe alone with no target was used as a negative control (NC). List of wild type padlock probes evaluated and templates used is shown in Table 8.

Table 8. List of templates and padlock probes used in specificity test for *rpoB* wild type padlock probes.

Codon	Padlock probes	Target	CO
516	P5285[b]	L11488[a]	L10918
		L11487[a]	L10918
		L10919[b]	L10918
526 (RS)	L11772[b]	L11881[a]	L11783
		L11882[a]	L11783
		L11885[a]	L11783
		L11884[a]	L11783
		L11883[a]	L11783
		L11800[b]	L11783
531 (RS)	P5268[b]	L11801[a]	L11783
		L11880[b]	L11783
533 (RS)	L11769[b]	L11879[a]	L11783
		L11880[b]	L11783

[a]: Mutated type [b]: Wild type

Sample A= Padlock probe[b] + Targets[a]
PC= Padlock probe[b] + Target[b]
NC= Padlock probe[b] + Water

RESULTS

Evaluation of *rpoB* specific padlock probes

Different versions of padlock probes designed to detect the mutated type codons in *rpoB* gene (Table 2) were evaluated using the templates and other oligonucleotides listed in Table 6. C2CA and the SMD methods were used in signal amplification and detection respectively.

rpoB 516 TAC v1 – v10: Versions 3, 4, 6, 8 and 9 yielded good number of RCPs (Table 9). Yet, version 8 (P5416) was preferred to detect this mutation for its highest yield of RCPs than that of the rest.

rpoB 526 TAC v1 - v9: Most of the designed versions of padlock probes except versions 5 and 6 yielded an efficient number of RCPs (Table 9). However, version 2 (P5427) that has yielded higher RCPs among than the others was selected for further analysis.

rpoB 526 GAC v1 – v7: All of the designed padlock probes were efficient in detecting this mutation (Table 9). Version 3 (P5421) was preferred among the others for further analysis for its relatively high yield of RCPs.

rpoB 531 TGG v1 – v12: Most of the designed versions of padlock probes were inefficient in recognizing the template except versions 8 and 12 (Table 9). *rpoB* 531 TGG RS v8 (P5466) was chosen for further analysis.

rpoB 533 CCG v1 – v6: Versions 1, 2 and 4 yielded decent number of RCPs (Table 9). However, version 1 (P5471) was selected for further analysis for its relatively high yield of RCPs.

Evaluation of *inhA, katG, gyrA and rrs* specific padlock probes

Various versions of padlock probes designed to detect the wild type as well as mutated codons in *inhA, katG, gyrA* and *rrs* genes (shown in Table 2) were evaluated in C2CA and SMD method. These padlock probes were evaluated using the templates and the oligonucleotides that are listed in Table 6. Wild type padlock probes *inhA* -15 (P5265, P5266), *katG* 315 (P5263, P5264), *gyrA* 94 (P5273, P5274) were observed to be poor recognizers of their respective targets. Padlock probe P5591 was able to detect *inhA* -15T target, and similarly, P5590, P5588, P5589 and L11785 were able to detect *katG* 315 AAC target , *gyrA* SW wt, *gyrA* 90 GTG target and *rrs* 1401 A-G target respectively. *gyrA* 94 GGC (P5587) yielded a poorer number of RCPs (Table 10).

Targets mabA-*inhA* SW wt (L11857), *katG* SW wt (L11859) and *gyrA* SW wt (L11877) specific for wild type padlock probes were shortened in their length to L12720, L12721 and L12722, respectively (shown in Table 3). Wild type padlock probes for upstream of *inhA* at -15 position, codon 315 of *katG* and codon 94 of *gyrA* were evaluated using the templates and oligonucleotides listed in Table 6. At least one wild type padlock probe for each target was identified to yield a normal number of RCPs after truncating the targets.

Padlock probes P5266, P5263 and P5273 have detected targets L12720, L12721 and L12722 respectively (Table 10).

Table 9. Result of the evaluated rpoB specific padlock probes.

Padlock probe (ID)	Number of RCPs	Padlock probe (ID)	Number of RCPs
rpoB 516 TAC v1 (P5409)	776	*rpoB* 531 TGG RS v1 (P5459)	6552
rpoB 516 TAC v2 (P5410)	1328	*rpoB* 531 TGG RS v2 (P5460)	4406
rpoB 516 TAC v3 (P5411)	26301	*rpoB* 531 TGG RS v3 (P5461)	7044
rpoB 516 TAC v4 (P5412)	35837	*rpoB* 531 TGG RS v4 (P5462)	5396
rpoB 516 TAC v5 (P5413)	3921	*rpoB* 531 TGG RS v5 (P5463)	478
rpoB 516 TAC v6 (P5414)	25134	*rpoB* 531 TGG RS v6 (P5464)	2418
rpoB 516 TAC v7 (P5415)	717	*rpoB* 531 TGG RS v7 (P5465)	9885
rpoB 516 TAC v8 (P5416)	74612	*rpoB* 531 TGG RS v8 (P5466)	17124
rpoB 516 TAC v9 (P5417)	68520	*rpoB* 531 TGG RS v9 (P5467)	11031
rpoB 516 TAC v10 (P5418)	3354	*rpoB* 531 TGG RS v10 (P5468)	8152
rpoB 526 TAC RS v1 (P5426)	36039	*rpoB* 531 TGG RS v11 (P5469)	9086
rpoB 526 TAC RS v2 (P5427)	45317	*rpoB* 531 TGG RS v12 (P5470)	12841
rpoB 526 TAC RS v3 (P5428)	37269	*rpoB* 533 CCG RS v1 (P5471)	17113
rpoB 526 TAC RS v4 (P5429)	28880	*rpoB* 533 CCG RS v2 (P5472)	15945
rpoB 526 TAC RS v5 (P5430)	9833	*rpoB* 533 CCG RS v3 (P5473)	14843
rpoB 526 TAC RS v6 (P5431)	5453	*rpoB* 533 CCG RS v4 (P5474)	15945
rpoB 526 TAC RS v7 (P5432)	15163	*rpoB* 533 CCG RS v5 (P5475)	10901
rpoB 526 TAC RS v8 (P5433)	27294	*rpoB* 533 CCG RS v6 (P5476)	5182
rpoB 526 TAC RS v9 (P5434)	25292	NC[a]	41
rpoB 526 GAC RS v1 (P5419)	23926	NC[b]	9
rpoB 526 GAC RS v2 (P5420)	33351	NC[c]	30
rpoB 526 GAC RS v3 (P5421)	42486	NC[d]	6
rpoB 526 GAC RS v4 (P5422)	20145	NC[e]	27
rpoB 526 GAC RS v5 (P5423)	16041		

[a]: Negative control for *rpoB* 516 TAC v1 - v10
[b]: Negative control for *rpoB* 526 TAC v1 – v9
[c]: Negative control for *rpoB* 526 GAC v1 – v7
[d]: Negative control for *rpoB* 531 TGG v1 – v12
[e]: Negative control for *rpoB* 533 CCG v1 – v6

Table 10. Result of the evaluated *inhA, katG, gyrA* and *rrs* specific padlock probes.

Padlock probe (ID)	Number of RCPs
inhA -15 wt (P5265)	920
inhA -15 wt (P5265)[a]	7002
inhA -15 wt_1 (P5266)	758
inhA -15 wt_1 (P5266)[a]	17078
inhA -15 T (P5591)	34440
katG 315 wt (P5263)	6196
katG 315 wt (P5263)[a]	24955
katG 315 wt_1 (P5264)	2764
katG 315 wt_1 (P5264)[a]	10028
katG 315 ACC (P5590)	18329
gyrA 90 wt (P5588)	16262
gyrA 90 GTG (P5589)	20747
gyrA 94 wt_1 (P5273)	6943
gyrA 94 wt_1 (P5273)[a]	14625
gyrA 94 wt_2 (P5274)	1283
gyrA 94 wt_2 (P5274)[a]	2959
gyrA 94 GGC (P5587)	8580
rrs 1401 A-G (L11785)	16227
NC	12
NC[a]	12

[a]: Evaluated on truncated targets

Padlock probes that have yielded the highest number of RCPs were selected from several tested padlock probes. At the end, we had one padlock probe each to detect wild type and mutated codons of *rpoB* codons 516, 526, 531 and 533; upstream of *inhA* promoter at position -15; *katG* codon 315; *gyrA* codons 90 and 94; and *rrs* codon 1401 (Table 11). Based on the number of RCPs yielded, padlock probes are categorized as very high, high, normal and low yielders of RCPs. Except the low yielding padlock probes all the others are further evaluated for their specificity.

Table 11. Summary of the selected padlock probes and their respective RCPs count.

Antibiotic	Gene	Mutation	Number of RCPs	
			Wildtype padlock probe	Mutated padlock probe
Rifampicin	*rpoB*	D516V (GAC/GTC)	P5285*	L11764*
	rpoB	D516Y (GAC/TAC)		P5417
	rpoB	H526Y (CAC/TAC)	L11772*	P5427
	rpoB	H526D (CAC/GAC)		P5421
	rpoB	S531L (TCG/TTG)	P5268*	L11768*
	rpoB	S531W (TCG/TGG)		P5466
	rpoB	L533P (CTG/CCG)	L11769*	P5471
Isoniazid	*katG*	S315T (AGC/ACC)	P5263	P5590
	inhA (= *mabA-inhA* promoter)	-15 (C/T)	P5266	P5591
Fluoroquinolones	*gyrA*	A90V (GCG/GTG)	P5588	P5589
	gyrA	D94G (GAC/GGC)	P5273	P5587
Amikacin, Kanamycin, Capreomycin	*rrs*	1401 A/G	L11784 *	L11785*

*= tested by Anna Engström and re-tested by me

Very high= RCPs>20.000	High=14.000<RCPs<20.000	Normal= 8000<RCPs<14.000	Low=RCPs<8000

Specificity tests for *rpoB* specific padlock probes

Specificity of *rpoB* mutated type padlock probes was evaluated by preparing duplicates of samples (A, B, PC and NC) as mentioned in materials and methods (Table 7). The yield of RCPs by PC of codon 533 was high while that by the PCs of codon 516 and 526 was a bit low. PC of codon 531 yielded a very low count of RCPs. The yield of RCPs by samples A of all codons was quite low while that by the samples B was close to its respective PC's count. NCs of all codons were just as expected to be (Figure 5).

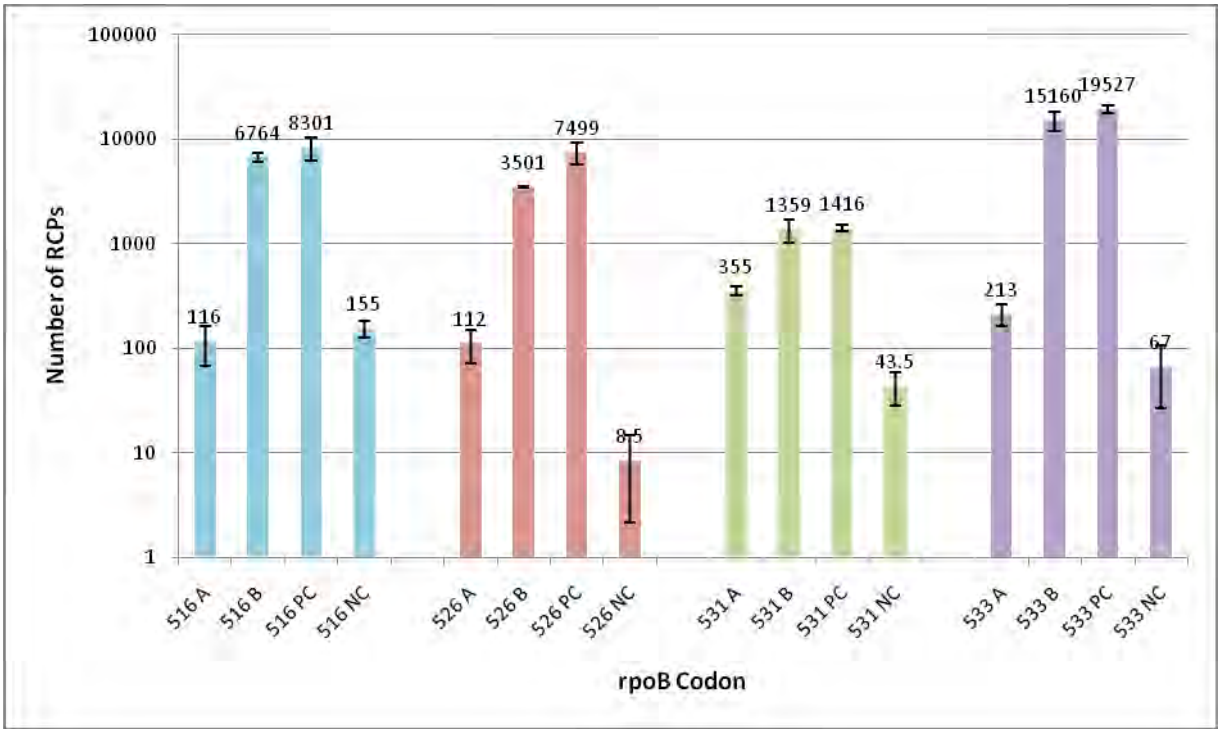

Figure 5. Specificity test for *rpoB* mutated type padlock probes. Samples A, B, PC and NC were prepared using the padlock probes and the targets as listed in Table 7. All the samples were evaluated in duplicates and their average RCP counts are represented in a logarithmic scale on Y-axis of the graphs. The standard deviations obtained by duplicate sample measurements are shown as the error bars in graphs.

Specificity of *rpoB* wild type padlocks was evaluated by preparing duplicates of samples (A, PC and NC) as explained in materials and methods (Table 8). Samples A for all codons except 526 yielded a very low count of RCPs. The yield of RCPs by the PC of codon 516 was high, while that in codon 526 and 533 was a bit low. PC of codon 531 yielded a very low count of RCPs. All NCs are low (Figure 6).

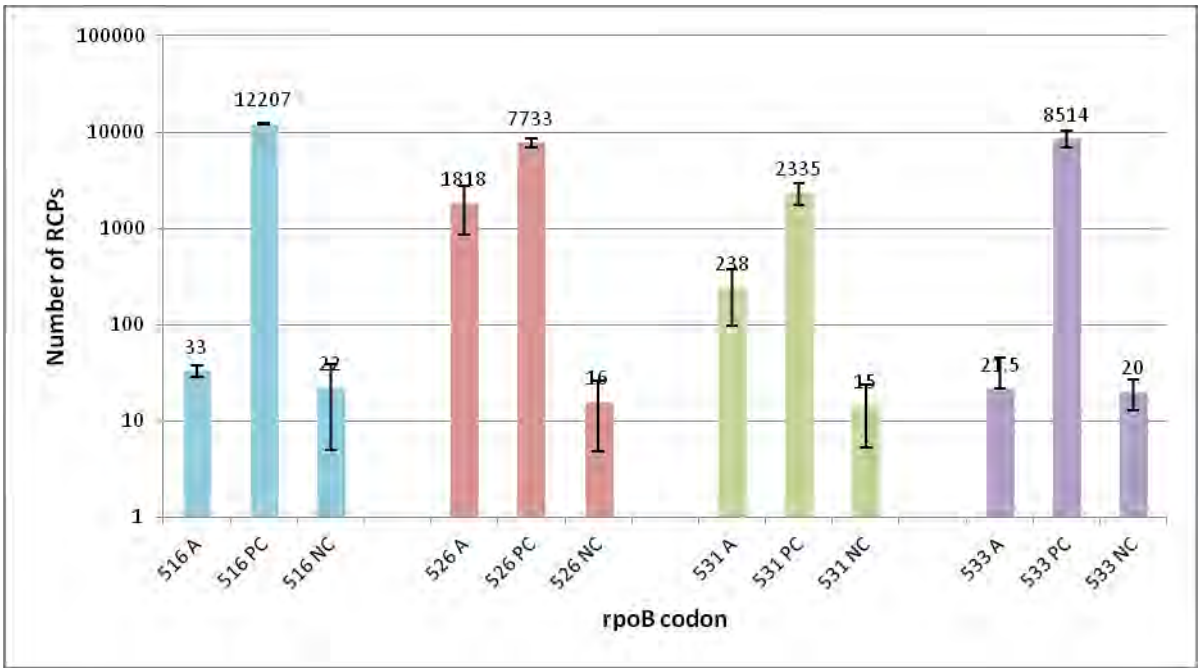

Figure 6. Specificity test for padlock probes specific to *rpoB* wild type codons. Samples A, PC and NC were prepared using the padlock probes and the targets as listed in Table 8. All the samples were evaluated in duplicates and their average RCP counts are represented in a logarithmic scale on Y-axis of the graphs. The standard deviations obtained by duplicate sample measurements are shown as the error bars in graphs.

DISCUSSION

Knowing that it is only the point mutations that confer drug resistance in MTB, various padlock probes were designed to detect these mutations. Padlock probes are generally designed considering many factors like secondary structures, melting temperatures of the binding arms, etc., that would affect the function of padlock probes. All the padlock probes used in this study were designed considering most of the above mentioned factors, and yet, each padlock probe has yielded different counts of RCPs (see Tables 9 and 10). Other than the factors that were considered in the padlock probe's design, experimental factors like ligation temperatures, secondary structures of the template, etc., might affect the functionality of the padlock probe. The secondary structure predicted by the in silico models might not be the true structure of the oligonucleotide. So, even if a padlock probe design is based on the secondary structure predicted by the in silico models it might not work as it is supposed to. We believe that there is a significant correlation between the secondary structures of the padlock probe and its functionality which we are trying to understand. We have noticed, fo example, that the padlock probes with the secondary structure within the binding arms yield low counts of RCPs. The secondary structure motifs in the binding arms of the padlock probes might probably limit their hybridization to the targets resulting in a low signal. We also believe that the strong GC secondary structures in the templates (Figure 7) might impair the intended hybridization of the padlock probes.

Padlock probes yielded different counts of RCPs with templates of different lengths (see Table 10). Shorter templates (≤120 nucleobases) seem to be better substrates for the padlock probes than the longer ones (>120 nucleobases). From the literature search we have found that, although the commercial oligonucleotide synthesis can generate long oligonucleotides (≥150 mers) the problems in the synthesis quality are expected as the oligonucleotide length exceeds 100 nucleobases[23–25]. The problems in synthesis of long oligonucleotides might somehow affect the hybridization of padlock probes to them.

In the specificity test for *rpoB* mutated type padlock probes, the yield of RCPs by mutated type padlock probes on wild type targets was low. Also, the mutated type padlock probes in competition with the wild type padlock probes for wild type targets (samples B) yielded RCPs as much as that by the wild type padlock probes on wild type targets (PCs). These indicate that mutated type padlock probes are highly specific. In the specificity test for *rpoB* wild type padlock probes, the yield of RCPs by wild type padlock probes on mutated type targets (samples A) was low and similar to the yields by the wild type padlock probe on no target (NCs). So, the wild type padlock probes do not exhibit any unspecific target recognition.

The positive controls used in both of the specificity tests were the same (i.e., the wild type padlock probes on wild type targets) but their count of RCPs was not high enough which might be due to strong secondary motifs in the wild type target (L11800, the same for codons 526, 531 and 533). We observed that the codon 531 was involved in a strong secondary structure[22] , which could explain an improper hybridization of the padlock probes to it. Sometimes the padlock probes targeting the same codon might have more or less similar 5'

binding arms but different 3' binding arms. So, when a wild type padlock probe for a codon is in competition with the mutated type padlock probes for the same codon, the mutated type padlock probes can hybridize to the template with their 5' binding arm but not with 3' binding arm. This partial hybridization of the mutated type padlock probes might limit the available templates for the wild type padlock probes to hybridize. This might be the reason for the lower yield of RCPs with sample B of codon 526 than for its positive control. Incubating the padlock probes with templates for longer times, using higher hybridization temperatures or temperature ramping might give a better chance for padlock probes to hybridize onto their targets.

The strong secondary motifs in the templates had made it difficult to design the padlock probes that could efficiently recognize them. By being keen while designing the padlock probes, their specificity and sensitivity to detect the single nucleotide variants can be further improved.

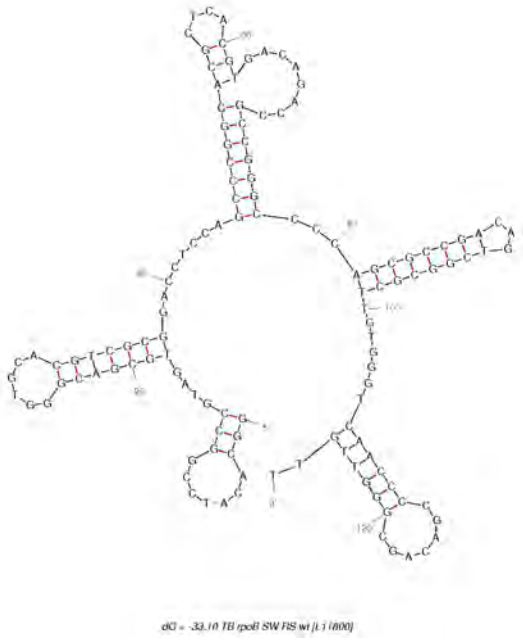

Figure 7. Secondary structure motifs in L11800. Secondary structures in the reverse strand of rpoB gene predicted by Mfold. The codon 531 (CAG) was involved in one of the secondary structures.

FUTURE PLANS

Wild type padlock probe for codons 526 and 531 have to be re-designed to generate better signals. Once we have at least one padlock probe to detect the wild type and mutated type sequences, they have to be tested on genomic TB DNA to confirm their activity. As of yet, the specificity of the padlock probes has been evaluated, next is to test multiplexibility (different padlock probes are tested at a time simultaneously on its respective synthetic target) using a Luminex assay. Based on the results from the multiplexibility experiments, a low density array with immobilized oligonucleotides capable of recognizing RCPs will be ordered and tested. Low density arrays might be preferred for their ease of use and convenient read out format.

TROUBLESHOOTING

At the onset of this thesis work, we got repeatedly lower yields of RCPs in C2CA experiments. Even the system that served as our positive control (padlock probe: P5400 and target: L10919) and known to yield a high number of RCPs did not work properly. Initially the suspicion was on the mishandling of the oligonucleotides or the other reagents that are used in the C2CA protocol. By performing some more C2CA experiments, the problems with the oligonucleotides and reagents were excluded. Then the enzymes that are used in C2CA experiments were tested for their activities. Enzymes that are used in our C2CA experiments are Ampligase and T4 DNA ligase for ligating the padlock probes, phi 29 DNA polymerase for amplifying the padlock probes, and AluI for restriction digestion of the concatemers.

In our lab, the stock of Ampligase was aliquoted and used in experiments, while the rest of the enzymes were used directly from the stock. A few members in the group share the enzymes while a few have their own. However, everyone in the group had low count of RCPs in their experiments. The activities of Ampligase and T4 DNA ligase were evaluated by ligating padlock probes using each of them separately followed by 20 min RCA using phi29 DNA polymerase. We had good number of RCPs and it was a bit higher with the Ampligase than that by the T4 DNA ligase (Figure 8). In this experiment, padlock probes were able to circularize and amplify without any problem. Heading to the next enzyme AluI, its activity was evaluated by performing C2CA with AluI from three different stocks, yet there was no improvement in yield of RCPs. The activities of three different stocks of AluI were later evaluated by restriction digesting the lambda (λ) DNA and running on a 2% agarose gel. Except one, the other two were able to digest the λ DNA in 1 min. However, for longer digestion times (3 and 5 min) all the three were able to digest the λ DNA in the same pattern (Figure 9). From the tests for enzyme activities, all of them were found to be flawless. But a C2CA experiment with these apparently flawless enzymes yielded a low number of RCPs.

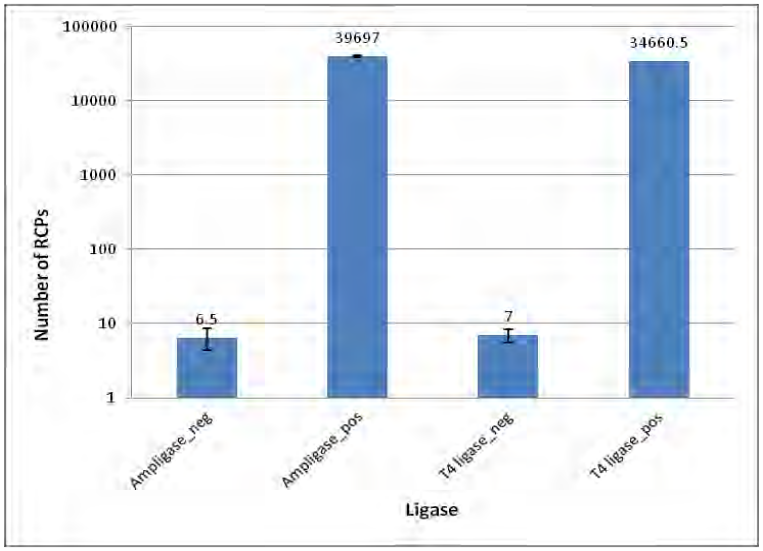

Figure 8. Test for the activities of Ampligase and T4 DNA ligase in 20 minutes RCA. 3µl of 100nM template and 1µl of 100nM padlock probe was used in making 50µl of ligation mix.

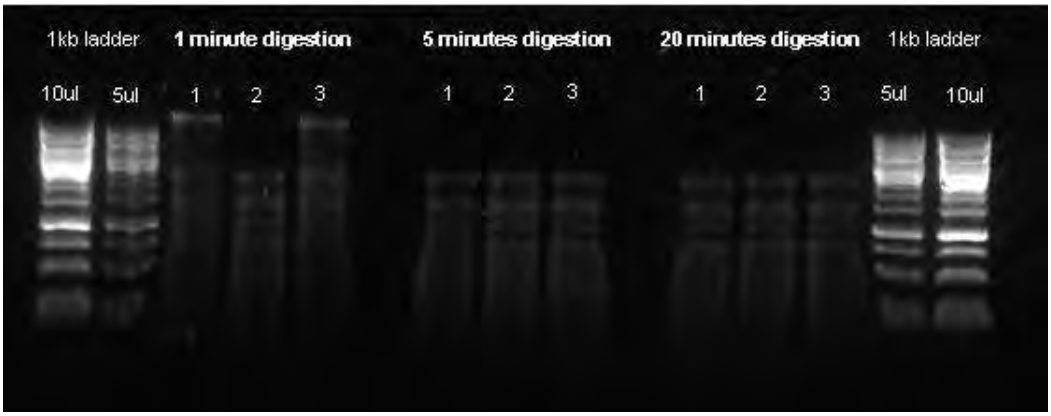

Figure 9. Quality test for AluI from three different stocks. λ DNA was digested at 37^{0}C with AluI for three different times (1,5 and 20min) followed by inactivation of the enzyme at 65^{0}C for 10min.

Q-linea, a company that performs C2CA experiments had also experienced the same low RCPs count. Q-linea had suggested us to try BSA from a different manufacturer in C2CA protocol. BSA is commonly used to stabilize some enzymes during digestion of DNA and to prevent adhesion of the enzyme to reaction tubes, pipette tips, and other vessels. BSA from New England Biolabs is generally used in our C2CA experiments and as suggested by Q-linea, BSA from Sigma and Fermentas BSA (own choice) were also tested in C2CA (Figure 10). But they did not show any improvement in the count of RCPs; instead it was even lower with Sigma BSA and Fermentas BSA than with NEB BSA. Then 1RCA was performed with T4 DNA ligase and in combination with different BSAs to see if the BSA is inhibiting the activity of the T4 DNA ligase. Interestingly, none of the BSAs showed any effect on the activity of the T4 DNA ligase. Then the combinational effect of different BSAs and AluI on monomerization of concatemers was investigated. It took us a few experiments to know the optimum monomers concentration required in order to see them on a 2% agarose gel. At the end we did not notice any effect of BSA on AluI during the digestion (Figure 11).

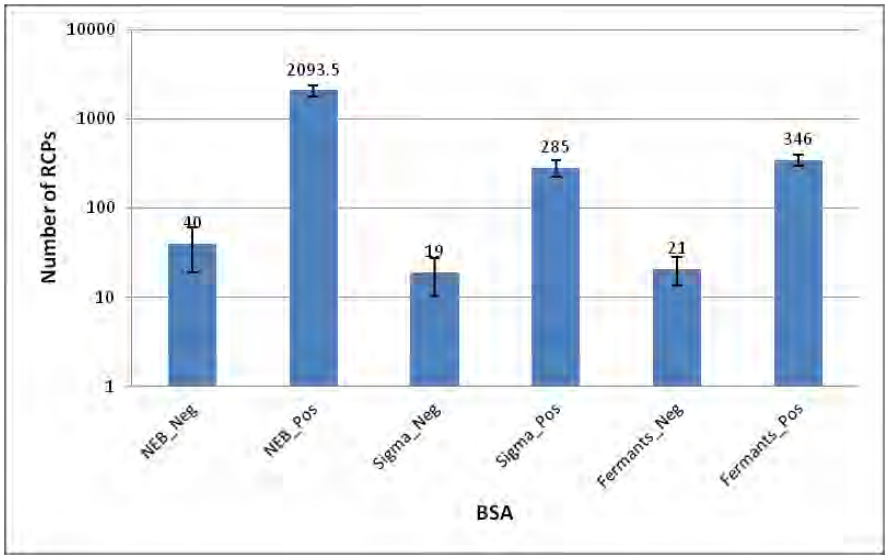

Figure 10. C2CA with BSA from NEB, Sigma and Fermentas (3 different manufacturers). C2CA (20 min) was performed with P5400-L10919 padlock system. 1amol of target was used.

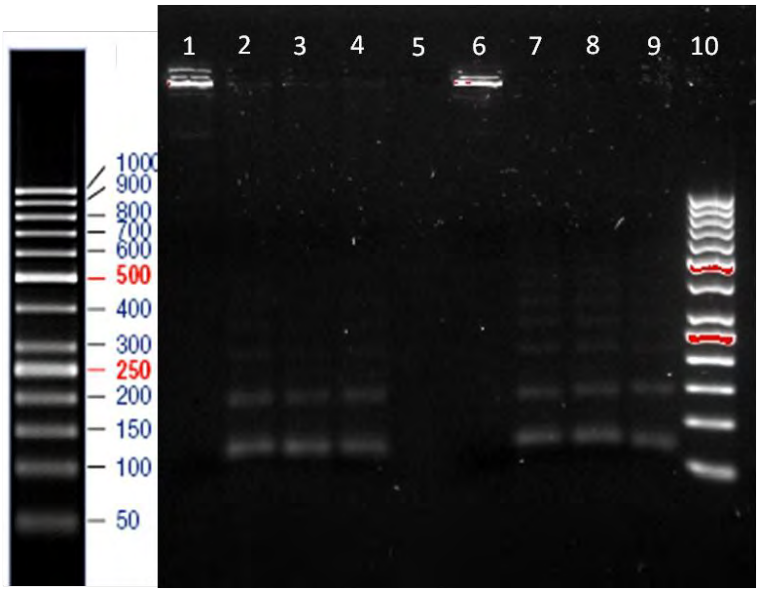

Figure 11. AluI digestion of RCPs in different BSAs (NEB and Fermentas).

Samples treated with NEB BSA during 1RCA & Digestion.
1: Uncut DNA
2: 0.1ul of Alu1
3: 0.24ul of Alu1
4: 0.5ul of Alu1
5: ----------

Samples treated with Fermentas BSA during 1RCA & Digestion
6: Uncut DNA
7: 0.1ul of Alu1
8: 0.24ul of Alu1
9: 0.5ul of Alu1
10: 50bp DNA ladder (5ul)

Once, aliquots of Ampligase were prepared from a new lot and one of them was used to ligate the padlock probes in a C2CA experiment. With new aliquot of Ampligase we had normal count of RCPs as we had before (Figure 12). The reason for Ampligase lowering the yield of RCPs was further investigated in an experiment.

Experiment: Padlock probes were incubated separately with the two different Ampligase enzymes, one was from a new lot and the other was from an old lot. Incubation of padlock probes with Ampligase was not to ligate them but to see if Ampligase had any nuclease activity. The RCPs yielding and non-yielding Ampligase enzymes were also heat inactivated (at 95°C for 10 min). After 1 hour of incubation of padlock probes with the normal and heat inactivated Ampligase enzymes, they were run on a 2% agarose gel (Figure 13). It was observed that the old stock of Ampligase had a nuclease contamination and it disappeared after heat inactivating the enzyme. Ampligase from new stock did not show any nuclease contamination in any of the cases.

Conclusion: Ampligase was nuclease contaminated. So, cleavage of padlock probes by the nucleases inhibited their circularization. Other oligonucleotides involved in the ligation: capture probes and templates could also probably be affected by this nuclease contamination. Though the activity of Ampligase was tested early in the troubleshooting by performing one

round of RCA, the experiment did not give any clue about the Ampligase nuclease contamination. The excessive template used in 1RCA protocol might have been the substrate for nuclease activity instead of the padlock probe; thus making the nuclease activity limiting.

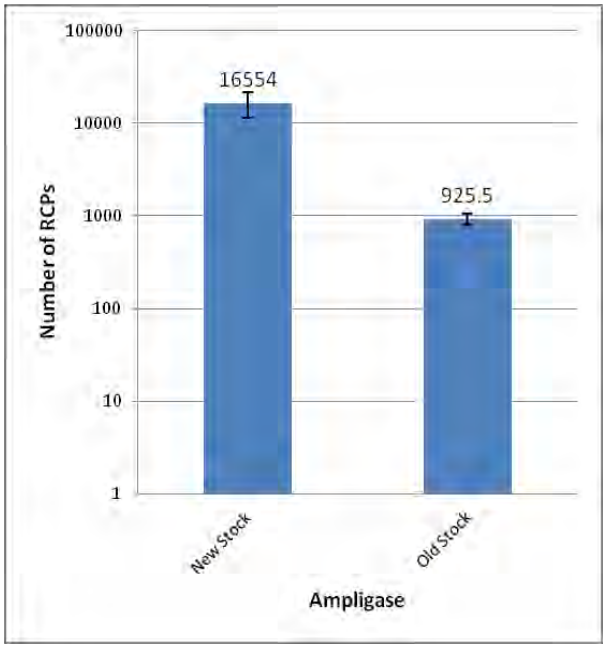

Figure 12. C2CA with new and old stocks of Ampligase. 1amol of template (L10919) and 1µ of padlock probe was used in 20 min C2CA.

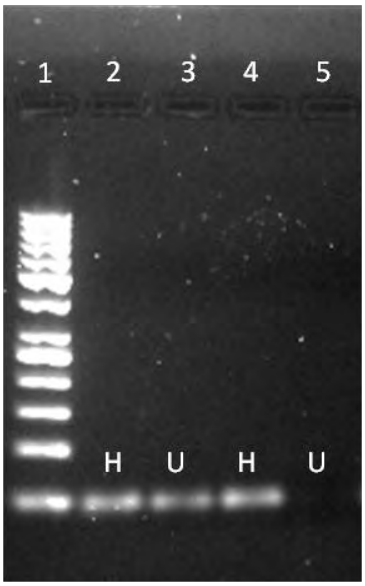

Figure 13. Nuclease contamination in Ampligase. Incubation of padlock probes with heat inactivated and normal Ampligase from two different lots for 1 hour.

Lane 1: GeneRuler™ 50bp DNA ladder
Lane 2 & 3: New lot-DNA ligase
Lane 4 & 5: Old lot-DNA ligase

H: Heat treated DNA ligase
U: Untreated DNA ligase

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