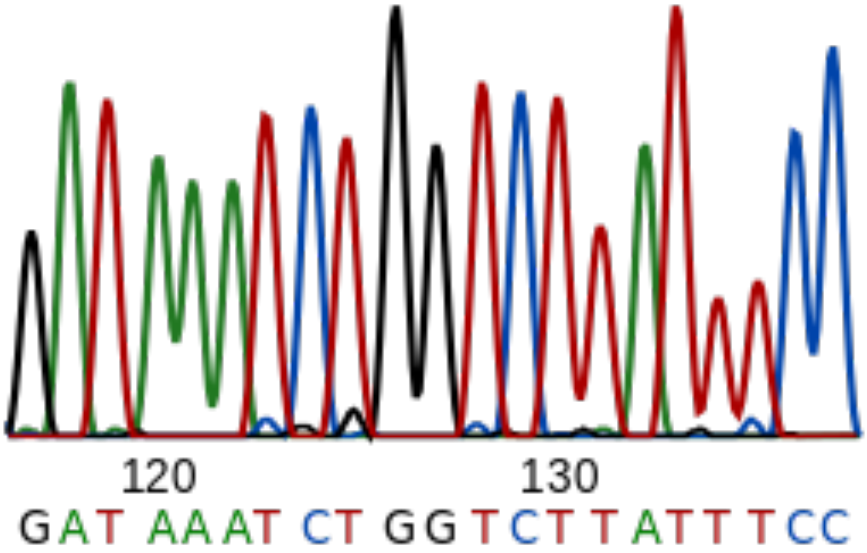
Antibiotic Resistance Sequencing



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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. *rpo*B for rifampicin (RIF)(3), *gyr*A for fluoroquinolones (FQ)(4), *kat*G 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

1.1 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 µl
  - Tris HCl 1M — 100 µl
  - H_2O to — 10 mL

1.2 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 200µl 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 µl 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 500µl 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 200µl 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 :
    - 5µL of supernatant for the first amplification,
    - 5µL 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.

2.1 In the “white room”, mix preparation

2.1.1 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™ with GC buffers réf. RR002AG (Used with GC Buffer I and dNTP mix provided)

Genes	Antibiotics	Primers	Sequences	Hybridization temperature	Amplification product size
gyrA	Fluoroquinolones	gyrA-F	5'-GATGACAGACACGACGTTGC-3'	55°C	398 bp
		gyrA-R	5'-GGGCTTCGGTGTACCTCAT-3'		
rrs	Aminoglycosides Capreomycine	rrs-F	5'-AAACCTCTTTCACCATCGAC-3'	59°C	1329 bp
		rrs-R	5'-GTATCCATTGATGCTCGC-3'		
rpoB	Rifampicin	TR1	5'-TACGGTCGGCGAGCTGATCC-3'	53°C	411 bp
		TR2	5'-TACGGCGTTTCGATGAACC-3'		
pncA	Pyrazinamide	pncA2	5'-CTGTCACCGGACGGATTTG-3'	60°C	950 bp
		pncA1	5'-ATCGCGATGGAACGTGATA-3'		
katG	Isoniazide	katG1	5'-TGGCCGCGGCGGTCGACATT-3'	60°C	330 bp
		katG2	5'-CCAGCAGGGCTCTTCGTCAG-3'		

Table 1. Primer sequences for antibiotic gene resistance

2.1.2 Amplification mix preparation

PCR products < 1000 pb:

For 1 PCR reaction: 45 µl (+ 5µl sample)		
Reagents	**Final Concentration**	**Quantity (µl)**
10X Buffer	1X	5
DMSO	10%	5
MgCl2 50mM	1.5 mM	1.5
Sens primer 10µM	0.3 µM	1.5
Antisens primer 10µM	0.3 µM	1.5
dNTP 25mM each	200 µM	0.4
Biotaq™ (5U/µl)	1 U	0.4
H_2O		29.70
Final mix volume		**45**

PCR products ≥ 1000 pb:

For 1 PCR reaction: 45 µl (+ 5µl sample)		
Reagents	**Final Concentration**	**Quantity (µl)**
2X Buffer	1X	25
dNTP 25 mM each	400 µM	8
Sens primer 10µM	0.4 µM	2
Antisens primer 10µM	0.4 µM	2
LA Taq™ (5U/µl)	1 U	0.4
H_2O		7.6
Final mix volume		**45**

Table 2. 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
- Close the tubes
- Carry them out of the “white room” on a disposable single-use test tube rack.

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

2.2.1 Material

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

2.2.2 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.

2.3 In the “dark room”, amplification and detection

2.3.1 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
- H_2O to	1	L

2.3.2 Amplification reaction

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

Biotaq™ DNA Polymerase
(PCR products < 1000 pb)

95°C	5mn	1 cycle
95°C	1mn	35 cycles
*hybridization T°	1mn	
72°C	1mn	
72°C	10mn	1 cycle
4°C	∞	

TaKaRa LA Taq™
(PCR products > 1000 pb)

94°C	5mn	1 cycle
94°C	30sec	30 cycles
*hybridization T°	30sec	
72°C	2mn	
72°C	10 mn	1 cycle
4°C	∞	

*hybridization T°: see primers table above (Table 1)

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. Sequençing

- 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-int2 :	5’-GGACTTCCATCCCAGTCTG-3’
pncA-int1 :	5’-CGAAGCCGCTGTACGCTCCG-3’

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

5. Chromatograms analysis

Received chromatograms are compared to reference sequences:

gyrA	*M. tuberculosis* H37Rv|Rv0006|gyrA: 2517 bp - DNA GYRASE (SUBUNIT A) GYRA (DNA TOPOISOMERASE (ATP-HYDROLYSING)) (DNA TOPOISOMERASE II) (TYPE II DNA TOPOISOMERASE)
rrs	*M. tuberculosis* H37Rv|MTB000033|rrs: 1537 bp - ribosomal RNA 16S
rpoB	*M. tuberculosis* H37Rv|Rv0667|rpoB: 3519 bp - DNA-DIRECTED RNA POLYMERASE (BETA CHAIN) RPOB (TRANSCRIPTASE BETA CHAIN) (RNA POLYMERASE BETA SUBUNIT)
pncA	*M. tuberculosis* H37Rv|Rv2043c|pncA: 561 bp - PYRAZINAMIDASE/NICOTINAMIDAS PNCA (PZase)
katG	*M. tuberculosis* H37Rv|Rv1908c|katG: 2223 bp CATALASE-PEROXIDASE-PEROXYNITRITASE T KATG

http://genolist.pasteur.fr/TubercuList/index.html

Compare to databases (11)

6. Bibliographic references

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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.
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Antibiotic resistance sequencing on Monday June 12 2017

Each group receives a strain (G to N) and will keep it at +4°C on ice during the test. After sequencing the strain will be kept at -20°C to use it again for the HAIN test.

Each group prepares 2 mixes for 3 tubes (negative, strain +1)

- One for the *rpoB* gene
- The second for the other antibiotic gene (see table)

"In the white room, without DNA":

Work on ice with the set of "**without DNA**" pipettes. Each group prepares

- 4 PCR tubes (the number is written on top of each PCR tube)
- 2 mixes : 1 mix for *rpoB* to divide into 2 PCR tubes :

Tube 1 : *rpoB*-negative control (named « group#/1 »)

Tube 2 : *rpoB*-strain (named « group#/2 »)

1 mix for the other gene to divide into 2 PCR tubes :

Tube 3 : other gene-negative control (named « group#/3 »)

Tube 4 : other gene-strain (named « group#/4 »)

See components of the mix (depends on the target) in the table 2 of the chapter

Choose the appropriate polymerase (BioTaq or LA Taq)

Place Buffer first and add components pipeting up and down into the buffer

"In the grey room, with DNA"

add 5 μL of DNA into tubes *rpoB* (group#/2) and other gene (group#/4),

add 5 μL of sterile water into the negative control tubes (named "group#/1") and (named "group#/3").

Place the 4 tubes in the appropriate amplification machine in the order of the plan laied beside the machine.

Sequencing						
Group	**Strain**	**Primer**	**Tube 2**	**Strain**	**Primer**	**Tube 4**
1	I	*rpoB*	*1/2*	I	*gyrA*	*1/4*
2	H	*rpoB*	*2/2*	H	*katG*	*2/4*
3	L	*rpoB*	*3/2*	L	*gyrA*	*3/4*
4	K	*rpoB*	*4/2*	K	*gyrA*	*4/4*
5	J	*rpoB*	*5/2*	J	*rrs*	*5/4*
6	F	*rpoB*	*6/2*	F	*gyrA*	*6/4*
7	M	*rpoB*	*7/2*	M	*pncA*	*7/4*
8	N	*rpoB*	*8/2*	N	*pncA*	*8/4*
9	K	*rpoB*	*9/2*	K	*pncA*	*9/4*
10	G	*rpoB*	*10/2*	G	*rrs*	*10/4*

Electrophoresis on Tuesday June 13 2017
Each group deposes his PCR products in 1% TAE agarose gel.
Two groups for one gel :

Prepare 3 μL blue + 5 μL of PCR product and deposes 8 μL of this mix
Ladder : 5 µL/well of a 0,1ug/ µL solution in loading buffer blue

Group	First				Second			
Gel wells	1	2	3	4	5	6	7	8
	Ladder	negative control	rpoB	other target	Ladder	negative control	rpoB	other target

Gel	Groups
1	1 and 2
2	3 and 4
3	5 and 6
4	7 and 8
5	9 and 10