

# Detection of Mutations in Rifampin-Resistant *Mycobacterium Tuberculosis* by Short Oligonucleotide Ligation Assay on DNA Chips (SOLAC)

Xian-En Zhang (✉) · Jiao-Yu Deng

State Key Laboratory of Virology, Wuhan Institute of Virology,  
Chinese Academy of Sciences and State Key Laboratory of Biomacromolecules,  
Institute of Biophysics, Chinese Academy of Sciences, 44 XiaoHongShan, 430071 Wuhan,  
P.R. China  
zhangxe@mail.most.gov.cn

<b>1</b>	<b>Introduction</b> . . . . .	171
1.1	Rifampin Resistance (Rif <sup>r</sup> ) in <i>M. Tuberculosis</i> . . . . .	171
1.2	Current Methods for Detection of Rif <sup>r</sup> <i>M. Tuberculosis</i> . . . . .	171
1.3	Overview of Ligase-based Methods for DNA Mutation Detection . . . . .	172
1.4	Short Oligonucleotide Ligation Assay on DNA Chips (SOLAC) . . . . .	172
<b>2</b>	<b>Experiment</b> . . . . .	176
2.1	Reagents . . . . .	176
2.2	Ligation Experiment . . . . .	176
2.3	SOLAC-LOS Experiment . . . . .	179
2.4	SOLAC-GOS Experiment . . . . .	180
<b>3</b>	<b>Results and Discussion</b> . . . . .	182
3.1	Ligation on Chips . . . . .	182
3.2	Ligation Efficiency of T4 DNA Ligase . . . . .	183
3.3	Detection of DNA Mutations by SOLAC-LOS Experiments . . . . .	184
3.4	Detection of DNA Mutations by SOLAC-GOS Experiments . . . . .	185
<b>4</b>	<b>Conclusions</b> . . . . .	188
	<b>References</b> . . . . .	189

**Abstract** A new approach, a short oligonucleotide ligation assay on DNA chips (SOLAC) is developed to detect DNA mutations. The SOLAC approach can be carried out through two experimental schemes: loss-of-signal (SOLAC-LOS) and gain-of-signal (SOLAC-GOS). In both experimental schemes, probes with a disulfide modification on their terminuses are immobilized onto mercaptosilane derivatized glass slides through a thiol/disulfide exchange reaction.

In SOLAC-LOS, the common probe is immobilized on the chip, and the allele-specific probe is used as the detecting probe; both probes are perfectly complementary to the wild-type target DNA. After hybridization of sample DNA with the immobilized common probe, T4 DNA ligase is applied to ligate the common probe and to detect the probe. Failure of ligation occurs if there is any mismatch between the sample DNA and the detecting probe. This nick-containing hybrid conjugate cannot withstand denaturing and washing

treatments, leading to the loss of signal, which indicates the presence of mutations in the target sample. Theoretically, with one pair of probes (one common probe and one pentamer) all mutations (substitutions, insertions, and deletions) in the five-nucleoside region of the target DNA can be detected.

By contrast, in SOLAC-GOS, the solid phase is the array of allele-specific probes, which are designed to be complementary to all of the known mutations of the target region of the sample DNA, while the common probes are detecting probes. After hybridization, ligation, and washing, the gain of signal is an indicator of the presence of mutations. For a five-base region of the target DNA, basically sixteen allele-specific pentamers and just one common probe are needed to detect all possible mutations.

In combination with an alkaline phosphatase reaction-linked assay, these two schemes have been used successfully for the identification of mutations in the *rpoB* gene of *Mycobacterium tuberculosis* from clinical isolates that show rifampin resistance (Rif<sup>r</sup>). The advantages and disadvantages of the new approach are discussed.

**Keywords** Enzyme-linked assay · *M. tuberculosis* · Mutation detection · Rifampin resistance · Short oligonucleotide ligation on DNA chips (SOLAC) · T4 DNA ligase

### Abbreviations

TB	Tuberculosis
MTB	<i>Mycobacterium tuberculosis</i>
RIF	Rifampin
Rif <sup>r</sup>	Rifampin resistant
INH	Isoniazid
MDR	Multidrug-resistant
RRDR	Rifampin resistance-determining region
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformation polymorphism
OLA	Oligonucleotide ligation assay
SOLAC	Short oligonucleotide ligation assay on DNA chips
bp	Base pair
PCR	Polymerase chain reaction
AS-PCR	Allele-specific PCR
LiPA	Line probe assay
<i>E. coli</i>	<i>Escherichia coli</i>
<i>Th</i>	<i>Thermus thermophilus</i>
BCIP	5-bromo-4-chloro-3-indolyl phosphate
NBT	Nitro-bluetetrazolium
MPTS	3-mercaptopropyl trimethoxysilane
BSA	Bovine serum albumin
AAP	Avidin-alkaline phosphatase

## 1

### Introduction

#### 1.1

##### Rifampin Resistance (Rif<sup>r</sup>) in *M. Tuberculosis*

*Mycobacterium tuberculosis* is still regarded as a causative agent of high morbidity and mortality throughout the world. Control of tuberculosis (TB) has been more difficult since the emergence of drug- and multidrug-resistant (MDR) *M. tuberculosis* strains [1, 2]. Rifampin (RIF) is an effective drug against *M. tuberculosis* and forms the backbone of short-course chemotherapy along with isoniazid (INH) [3]. As reported by the WHO, the median prevalence of Rif<sup>r</sup> in new cases and previously treated cases are 1.4% and 8.7%, respectively [4]. More than 95% of rifampin-resistant (Rif<sup>r</sup>) *M. tuberculosis* strains carry mutations in an 81-bp Rif<sup>r</sup>-determining region (RRDR) in the *rpoB* gene [5, 6], making it a good target for molecular diagnosis. Moreover, more than 90% of Rif<sup>r</sup> isolates are also resistant to INH; therefore, Rif<sup>r</sup> can be regarded as a surrogate marker for multi-drug resistance in *M. tuberculosis* [7].

#### 1.2

##### Current Methods for Detection of Rif<sup>r</sup> *M. Tuberculosis*

Early diagnosis of Rif<sup>r</sup> is essential for the efficient treatment and control of drug-resistant tuberculosis. Culture-based methods for drug susceptibility testing usually take more than one month, and therefore, a reduction of the detection period by more rapid means of drug susceptibility testing is called for. To date, the most promising methods are those based on molecular biology, including direct sequencing [8], PCR single-strand conformational length polymorphism (PCR-SSCP) [9, 10], line probe assay (LiPA) [11, 12], allele-specific PCR (AS-PCR) [13], oligonucleotide microarray-based methods [14–17], etc. With these means, rifampin resistance detection can be done in one day or even a few hours, excluding time needed for sample preparation.

Among these methods, DNA sequencing and PCR-SSCP are based on DNA electrophoresis. DNA sequencing has always been recognized as the “golden” standard method, while PCR-SSCP may be the most cost-effective method for detecting point mutations within the RRDR. However, these methods are usually technically difficult and time-consuming. LiPA is a commercially available kit-based method for use on isolates, which is based on reverse hybridization between *rpoB* amplicon and immobilized membrane-bound probes. It is easy to perform and allows the rapid detection of rifampin resistance. However, the number of probes in LiPA is limited. The type of mutation cannot be determined if the mutations are not among those included on the LiPA strip. AS-PCR is based directly on PCR amplification, it is rapid and easy to perform, and the

results are easy to interpret. The procedure is inexpensive and requires only standard PCR and electrophoresis equipment. It has the potential to be used for direct analysis of sputum slides. However, stringency of AS-PCR is difficult to be stably adjusted, especially in multiplex mutation detection, and the number of primers in a single PCR reaction is limited. Thus, the method can detect a considerable proportion of Rif<sup>r</sup> *M. tuberculosis* isolates, but not all.

An oligonucleotide microarray is composed of oligonucleotides synthesized onto a silica slide or pre-synthesized probes spotted onto a glass slide. This technique allows for the parallel analysis of many genetic targets in a single reaction. Based on this technique, researchers have developed many powerful methods for detecting rifampin resistance determinants in *M. tuberculosis* strains, including hybridization on oligonucleotide microarray [14–16], AS-PCR on oligonucleotide microarray [16] and minisequencing [17]. The principle of these methods is the same as conventional molecular techniques. The difference is that they are carried out on solid supports and can be easily automated and standardized, suitable for large-scale diagnosis. For example, to detect possible substitution mutations in the RRDR of *rpoB* gene by AS-PCR, 324 allele-specific primers are needed. In theory, using AS-PCR on an oligonucleotide microarray, a single PCR reaction containing two outer primers that flank the region under study can detect all possible substitutions, which cannot be achieved by conventional AS-PCR.

### 1.3

#### Overview of Ligase-based Methods for DNA Mutation Detection

In the late 1980s, it was found that when two oligonucleotides were annealed immediately adjacent to each other on a complementary target DNA molecule, single nucleotide substitutions at the junction could be detected by T4 phage DNA ligase [18]. A later approach was the combination of PCR and an oligonucleotide ligation assay, called PCR/OLA [19]. Application of thermostable DNA ligase makes the method more practical [20].

By the principle of OLA, any nucleotide variation at the ligation junction can be detected using a single set of assay conditions. Other advantages include high specificity, speed, and automation, as well as compatibility with PCR, suitable for genotyping on large-scale detections [21–23]. However, these assays can only detect mutations at the ligation junction. A false negative could arise if the mutations occur in a nearby sequence. Therefore, a new approach is needed to circumvent this drawback.

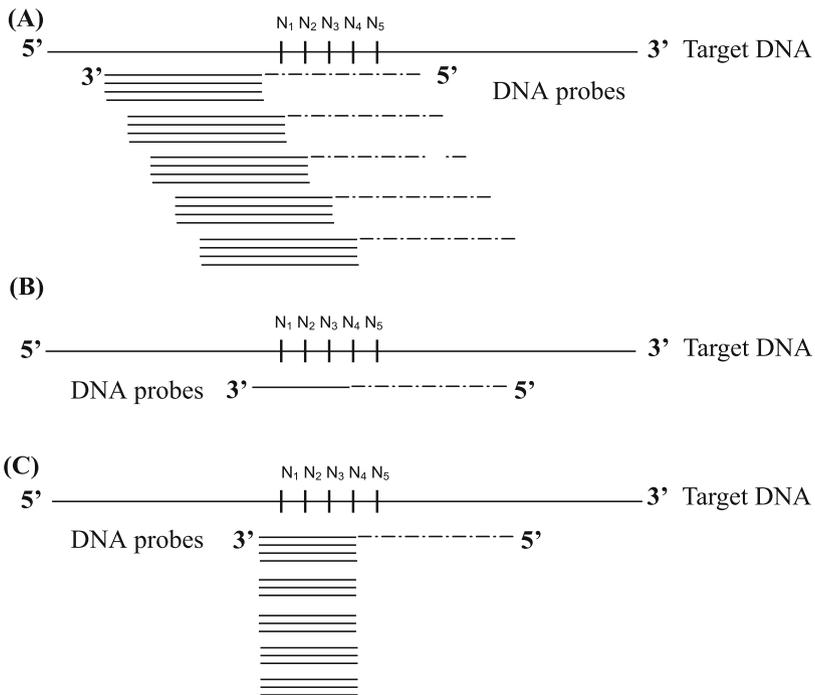
### 1.4

#### Short Oligonucleotide Ligation Assay on DNA Chips (SOLAC)

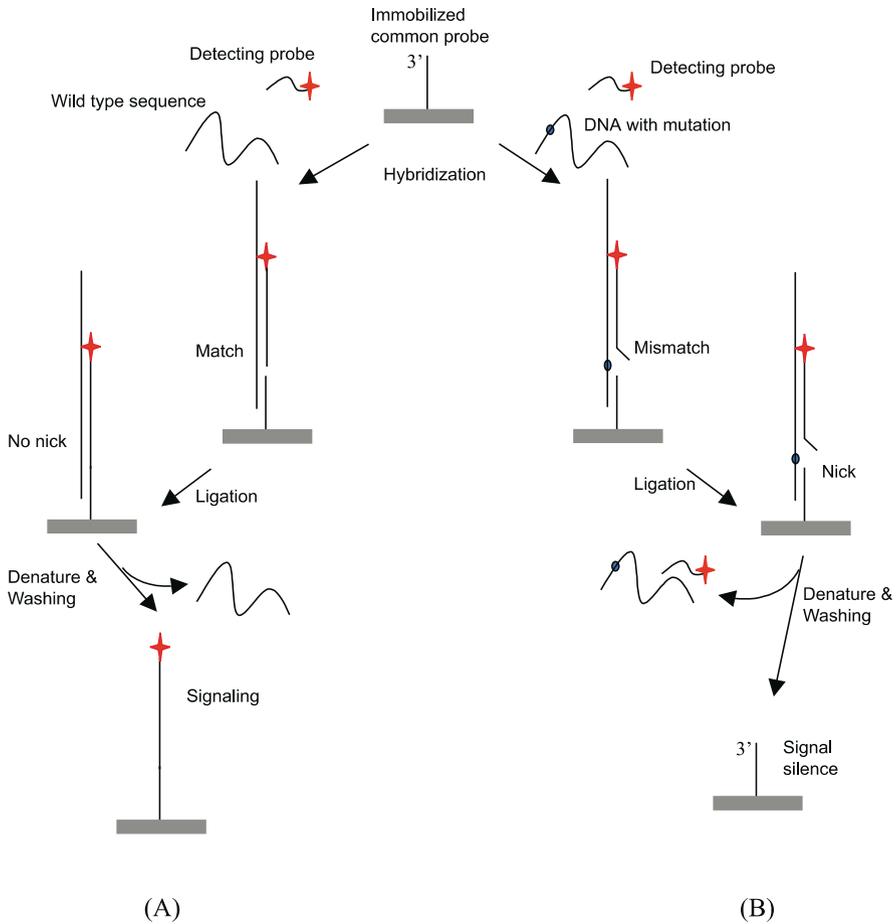
It was known that T4 DNA ligase could ligate pentamers efficiently in solution [24]. In addition, Pritchard and Southern [25] studied the ligation

specificity of *Thermus thermophilus* (*Tth*) DNA ligase, and found that even mismatches at the distal position from the ligation junction site were able to completely inhibit the ligation of octamers. Based on these findings, we developed a new method termed short oligonucleotide ligation assay on DNA chips (SOLAC) for mutation detection. The proposed method combines OLA, DNA chip technologies, and T4 DNA ligase catalysis [26]. It was found that any mismatch between the pentamer and the target DNA, not only those at an end position such as that of a junction point, could lead to a dramatic decrease of ligation efficiency.

The SOLAC approach can be carried out via two experimental schemes: loss-of-signal (SOLAC-LOS) and gain-of-signal (SOLAC-GOS). Figure 1 is a comparison of the probe design scheme of SOLAC with that of an existing OLA method. In the OLA scheme, 25 probes are required to detect all possible substitutions in a five-base region of the target DNA, including 20



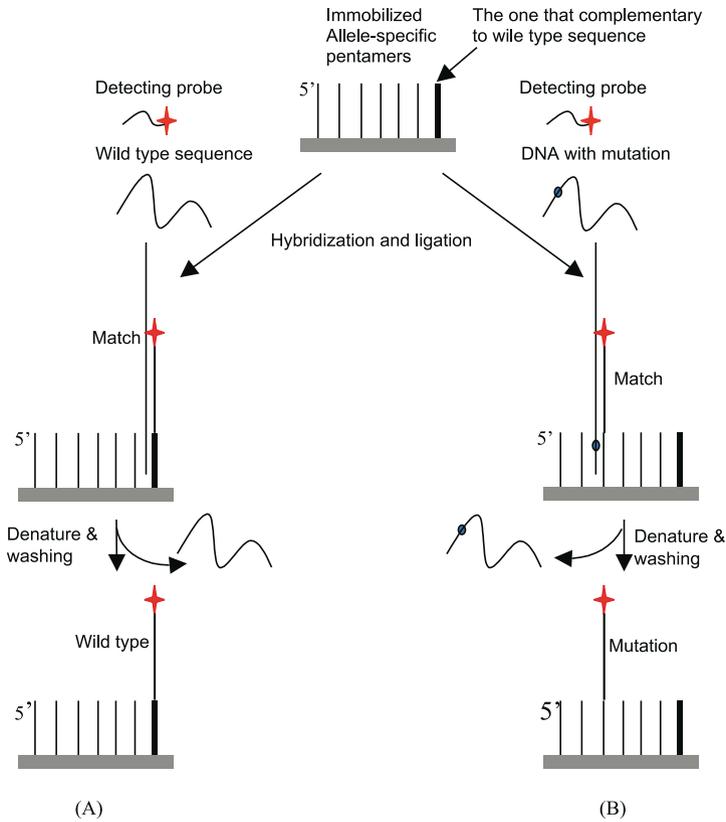
**Fig. 1** Probe design scheme. Probes were designed to detect possible substitutions in a 5 bp region of the target DNA. Conventional OLA and pentamer-based OLA (SOLAC) are compared. **A** In conventional OLA, five common probes (*broken line*) and 20 allele-specific probes (*solid line*) are needed to detect all possible 15 single mutants in the 5 bp region. **B** In SOLAC-LOS, only one common probe and one allele-specific pentamer are needed for the same purpose. **C** In SOLAC-GOS, 17 probes are needed, including 16 allele-specific probes and one common probe



**Fig. 2** SOLAC-LOS procedure. One pair of probes is designed to detect all possible substitutions in a 5 bp region of the target DNA. The common probe is immobilized on a DNA chip. The allele-specific pentamer is in the reaction solution. By comparing perfectly matched hybridization, the loss of signal indicates the existence of a mutation in the target DNA

allele-specific probes and five common probes (Fig. 1A), whereas in the SOLAC scheme, fewer probes are needed for the same purpose.

In the SOLAC-LOS scheme (Figs. 1B and 2), the common probe is immobilized, and the allele-specific probe is the detecting probe; both probes are perfectly complementary to the wild-type target DNA. After hybridization, ligation, denaturing, and washing, the loss of signal indicates the presence of mutations in the target sample. Theoretically, with one pair of probes (one common probe and one pentamer) it can detect all mutations (substitutions, insertions, and deletions) in the five-nucleoside region of the target



**Fig. 3** SOLAC-GOS procedure. Seventeen probes were designed to detect all possible substitutions in a 5 bp region of the target DNA, including 16 allele-specific pentamers and one common probe. All allele-specific pentamers are immobilized on a DNA chip, while the common probe is in the reaction solution. A gain of signal indicates a mutation

DNA. Four common mutations found in Rif<sup>r</sup> *M. tuberculosis* (513CAA > CCA, 516GAC > GTC, 526CAC > TAC, 531TCG > TTG) were identified using this scheme (unpublished data).

By contrast, in SOLAC-GOS (Figs. 1B and 3), the solid phase is the array of allele-specific probes, which are designed to be complementary to the all known mutations of the target region of the sample DNA, while the common probes are labeled as the detecting probes. After hybridization, ligation, denaturing, and washing, the gain of signal is an indication of the presence of mutations. For a five-base region of the target DNA, only sixteen allele-specific pentamers and just one common probe are required to detect all possible substitutions. For example, with four common probes, we successfully scanned fifteen mutant variants in the *rpoB* gene of Rif<sup>r</sup> *M. tuberculosis* clinical isolates [27].

## 2 Experiment

### 2.1 Reagents

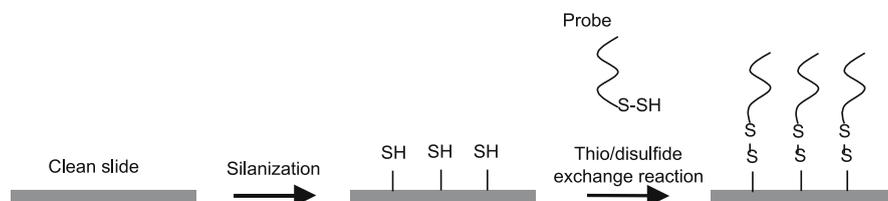
All oligonucleotides were synthesized and purified by Sangon Company Limited (Shanghai, China). T4 phage DNA ligase and *Escherichia coli* (*E. coli*) DNA ligase were purchased from Promega Company (U.S.A). Avidin alkaline-phosphatase conjugates (AAP) were purchased from Calbiochem-Novabiochem Corporation (U.S.A). Glass slides (1 mm × 25 mm × 75 mm) were purchased from Fanchuan Company (Shanghai, China). (3-mercaptopropyl) trimethoxysilane (MPTS) was purchased from Sigma (USA). E.N.Z.A cycle-pure kits were purchased from Omega Bio-tek (USA). All chemicals used were of analytical degree.

### 2.2 Ligation Experiment

#### Protocol I Preparation of slides and probe immobilization

The slides were prepared as previously described by Rogers et al. [28] with some modifications (Fig. 4).

- Step 1. Etch arrays on the glass slides (1 mm × 25 mm × 75 mm) with fluohydric acid (40%) at room temperature for 2 h and wash thoroughly with Milli-Q water.
- Step 2. Treat the etched slides with 25% ammonia water overnight and wash thoroughly with Milli-Q water.
- Step 3. Wash the slides with ethanol once and immerse them in 1% MPTS (dissolved in 95% ethanol containing 16 mM acetic acid) at room temperature for 1 h.
- Step 4. Wash the slides with a washing buffer (95% ethanol, 16 mM acetic acid) once.
- Step 5. Store the slides in dry nitrogen gas for at least 24 h before use.
- Step 6. Dissolve the oligonucleotides with disulfide modifications on their terminuses in 0.5 mol/L Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> (pH 9.0) at a concentration of 15 μM.
- Step 7. Add the oligonucleotides to the wells, and put the slides into humid chambers at 20 °C overnight. The oligonucleotides are immobilized into the wells through disulfide bonds.
- Step 8. Wash the slides with TNTW (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) three times, and finally, rinse them with Milli-Q water once.



**Fig. 4** Slide preparation and probe immobilization scheme. A clean slide is treated with mercaptosilane (MPTS), and an intermediate mercaptosilane layer is formed on the surface of the slide. 5' disulfide-modified probes are immobilized onto the slide through thiol/disulfide exchange reactions

### Protocol II Short oligonucleotide ligation on DNA chips

- Step 1. Design the eight oligonucleotides shown in Table 1. Get the G:T mismatch at each position from the 5' of the pentamer through an appropriate combination of the template and the pentamer.
- Step 2. Immobilize oligonucleotide Com (this is an abbreviation of common, as indicated in Table 1) into the wells as solid-state probes as previously described.
- Step 3. Distinguish these mismatches by T4 DNA ligase. The ligation reaction mixture contains 2  $\mu$ l 10x T4 DNA ligase reaction buffer, 0.6  $\mu$ l Template (20  $\mu$ M), 0.5  $\mu$ l pentamer (20  $\mu$ M), 1  $\mu$ l T4 DNA ligase (3 U/ $\mu$ l), and H<sub>2</sub>O to 20  $\mu$ l. Ligation reactions are performed directly on slides at 30 °C for 1 h.

### Protocol III Detection of ligation on chips via an enzyme-linked assay

An enzyme-linked assay was used to detect chip ligation products.

- Step 1. Block the slides with blocking buffer (100 mM Tris-HCl, pH 7.8, 150 mM NaCl, 50 mg/ml BSA) for 10 min at 37 °C.
- Step 2. Add Avidin-alkaline phosphatase (AAP) (1 ng/ $\mu$ l in 100 mM Tris-HCl, pH 7.3, 150 mM MgCl<sub>2</sub>, 10 mg/ml BSA) into the wells and incubate the slides at 37 °C for 10 min.
- Step 3. Wash the slides with TNTW three times and air-dry.
- Step 4. Add NBT/BCIP mixture (100  $\mu$ g/ml in 100 mM Tris-HCl pH 9.5, 150 mM NaCl and 50 mM MgCl<sub>2</sub>) into the wells, and incubate the slides at 37 °C for 30 min to 2 h. Purple color developed during incubation indicates a positive result.

**Table 1** Oligonucleotides designed for evaluating the effects of mismatches on ligation

Oligonucleotide <sup>a</sup>	Size (mer)	Sequence (5' → 3') <sup>b</sup>
Temp1	25	ATTGGCTCAGCTGGCTGGTGCCCAA
Temp2	25	ATTGGCTCGGCTGGCTGGTGCCCAA
Temp3	25	ATTGGCTTAGCTGGCTGGTGCCCAA
Temp4	25	ATTGGTTCAGCTGGCTGGTGCCCAA
Com	19	TTTTTTTTTTTACCAGCCAG
Penta1	5	pCTGAG-biotin
Penta2	5	pCTGGG-biotin
Penta3	5	pTTGAG-biotin

<sup>a</sup> Temp, template; Com, common probe; Penta, pentamer. <sup>b</sup> The Italic “T” was modified with (CH<sub>2</sub>)<sub>6</sub> – S – S – (CH<sub>2</sub>)<sub>6</sub> – (PO<sub>4</sub>) on its 5'-end. The four templates shared a 9-mer sequence (*underlined*) complementary to the common probe near their 3'-ends (*underlined*) and 5-mer sequences complement with the pentamers (*bold*). The common probe contained a ten-thymine sequence used as spacer in oligonucleotide immobilization. The pentamers contained 5'-phosphate groups and biotin labels on their 3'-ends.

**Table 2** G:T mismatch between the template oligonucleotides and pentamers containing oligonucleotides during the evaluation of ligation reaction

Template	Pentamer	Mismatch
Temp1	Penta3	5'-ATTGGCTCAGCTGGCTGGTGCCCAA-3' 3'-GAGT <b>T</b> GACCGACCATTTTTTTTTT-5'
Temp2	Penta1	5'-ATTGGCTCGGCTGGCTGGTGCCCAA-3' 3'-GAG <b>T</b> CGACCGACCATTTTTTTTTT-5'
Temp3	Penta1	5'-ATTGGCT <b>T</b> AGCTGGCTGGTGCCCAA-3' 3'-GAG <b>T</b> CGACCGACCATTTTTTTTTT-5'
Temp3	Penta2	5'-ATTGGCT <b>T</b> AGCTGGCTGGTGCCCAA-3' 3'-GGG <b>T</b> CGACCGACCATTTTTTTTTT-5'
Temp4	Penta1	5'-ATTGG <b>T</b> TCAGCTGGCTGGTGCCCAA-3' 3'-GAG <b>T</b> CGACCGACCATTTTTTTTTT-5'

The bases underlined represent both the feature and the position of the mismatches formed through different combinations of oligonucleotides listed in Table 1.

## 2.3

### SOLAC-LOS Experiment

#### Protocol IV Detection of mutations in clinical isolates of Rif<sup>r</sup> *M. tuberculosis*

- Step 1. Isolate all clinical isolates at Wuhan Tuberculosis Prevention and Cure Institute. Perform the rifampin susceptibility as previously described [29].
- Step 2. Prepare genome DNA from *M. tuberculosis* cultures as previously described [16].
- Step 3. Amplify a 130 bp segment of the *rpoB* gene that contains RRDR from clinical isolates of Rif<sup>r</sup> *M. tuberculosis* by PCR (forward primer: 5'-GCCGCGATCAAGGAGTTCTTC-3', reverse primer: 5'-GCACGTTACGTCGACAGACC-3'). The sequences of RRDR in these isolates are determined before detection by SOLAC. The PCR reaction mixture contains 10 µl 10X Taq DNA polymerase reaction buffer, 8 µl dNTPs (2.5 mM), 3 µl forward primer (20 µM), 3 µl reverse primer (20 µM), 1 µl BSA (10 mg/ml), 0.5 µl Taq DNA Polymerase (5 U/µl), and H<sub>2</sub>O to 100 µl. The amplification is carried out as follows: 3 min at 94 °C; 30 cycles of 45 s at 94 °C, 45 s at 57 °C, 30 s at 72 °C and 5 min at 72 °C. The PCR products are verified by DNA electrophoresis.
- Step 4. Design the ten oligonucleotides shown in Table 3 according to the sequence of the *rpoB* gene, including four common probes (Com513, Com516, Com526, and Com531), four detection probes (A513, A516, A526, and A531), and two extra probes (E26 and E31).
- Step 5. Immobilize all common probes in the wells as previously described.
- Step 6. Denature the 130 bp PCR products at 100 °C for 5 min, and then cool on ice for another 5 min.
- Step 7. Mix the denatured PCR products (about 200 ng per 10 µl reaction mixture) with 1/2 volume of 20 X SSC (3 M NaCl, 0.3 M Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> · 2H<sub>2</sub>O, pH 7.0).
- Step 8. Add the mixture to the slides. Perform the hybridization between the denatured PCR products and the immobilized common probes at room temperature for two hours.
- Step 9. Wash the slides with TNTW twice and then rinse them with Milli-Q water once; air-dry the slides.
- Step 10. Prepare four sets of oligonucleotides in tubes: the first set includes 2.0 µM A513; the second set includes 0.75 µM A516; the third set includes 1.0 µM A526, 1.5 µM E26; the fourth set includes 1.0 µM A531, 2.0 µM E31.
- Step 11. Add the ligation reaction mixtures that contain different sets of oligonucleotides to four separate positions, and perform the four ligation reactions simultaneously at 16 °C for 1 h.
- Step 12. Visualize the ligation products by AAP as previously described.

**Table 3** Oligonucleotides designed in the detection of mutations by SOLAC

Oligonucleotide <sup>a</sup>	Size(mer)	Sequence (5' → 3') <sup>b</sup>
Com513	26	TTTTTTTTTTTACCAGCCAGCTGAGCC
Com516	26	TTTTTTTTTTTCAGCGGGTTGTTCTGG
Com526	26	TTTTTTTTTTTCTGTGCGTGGTTGACCC
Com531	22	TTTTTTTTTTGTCCCCAGCGCC
A513	5	pAATTC-biotin
A516	5	pTCCAT-biotin
A526	5	pACAAG-biotin
A531	5	pGACAG-biotin
E26	15	TCGGCGCTGGGGACC
E31	15	CGCTTGTGGGTCAAC

<sup>a</sup> com, common probe; A, allele specific probe; E, extra probe. <sup>b</sup> The italic "T" was modified with (CH<sub>2</sub>)<sub>6</sub>-S-S-(CH<sub>2</sub>)<sub>6</sub>-(PO<sub>4</sub>) on its 5'-end. Each four common probe contains a ten-thymine sequence used as the spacer for immobilization. The pentamers contain 5'-phosphate groups and biotin labels on their 3'-ends.

## 2.4

### SOLAC-GOS Experiment

#### Protocol V Multiplex detection of mutations in clinical isolates of Rif<sup>r</sup>

##### *M. tuberculosis*

- Step 1. Prepare genome DNA from clinical isolates of Rif<sup>r</sup> *M. tuberculosis* cultures as previously described.
- Step 2. Amplify a 130 bp segment of the *rpoB* gene that contains the RRDR region by PCR and purify the product.
- Step 3. Design the 25 oligonucleotides shown in Table 4, including four common probes, three extra probes, and 18 allele-specific probes. Divide all these probes into three groups (groups 16, 26, and 31) (Table 4).
- Step 4. Immobilize all allele-specific probes in the wells.
- Step 5. Prepare three sets of oligonucleotides corresponding to the three groups of allele-specific probes in tubes. The first set contains 1.0 μM Com516-1. The second set includes 0.75 μM Com526, 1 μM 5 Ex31-1, and 0.5 μM Ex526. The third set includes 1.0 μM 516Com-2, 1.5 μM Com531, and 1.5 μM Ex531-2.
- Step 6. Denature the 130 bp PCR products at 100 °C for 5 min; cool on ice for another 5 min.
- Step 7. Mix the denatured PCR products (about 200 ng per 10 μl reaction mixture) with the other components and the three sets of oligonucleotides, respectively.

**Table 4** Probes designed to detect mutations in the *rpoB* gene

Amino acid position	Probe <sup>a</sup>	Size	Group	Sequence (5' → 3') <sup>b</sup>
516	Com516-1	9		Biotin-GTTGTTCTG
516	Com516-2	15		Biotin-CAGCGGGTTGTTCTG
516	Wt516	15	16	<i>p</i> GTCATTTTTTTTTTT
516	A516-1	15	16	<i>p</i> GACCATTTTTTTTTTT
516	A516-2	16	31	<i>p</i> GTACAATTTTTTTTTTT
516	A516-3	15	16	<i>p</i> GCCCATTTTTTTTTTT
516	A516-4	15	16	<i>p</i> CACCATTTTTTTTTTT
526	Com526	15		Biotin-CGACAGTCGGCGCTT
526	Wt526	15	26	<i>p</i> GTGGGATTTTTTTTTTT
526	A526-1	15	26	<i>p</i> GTAGGTTTTTTTTTTTT
526	A526-2	15	26	<i>p</i> GTCGGTTTTTTTTTTTT
526	A526-3	16	26	<i>p</i> GTGGTTTTTTTTTTTTT
526	A526-4	16	26	<i>p</i> GAGGTTTTTTTTTTTTT
526	A526-5	16	26	<i>p</i> GCGGTTTTTTTTTTTTT
526	A526-6	16	26	<i>p</i> TGGGTTTTTTTTTTTTT
526	A526-7	16	26	<i>p</i> CTGGTTTTTTTTTTTTT
	Ex526	15		TCAACCCGACAGCG
531	Com531	15		Biotin-CCCCAGCGC
531	Wt531	15	31	<i>p</i> CGACATTTTTTTTTTT
531	A531-1	15	31	<i>p</i> CAACATTTTTTTTTTT
531	A531-2	15	31	<i>p</i> CCACATTTTTTTTTTT
531	A531-3	15	31	<i>p</i> CGGCATTTTTTTTTTT
531	A531-4	16	31	<i>p</i> AGACAGTTTTTTTTTTT
	Ex531-1	11		AGACCGCCGGG
	Ex531-2	15		CGCTTGTTGGTCAA

<sup>a</sup> Com, common probe; Wt, wild type probe; A, allele-specific probe; Ex, extra probe.

<sup>b</sup> The italic "T" was modified (CH<sub>2</sub>)<sub>6</sub>-S-S-(CH<sub>2</sub>)<sub>6</sub>-(PO<sub>4</sub>) on its 3'-end. The common probes contained biotin labels on their 5'-ends. The allele-specific probes contained 5'-phosphate groups and the discriminating bases near their 5'-ends (*bold*).

Step 8. Perform the three ligation reactions at three different temperatures. Optimal reaction temperature for the reactions containing the first, second, and third set of oligonucleotides are 26 °C, 30 °C and 21 °C, respectively.

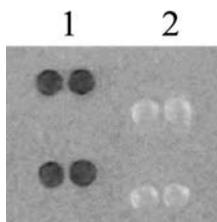
Step 9. Visualize the ligation products by AAP conjugates.

### 3 Results and Discussion

#### 3.1 Ligation on Chips

Ligation reaction in the solid phase is different from that in the liquid phase, since factors such as the surface characteristics of the support may influence the ligation. The advantage of ligation in the solid state is that the products can be detected directly on solid supports without further separation.

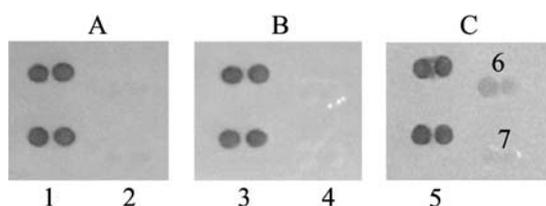
It has been reported that pentamers can be efficiently ligated to probes immobilized in gel pads by T4 DNA ligase [30]. However, such ligation needs 4 h, far longer than the time needed for ligation of hexamers and pentamers in the liquid phase [31]. In our experiment, ligation products can be visualized easily with AAP conjugates after 30 min of ligation. This difference can be explained by the difference of probe immobilization strategies. There are many advantages of using gel pads as the matrix of probe immobilization including high capacity, long spacing between immobilized molecules, and rather homogeneous water surrounding the immobilized molecules [32]. But, in gel pads, mobility of the enzyme and oligonucleotides might be reduced; thus, a longer time is needed for the ligase and oligonucleotides as well as other components to diffuse and interact. When the oligonucleotides are immobilized on a chip surface, they are exposed directly to other components of the reaction mixture, so the reaction is much faster. However, on the glass slides, non-specific protein adsorption is a problem. For example, AAP conjugates could be absorbed when loaded on slides to detect ligation products, causing false positive results. We found that blocking the spare surface with BSA could largely reduce the non-specific binding, yielding ideal signals (Fig. 5).



**Fig. 5** Ligation of pentamers to solid-state probes. Oligonucleotide Com was immobilized in all wells. T4 DNA ligase was added to the reaction mixture; (1) with the ligation mixture and (2) without the ligation mixture. All reactions were carried out at 25 °C for 30 min

### 3.2 Ligation Efficiency of T4 DNA Ligase

It is known that the specificities of the ligases from various sources are different. For example, *Tth* DNA ligase, which is most frequently used in ligase-mediated mutation detection methods, can distinguish a single mismatch at the seventh or eighth position from the 5' of the octamers, but T7 DNA ligase cannot [25]. T4 DNA ligase is another widely used ligase in mutation detection. However, the effect of mismatch on T4 DNA ligase-catalyzed ligation of short oligonucleotides has not been well studied. With an OLA method using T4 DNA ligase, two phenomena were reported [33]: the mismatch ligation occurred with greater probability if the mismatch occurred near the 3' end, and distinguishing G:T mismatch was more difficult than other types of mismatches. Accordingly, the pentamers with G:T mismatch at various positions were tested in the present study. As predicted, mismatch at the first position (ligation site) was the easiest to distinguish, and in general, the closer to the ligation junction site the mismatch was, the easier it was to distinguish, and vice versa. As an exception, the mismatch at the distal position was easier to discriminate than that at the fourth position when the reactions were carried out at 25 °C (Fig. 6C). This may be explained by the fact that if the mismatch were at the fifth base, the length of the duplex formed between the pentamer and the DNA template decreases to four bases. If the G:T mismatch were at the fourth base, it would only destabilized the duplex between the pentamer and the template. Under the specific reaction condition, the effect of duplex instability on ligation efficiency was less than that of the duplex length decrease. After optimization of ligation conditions, all G:T mismatches were



**Fig. 6** Effect of mismatches on the ligation of pentamers. Oligonucleotide Com was immobilized in all wells. **A** G:T mismatch at the fourth base of the pentamer's 5' terminus. The ligation reactions contained probes (1) Tem1 and Penta1 or (2) Tem1 and Penta2, and were performed at 30 °C for 1 h. **B** G:T mismatch at the fifth base of the pentamer's 5' terminus. The ligation system contained probes (3) Tem1 and Penta1 or (4) Tem4 and Penta1, and the reactions were performed at 30 °C for 1 h. **C** G:T mismatches at the fourth and fifth base of the pentamer's 5' terminus. The ligation system contained probes (5) Tem1 and Penta1, (6) Tem1 and Penta2, or (7) Tem4 and Penta1, and the reaction were performed at 25 °C for 1 h

detected successfully with T4 DNA ligase (Figs. 6A and B), and no false ligation was observed.

The influence of temperature on the specificity of T4 DNA ligase was carefully investigated. Ligation reactions were performed at various temperatures from 15 °C to 30 °C. The specificity of T4 DNA ligase was found to increase as the temperature increased. When ligations were carried out at room temperature (25 °C), G:T mismatch at the fourth position of the pentamer was difficult to discern. The background of mismatch ligation was rather high (Fig. 6C). When the temperature of ligation was raised to 30 °C, no detectable background signal was observed.

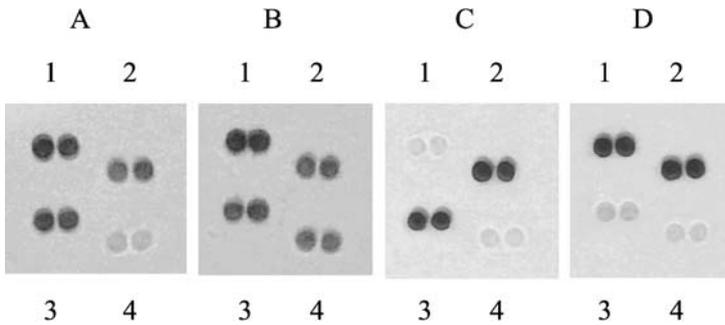
The efficiency of mismatch discrimination was also influenced by the concentration of allele-specific pentamers, and it is interesting that the influence of this factor varied with the mismatch position. For instance, once the mismatch occurred at the first position, it could be distinguished effectively even if the concentration of the pentamer was as high as 10  $\mu$ M. But the concentration of pentamer had to be decreased to 0.5  $\mu$ M to successfully distinguish a mismatch if it occurred at the fourth base of the 5' terminus.

### 3.3

#### Detection of DNA Mutations by SOLAC-LOS Experiments

The loss-of-signal scheme of SOLAC was used to scan the RRDR of the *rpoB* gene to detect four substitutions that happen frequently in Rif<sup>r</sup> *M. tuberculosis*. In the first step, the 130 bp target DNA was captured effectively by the four immobilized common probes through chip hybridization. This step ensures the efficiency of short oligonucleotide ligation, which is the next step in the assay. However, the ligation efficiency in codon 526 and 531 was very low. It was previously described that the efficiency of oligonucleotide hybridization could be enhanced through the addition of another adjacent oligonucleotide [34], so we designed two extra probes at the adjacent region of 526Com and 531Com. As expected, the efficiency of ligation was enhanced (data not shown).

In this assay, all detection probes were designed according to a wild-type gene; thus, the loss of ligation signal signifies the presence of mutations (Fig. 2). To detect the four common mutations in four codons, four separate ligation reactions were needed. The optimal ligation temperature of each ligation reaction was optimized by experiment. After optimization, the ligation temperature of all four reactions was adjusted to 16 °C; thus, these substitutions were detected simultaneously on one chip (Fig. 7). The advantage of this scheme is that, in theory, only one pair of oligonucleotides is needed to detect all possible mutations (including substitutions, deletions, and insertions) in a five-nucleotide region. As for the detection of mutations in RRDR of Rif<sup>r</sup> *M. tuberculosis*, four pairs of oligonucleotides are enough to detect 18 substitutions found in the four codons. Thus, this scheme has the potential of



**Fig. 7** Detection of DNA mutations in Rif<sup>r</sup> *M. tuberculosis* by SOLAC-LOS. The 130 bp amplification products obtained from four clinical isolates of Rif<sup>r</sup> *M. tuberculosis* with known mutations in the RRDR were used as target DNAs. In **A** and **B**, oligonucleotides Com513, Com516, Com526, and Com531 were immobilized at 1, 2, 3, and 4 respectively; in **C** and **D**, oligonucleotides Com513, Com516, and Com526 were immobilized at 1, 2, and 3 respectively; position 4 was the negative control. Known point mutations in codon 531, codon 513, and codon 526 were detected in **A**, **C**, and **D** in three of the four isolates. **B** No mutation was detected in one isolate. The results were identical with previously sequencing results of RRDR

detecting mutations at a large scale. However, the loss of signal is sometimes caused by a failure of operation, and a reduced signal level or a lack of signal is not necessarily indicative of a mutation but may be due to missing or a failure of experiment steps. For this reason, the loss-of-signal scheme is not the first choice for setting up a routine method. Furthermore, the scheme produces results of a positive detection of mutations but gives no information on the nature of the associated mutation. For example, silent mutations in the *rpoB* gene that were not actually associated with Rif<sup>r</sup> could be identified, which underlines the need for caution in interpreting results and phenotypic or genotypic correlation.

### 3.4

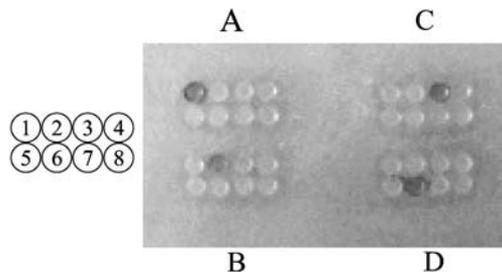
#### Detection of DNA Mutations by SOLAC-GOS Experiments

To circumvent the disadvantages of SOLAC-LOS, SOLAC-GOS was developed. In this scheme, all allele-specific short oligonucleotides were immobilized on chips, hybridization and ligation were incorporated into a single step, and the gain of signal signifies the presence of mutations (Fig. 3).

The scheme was first used to scan the 130 bp *rpoB* gene PCR products and to detect mutations occurring in codon 531, which arise frequently in Rif<sup>r</sup> *M. tuberculosis*. In this scheme, only one common probe is needed in the reaction to detect four alleles located at different positions of codon 531. Two substitutions (CGA > CAA and CGA > CCA) were correctly identified by this scheme [26].

So far, more than 40 substitutions have been identified in the *rpoB* gene of Rif<sup>r</sup> *M. tuberculosis* [35]; among them, 15 substitutions in three codons (516, 526, and 531) are found in nearly 85% of all mutants whose rifampin resistance is caused by mutations in the RRDR. To detect these 15 mutations by SOLAC-GOS, 25 probes were designed, which contained three extra probes and four common probes. The optimal ligation temperatures of short allele-specific oligonucleotides cannot be estimated by bio-software. To optimize the reaction temperature for multiplex ligation, three common mutants (516 GAC > GTC, 526 CAC > TAC, 531 TCG > TTG) were constructed. In addition, DNA samples from 15 Rif<sup>r</sup> strains with known sequences (obtained from the Wuhan Tuberculosis Prevention and Cure Institute) were also used in the optimization process. The G:C content, the length of allele-specific oligonucleotides, and the length of the common probes were the main factors being adjusted in optimization, especially the second factor. As a result, the length of many allele-specific probes has to be elongated to six nucleotides (hexamers). After optimization, the allele-specific probes were divided into three groups according to their optimal ligation temperatures. The optimal ligation temperature for each group of oligonucleotides in this experiment was not very stringent, though a defined ligation temperature was chosen for each group in the experiment (unpublished data).

The 130 bp PCR products from 60 clinical isolates of *M. tuberculosis*, 55 known as rifampin-resistant and five known as rifampin-sensitive, were scanned for mutations by the SOLAC-GOS assay. Among the 55 rifampin-resistant isolates, 47 were found to have point mutations belonging to seven



**Fig. 8** Multiplex detection of point mutations in codon 526 of the *rpoB* gene in Rif<sup>r</sup> *M. tuberculosis* by SOLAC-GOS. Wt526, A526-1, A526-2, A526-3, A526-4, A526-5, A526-6, and A526-7 were immobilized onto loci 1, 2, 3, 4, 5, 6, 7, and 8 respectively. The 130 bp amplification products obtained from the four clinical isolates of rifampin-resistant *M. tuberculosis* were used as a target DNA template in ligation. Reactions were performed at 30 °C for 2 h. **A** The ligation contained the 130 bp segment from isolate 1; no mutation was found in this codon. **B** The ligation contained the 130 bp segment from isolate 8; it contained the C > T substitution in codon 526. **C** The ligation contained the 130 bp segment from isolate 13; it contained the C > G substitution in codon 526. **D** The ligation contained the 130 bp segment from isolate 54; an A > G substitution was found in codon 531. All the results were verified by DNA sequencing

**Table 5** Detection of *rpoB* gene mutations of *M. tuberculosis* in Wuhan by SOLAC compared with DNA sequencing results

Number of Strains	Rifampin resistance <sup>a</sup>	Mutations <sup>b</sup> CSOLA assay	DNA sequencing
26	R	531 TCG→TTG	531 TCG→TTG
3	R	531 TCG→TGG	531 TCG→TTG
7	R	526 CAC→TAC	526 CAC→TAC
3	R	526 CAC→GAC	526 CAC→GAC
3	R	526 CAC→CGC	526 CAC→CGC
2	R	516 GAC→TAC	516 GAC→TAC
3	R	516 GAC→GTC	516 GAC→GTC
1	R	531 TCG→TTG	531 TCG→TTG
			511 CTG→TTG
1	R	526 negative <sup>c</sup>	526 CAC→AC, one base deletion
1	R	WT	511 CTG→CGG
2	R	WT	513 CAA→CTA
1	R	WT	533 CTG→CCG
3	R	WT	WT
1 (H37Rv)	S	WT	WT
5	S	WT	WT

<sup>a</sup> Con, control. <sup>b</sup> R, resistant, S, sensitive. <sup>c</sup> WT, wild type. <sup>d</sup> negative, no results of ligation could be seen.

types (Fig. 8). A deletion mutation in one isolate was also indicated by this method. When the 130 bp fragment from isolate 30 was scanned, a negative result appeared at codon 526 (data not shown), and it was found by DNA sequencing to have a one-base deletion in this codon. No mutations were found in the five rifampin-sensitive isolates.

The RRDR of the *rpoB* gene was subsequently sequenced to analyze the mutations associated with rifampin resistance and to verify the detection results of the SOLAC-GOS assay. DNA sequencing analysis of the 55 Rif<sup>r</sup> isolates showed that 50 strains had point mutations, one strain had a double mutation, and one strain had a one-base deletion in the 81-bp RRDR of the *rpoB* gene. Three strains were found to have no mutation. A total of 52 mutations of 12 different types including 11 point mutations and one deletion were identified (Table 5). The most frequent mutations were found at codons 531, 526, and 516, with frequencies of 52.7, 23.6, and 9.1%, respectively. Similar results have been reported by other investigators [36–38].

Two types of point mutations were found at codon 531 (S531L and S531W), three different types of point mutations (H526Y, H526D and H526N) were

found at codon 526, and two types of point mutations were found at codon 516 (D516V and D516E). All of these mutations were identified by the SOLAC-GOS assay, resulting in an 83.6% concordance between SOLAC-GOS and DNA sequencing. One isolate with a one-base deletion at codon 526, and one isolate with double mutations (S531L and L511L) were also detected by SOLAC-GOS, resulting in 87.3% detection accuracy.

Seven Rif<sup>r</sup> isolates that were verified to be rifampin-resistant by conventional susceptibility testing gave false wild-type results by SOLAC-GOS, which reveals the limitations of this scheme: (1) some mutations, e.g., L511N, Q513L, and L533P, are outside the detection range; (2) no mutation exists in the RRDR of the *rpoB* gene even though the isolates are resistant to rifampin. There probably exist other resistance mechanisms, such as a permeability barrier or drug efflux pumps. Similar results were reported from independent investigations using different methods [37–41].

In spite of its limitations, SOLAC-GOS obviously has many advantages. First, the ligation condition is not stringent, enabling the typing of multiple-nucleotide substitutions in a single assay yet requiring specificity at the site of interest. Second, the scheme is highly adaptable: additional oligonucleotides can be easily incorporated to detect more mutations in the target DNA. Third, SOLAC-GOS needs fewer common probes than the conventional OLA assay in its detection of multiplex DNA mutations. This feature further facilitates multiplex detection of DNA mismatches in a single reaction in combination with DNA chips. Furthermore, SOLAC-GOS is simple to perform and interpret and does not require expensive equipment or technical expertise. Finally, either enzyme labeling or fluorescent labeling can be incorporated into the common probes to generate positive signals, allowing sensitive and automatic scans of large numbers of clinical samples, with or without a specific machine.

## 4

### Conclusions

A new technique, SOLAC, including SOLAC-LOS and SOLAC-GOS, has been developed to detect Rif<sup>r</sup> *M. tuberculosis*. In this assay, mismatches at or close to the ligation junction site (i.e., one to four bases away) can be successfully distinguished by using short oligonucleotides (pentamers or hexamers) and T4 DNA ligase on the chip, with fewer probes than the conventional OLA method. Obviously, it has great potential in multiplex/parallel detection and high-throughput preliminary screening of gene mutations in a variety of genes or genomes.

**Acknowledgements** This research is jointly supported by the Ministry of Science & Technology, the Wuhan Science & Technology Bureau, and the Chinese Academy of Science.

## References

1. Cohn DL, Bustreo F, Raviglione MC (1997) *Clin Infect Dis* 24:S121
2. Frieden TR, Sherman LF, Maw KL, Fujiwara PI, Crawford JT, Nivin B, Sharp V, Hewlett D, Brudney K Jr, Alland D, Kreisworth BN (1996) *J Am Med Assoc* 276:1229
3. Kochi A, Varelzdis B, Styblo K (1993) *Res Microbiol* 144:104
4. World Health Organization (2004) Anti-tuberculosis drug resistance in the world: third global report. World Health Organization, Geneva
5. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T (1993) *Lancet* 341:647
6. Telenti A, Imboden P, Marchesi F, Schmidheini T, Bodmer T (1993) *Antimicrob Agents Chemother* 37:2054
7. Drobniewski FA, Wilson SM (1998) *J Med Microbiol* 47:189
8. Kapur V, Li LL, Hamrick MR, Plikaytis BB, Shinnick TM, Telenti A (1995) *Arch Pathol Lab Med* 119:131-138
9. Kim BJ, Lee K, Park H, Kim SJ, Park EM, Park YG, Bai GH, Kim SJ, Kook YH (2001) *J Clin Microbiol* 39(7):2610
10. Felmler TA, Liu Q, Whelen AC, Williams D, Sommer SS, Persing DH (1995) *J Clin Microbiol* 33(6):1617
11. Williams DL, Spring L, Salfinger M, Gillis TP, Persing DH (1998) *Clin Infect Dis* 26:446
12. Cooksey RC, Morlock GP, Glickman S, Crawford JT (1997) *J Clin Microbiol* 35:1281
13. Mokrousov I, Otten T, Vyshnevskiy B, Narvskaya O (2003) *Antimicrob Agents Chemother* 47(7):2231
14. Gingerasm TR, Ghandour G, Wang E, Berno A, Small PM, Drobniewski F, Alland D, Desmond E, Holodniy M, Drenkow J (1998) *Genome Res* 8:435
15. Troesch H, Nguyen GC, Miyada S, Desvarenne TR, Gingeras PM, Kaplan PC, Mabitlat C (1999) *J Clin Microbiol* 37:49
16. Mikhailovich V, Lapa S, Gryadunov D, Sobolev A, Strizhkov B, Chernyh N, Skotnikova O, Irtuganova O, Moroz A, Litvinov V, Vladimirkii M, Perelman M, Chernousova L, Erokhin V, Zasedatelev A, Mirzabekov A (2001) *J Clin Microbiol* 39:2531
17. Head SR, Parikh K, Rogers YH, Bishai W, Goelet P, Boyce-Jacino MT (1999) *Mol Cell Probes* 13:81
18. Landegren U, Kaiser R, Sanders J, Hood L (1988) *Science* 241:1077
19. Nickerson DA, Kaiser R, Lappin S, Stewart J, Hood L, Landegren U (1990) *Proc Natl Acad Sci USA* 87:8923
20. Barany F (1991) *Proc Natl Acad Sci USA* 88:189
21. Chen X, Livak KJ, Kwok PY (1998) *Genome Res* 8:549
22. Gerry NP, Witowski NE, Day J, Hammer RP, Barany G, Banany F (1999) *J Mol Biol* 292:251
23. Zhong XB, Reynolds R, Kidd JR, Kidd KK, Jenison R, Marlar RA, Ward DC (2003) *Proc Natl Acad Sci USA* 100:11559
24. Kotler LE, Zevin-Sonkin D, Sobolev IA, Beskin AD, Ulanovsky LE (1993) *Proc Natl Acad Sci USA* 90:4241
25. Pritchard CE, Southern EM (1997) *Nucleic Acids Res* 25:3403
26. Deng JY, Zhang XE, Mang Y, Zhang ZP, Zhou YF, Liu Q, Lu HB, Fu ZJ (2004) *Biosens Bioelectron* 19:1277
27. Deng JY, Zhang XE, Lu HB, Liu Q, Zhang ZP, Zhou YF, Xie WH, Fu ZJ (2004) *J Clin Microbiol* 42:4850

28. Rogers YH, Jiang-Baucom P, Huang ZJ, Bogdanov V, Anderson S, Boyce-Jacino MT (1999) *Anal Biochem* 266:23
29. Kim SJ, Hong YP (1992) *Tuber Lung Dis* 73:219
30. Dubiley S, Kirillov E, Lysov Y, Mirzabecov A (1997) *Nucleic Acids Res* 25:2259
31. Kaczorowski T, Szybalski W (1996) *Gene* 179:189
32. Wu DY, Wallace RB (1989) *Gene* 76:245
33. Rubina AY, Pan'kov SV, Dementieva EI, Pen'kov DN, Butygin AV, Vasiliskov VA, Chudinov AV, Mikheikin AL, Mikhailovich VM, Mirzabekov AD (2004) *Anal Biochem* 325:92
34. Maldonado-Rodriguez R, Espinosa-Lara M, Calixto-Suarez A, Beattie WG, Beattie KL (1999) *Mol Biotechnol* 11:1
35. Rattan A, Kalia A, Ahmad N (1998) *Emerg Infect Dis* 4:195
36. Hirano K, Abe C, Takahashi M (1999) *J Clin Microbiol* 37:2663
37. Yuen LK, Leslie D, Coloe PJ (1999) *J Clin Microbiol* 37:3844
38. Fan XY, Hu ZY, Xu FH, Yan ZQ, Guo SQ, Li ZM (2003) *J Clin Microbiol* 41:993
39. Bártfai Z, Somoskövi Á, Ködmön C, Szabó N, Puskás E, Kosztolányi L, Faragó E, Mester J, Parsons LM, Salfinger M (2001) *J Clin Microbiol* 39:3736
40. Watterson SA, Wilson SM, Yates MD, Drobniewski FA (1998) *J Clin Microbiol* 36:1969
41. Yue J, Shi W, Xie J, Li Y, Zeng E, Liang L, Wang H (2004) *Diagn Microbiol Infect Dis* 48:47