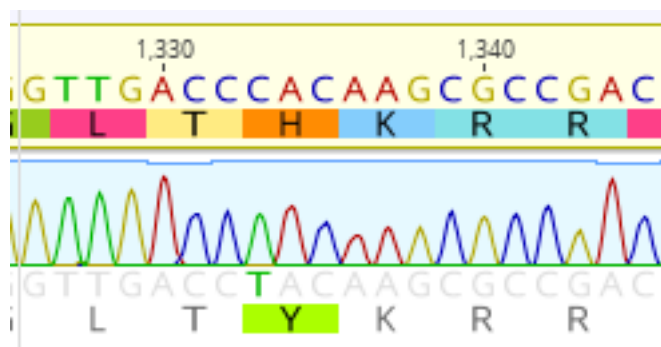


Antibiotic Resistance Sequencing



Principle

Sequencing of specific genes allows to detection of mutations known to be associated to antibiotic resistance and the prediction of their resistance level (1, 2). Target genes – or part of them – are here amplified and sequenced i.e. *rpoB* for rifampicin (RIF)(3), *gyrA* for fluoroquinolones (FQ)(4), *katG* for isoniazide (INH)(5), *rrs* for amikacine (AMK), kanamycine (KAN), capreomycine (CAP), viomycine (VIO)(6, 7), *pncA* for pyrazinamide (PZA) (8-10). Results are compared to wild type sequence of each target gene. Mutations are compared to mutations known to be associated with antibiotic resistance.

Bacilli from clinical samples or cultures will be inactivated. Enough DNA will be extracted from the bacilli and suitable for amplifications. Amplified products will be sequenced. Mutations associated with resistance will be identified.

1. Sample preparation for amplification

1. Material

- Eppendorf tubes
- 0.5M EDTA solution
- 1M Tris HCl solution
- Sterile distilled water
- 1000µl, 200 µl, 100µl, 20µl and 2 µl pipettes
- Disposable sterile pipette tips with filter
- Sterile disposable Pasteur pipettes
- 1µl disposable sterile loops (for solid medium culture)
- Container for infectious clinical waste
- Dry bath
- Ultra-sonic water bath
- Table top micro-centrifuge with aerosol tight rotor

TE [Tris-EDTA] - EDTA 0.5M pH8 20 μ l
 - Tris HCl 1M 100 μ l
 - H₂O to 10 mL

2. Sample preparation

- ***When using bacteria grown in liquid media***
 - Transfer 1 mL of liquid medium into a labeled 1.5 ml screw cap tube
 - Centrifuge for 15 min at 10 000 rpm
 - Discard supernatant
 - Wash pellet twice with 1 mL of TE (10 mM Tris-HCl, 1 mM EDTA (pH 7.0))
 - Centrifuge for 5 min at 10000 rpm
 - Discard supernatant
 - Re-suspend pellet in 200 μ l TE
 - Inactivate bacteria at 95°C for 15 min
 - Centrifuge for 5 min at 10000 rpm
 - Keep supernatant containing the DNA and transfer it into a new tube
- ***When using bacteria grown on solid medium***
 - Collect bacteria with an inoculation loop and suspend into a labeled 1.5 ml screw cap tube in 200 μ l of TE (10 mM Tris-HCl, 1 mM EDTA pH 7.0)
 - Inactivate bacteria at 95°C for 15 min
 - Centrifuge for 5 minutes at 10000 rpm
 - Keep supernatant containing the DNA and transfer it into a new tube
- ***When using patient specimens***
 - Transfer 500 μ l of decontaminated sample material into a labeled 1.5ml screw cap tube
 - Centrifuge for 15 min at 10 000 rpm
 - Discard supernatant and re-suspend pellet in 200 μ l TE
 - Inactivate bacteria at 95°C for 15 min
 - Sonic during 15 min in an ultra-sonic water bath
 - Centrifuge for 5 minutes at 10 000 rpm
 - Keep supernatant containing the DNA and transfer it into a new tube
 - Plan 2 amplification tubes and add :
 - 5 μ L of supernatant for the first amplification,
 - 5 μ L of 1/10th diluted supernatant for the second amplification, to PCR mix.

2. Amplification mix preparation

Carefully read the procedure « Amplification set up » and rigorously follow it to avoid contaminations.

1. In the “white room”, mix preparation

a) *Material*

- Eppendorf 1.5 mL tubes for mix preparation
- 200 µL PCR micro tubes (or strips of 8 PCR tubes or 96 wells PCR plates if needed)
- Disposable single-use test micro tube racks
- Sterile distilled water
- 1000 µl, 200 µl, 100 µl and 20 µl pipettes
- Disposable sterile pipette tips with filter
- Table top micro-centrifuge with aerosol tight rotor
- Refrigerator and -20°C freezer for PCR reagents conservation
- Taq polymerase and buffer (see below)

Different Taq polymerases are used depending on amplification product size:

PCR products < 1000 pb:

- Biotaq™ DNA Polymerase réf. BIO-21040. BIOLINE
- dNTP 25mM (dNTPs are not provided with Biotaq)
- DMSO D2650 SIGMA

PCR products ≥ 1000 pb:

- TaKaRa LA Taq™ + GC buffers réf. RR002AG (GC Buffer I and dNTP mix provided)

Genes	Antibiotics	Primers	Sequences	Hybridization temperature	Amplification product size
<i>gyrA</i>	Fluoroquinolones	gyrA-F	5'-GATGACAGACACGACGTTGC-3'	55°C	398 bp
		gyrA-R	5'-GGGCTTCGGTGTACCTCAT-3'		
<i>rrs</i>	Aminoglycosides Capreomycin	rrs-F	5'-AAACCTCTTTCACCATCGAC-3'	59°C	1329 bp
		rrs-R	5'-GTATCCATTGATGCTCGC-3'		
<i>rpoB</i>	Rifampicin	TR1 (<i>rpoB</i> -F)	5'-TACGGTCGGCGAGCTGATCC-3'	53°C	411 bp
		TR2 (<i>rpoB</i> -R)	5'-TACGGCGTTTCGATGAACC-3'		
<i>pncA</i>	Pyrazinamide	pncA-F	5'- CTGTCACCGGACGGATTTG -3'	60°C	950 bp
		pncA-R	5'- ATCGCGATGGAACGTGATA -3'		
<i>katG</i>	Isoniazid	katG-1 (<i>katG</i> -F)	5'-TGGCCGCGGCGGTCGACATT-3'	60°C	330 bp
		katG-2 (<i>katG</i> -R)	5'-CCAGCAGGGCTCTTCGTCAG-3'		

Primer sequences for antibiotic gene resistance

b) Amplification mix preparation

PCR products < 1000 bp			PCR products ≥ 1000 bp		
For 1 PCR reaction : 45 µL (+5 µL sample)			For 1 PCR reaction : 45µL (+5µL sample)		
Reagents	Final concentration	Quantity (µL)	Reagents	Final concentration	Quantity (µL)
10X Buffer	1 X	5.0	2X Buffer	1X	25
DMSO	10 %	5.0			
MgCl ₂ 50 mM	1.5 mM	1.5			
Sens primer 10 µM	0.3 µM	1.5	Sens primer 10 µM	0.4 µM	2
Antisens primer 10 µM	0.3 µM	1.5	Antisens primer 10 µM	0.4 µM	2
dNTP 25 mM each	200 µM	0.4	dNTP 2,5 mM each	400 µM	8
Biotaq™ 5U/µL	2 U	0.4	LA Taq™ 5U/µL	2 U	0.4
H ₂ O		29.7	H ₂ O		7.6
Final mix volume		45.0	Final mix volume		45.0

PCR mix for antibiotic gene resistance sequencing

- Completely thaw reagents, homogenise by vortexing
- Briefly centrifuge and keep on ice
- Prepare a sufficient mix volume: n PCR reaction tube + 1
- Distribute 45 µl of the mix in reaction micro tubes
- Add 5 µL water in negative control
- Close the tubes
- Carry them out of the “white room” on a disposable single-use test tube rack.

2. In the “grey room”, add DNA preparation to PCR mix

a) Material

- Clean working surface or laminar flow safety cabinet
- Lab coat and gloves
- 20 µl pipet
- Disposable sterile pipet tips with filter
- Table top micro-centrifuge with aerosol tight rotor

b) Introduction of DNA in PCR mix

- 5µL of supernatant prepared in step 1.2 into the first reaction tube and pipet up and down

- Briefly centrifuge.
- Close the tubes
- Carry them out of the “grey room” on a disposable single-use test tube rack.

3. In the “dark room”, amplification and detection

a) *Material*

- Clean working surface
- 1000 µl, 200 µl, 100 µl and 20 µl pipettes
- Disposable sterile pipette tips with filter
- Refrigerator and -20°C freezer for reagents conservation
- Thermocycler
- Electrophoresis chamber, gel form and comb, Power supply
- Agarose
- Masking tape, if needed to seal gel form
- Loading buffer
- Molecular Weight marker. Ref.: SmartLadder MW-1700-10 Eurogentec
- Sterile distilled water
- TAE 50X pH 8
 - Tris base 2M 108 g
 - EDTA 0,5M pH 8 40 mL
 - H₂O to 1 L

b) *Amplification reaction*

Place the PCR reaction tubes in a thermocycler programmed as follows:

PCR products < 1000 bp			PCR products ≥ 1000 bp		
Biotaq™ DNA Polymerase			TaKaRa LA Taq™		
95°C	1 min	1 cycle	95°C	5 min	1 cycle
95°C	1 min	35 cycles	95°C	30 sec	30 cycles
* hybridization T°	1 min		* hybridization T°	30 sec	
72°C	1 min		72°C	2 min	
72°C	10 min	1 cycle	72°C	10 min	1 cycle
4°C	∞		4°C	∞	

* hybridization T° : see primers table

Amplification reactions for antibiotic gene resistance sequencing

Amplification products may be stored at +4°C or at -20°C

3. PCR product analysis using agarose gel electrophoresis

- Depose 5 µl of PCR product on a 1 % in 1X TAE agarose gel (See electrophoresis protocol)
- Migrate 30 min at 120V

4. Sequencing

- Prepare aliquots of sequencing primers in the “white room”. Primers used for sequencing are the same as those used for amplification. See primers table above (Table 1).
- To ensure the sequencing of all *pncA* gene (561 bp), the PCR primers were designed to amplify a fragment of 950 bp and internal primers were also used for sequencing reaction.

pncA-int-F :	5'-GGACTTCCATCCCAGTCTG-3'
pncA-int-R :	5'-CGAAGCCGCTGTACGCTCCG-3'

- Send amplification products and sequencing primers to a sequencing platform (see platform procedure).
- Send amplification products and sequencing primers to a sequencing platform (see platform procedure)

5. Chromatograms analysis

Received chromatograms are compared to reference sequences:

<i>gyrA</i>	<i>M. tuberculosis</i> H37Rv Rv0006 gyrA: 2517 bp DNA GYRASE (SUBUNIT A) GYRA (DNA TOPOISOMERASE (ATP-HYDROLYSING)) (DNA TOPOISOMERASE II) (TYPE II DNA TOPOISOMERASE)
<i>rrs</i>	<i>M. tuberculosis</i> H37Rv MTB000019 rrs: 1537 bp ribosomal RNA 16S
<i>rpoB</i>	<i>M. tuberculosis</i> H37Rv Rv0667 rpoB: 3519 bp DNA-DIRECTED RNA POLYMERASE (BETA CHAIN) RPOB (TRANSCRIPTASE BETA CHAIN) (RNA POLYMERASE BETA SUBUNIT)
<i>pncA</i>	<i>M. tuberculosis</i> H37Rv Rv2043c pncA: 561 bp PYRAZINAMIDASE/NICOTINAMIDAS PNCA (PZase)
<i>katG</i>	<i>M. tuberculosis</i> H37Rv Rv1908c katG: 2223 bp CATALASE-PEROXIDASE-PEROXYNITRITASE T KATG

Compare to databases (11) <http://tuberculist.epfl.ch>

6. Bibliographic references

1. **Riska PF, Jacobs WR, Jr., Alland D.** 2000. Molecular determinants of drug resistance in tuberculosis. *Int J Tuberc Lung Dis* **4**:S4-10.
2. **Zhang Y, Yew WW.** 2009. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis* **13**:1320-1330.
3. **Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T.** 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* **341**:647-650.
4. **Mayer C, Takiff H.** 2014. The Molecular Genetics of Fluoroquinolone Resistance in *Mycobacterium tuberculosis*. *Microbiol Spectr* **2**:MGM2-0009-2013.
5. **Zhang Y, Heym B, Allen B, Young D, Cole S.** 1992. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* **358**:591-593.
6. **Maus CE, Plikaytis BB, Shinnick TM.** 2005. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **49**:3192-3197.
7. **Georghiou SB, Magana M, Garfein RS, Catanzaro DG, Catanzaro A, Rodwell TC.** 2012. Evaluation of genetic mutations associated with *Mycobacterium tuberculosis* resistance to amikacin, kanamycin and capreomycin: a systematic review. *PLoS One* **7**:e33275.
8. **Scorpio A, Zhang Y.** 1996. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in *tubercle bacillus*. *Nat Med* **2**:662-667.
9. **Chang KC, Yew WW, Zhang Y.** 2011. Pyrazinamide susceptibility testing in *Mycobacterium tuberculosis*: a systematic review with meta-analyses. *Antimicrob Agents Chemother* **55**:4499-4505.
10. **Simons SO, van Ingen J, van der Laan T, Mulder A, Dekhuijzen PN, Boeree MJ, van Soolingen D.** 2012. Validation of *pncA* gene sequencing in combination with the mycobacterial growth indicator tube method to test susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *J Clin Microbiol* **50**:428-434.
11. **Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB.** 2009. Tuberculosis drug resistance mutation database. *PLoS Med* **6**:e2.

Annexe

Table 1

Conversion table between the two co-existing numbering systems for the most frequent *rpoB* mutations associated with rifampicin resistance among *Mycobacterium tuberculosis* complex, *Mycobacterium leprae* and *Mycobacterium kansasii*

<i>M. tuberculosis</i> H37Rv (GenBank AL123456)		<i>M. leprae</i> TN (GenBank AL583923)		<i>M. kansasii</i> ATCC 12478 (GenBank CP006835)		<i>Escherichia coli</i> (GenBank V00339)	
WT nucleotide sequence (amino acid)	Codon position (conversion from <i>E. coli</i> numbering)	WT nucleotide sequence (amino acid)	Codon position (conversion from <i>E. coli</i> numbering)	WT nucleotide sequence (amino acid)	Codon position (conversion from <i>E. coli</i> numbering)	WT nucleotide sequence (amino acid)	Codon position
GTC (Val)	170 (+24)					GTT (Val)	146
GGC (Gly)	426 (–81)	GGC (Gly)	432 (–75)			GTT (Gly)	507
CTG (Leu)	430 (–81)	CTG (Leu)	436 (–75)			CTG (Leu)	511
CAA (Gln)	432 (–81)	CAG (Gln)	438 (–75)	CAG (Gln)	442 (–71)	CAG (Gln)	513
GAC (Asp)	435 (–81)	GAT (Asp)	441 (–75)	GAC (Asp)	445 (–71)	GTC (Val)	516
CAC (His)	445 (–81)	CAC (His)	451 (–75)	CAC (His)	455 (–71)	CAC (His)	526
TCG (Ser)	450 (–81)	TGC (Ser)	456 (–75)	TCG (Ser)	460 (–71)	TCC (Ser)	531
CTG (Leu)	452 (–81)	CTG (Leu)	458 (–75)			CTC (Leu)	533
ATC (Ile)	491 (–81)					ATC (Ile)	572

WT, wild-type. The homology in regard to the nucleotide and the amino acid sequences of the different mycobacteria and *E. coli* is highlighted in green. Differences are highlighted in red.

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