Antibiotic Resistance Sequencing

Principle

Sequencing of specific genes allows for the detection of mutations known to be associated with antibiotic resistance and the prediction of their resistance level (1, 2). Target genes – or parts of them – are here amplified and sequenced, e.g., 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 sequences 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
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.

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)
<table>
<thead>
<tr>
<th>Genes</th>
<th>Antibiotics</th>
<th>Primers</th>
<th>Sequences</th>
<th>Hybridization temperature</th>
<th>Amplification product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrA</td>
<td>Fluoroquinolones</td>
<td>gyrA-F</td>
<td>5'-'GATGACAGACACGCACGTTG-3'</td>
<td>55°C</td>
<td>398 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gyrA-R</td>
<td>5'-'GGCCTCGGTGACTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rrs</td>
<td>Aminoglycosides</td>
<td>rrs-F</td>
<td>5'-'AAACCTCTTTACACCTGAC-3'</td>
<td>59°C</td>
<td>1329 bp</td>
</tr>
<tr>
<td></td>
<td>Capreomycin</td>
<td>rrs-R</td>
<td>5'-'GTATCCATTGAGTTGCTGC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rpoB</td>
<td>Rifampicin</td>
<td>TR1 (rpoB-F)</td>
<td>5'-'TACGCTGGCGAGCGTAT-3'</td>
<td>53°C</td>
<td>411 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TR2 (rpoB-R)</td>
<td>5'-'TGACCTGCTTCGATGAACC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pncA</td>
<td>Pyrazinamide</td>
<td>pncA-F</td>
<td>5'-'CTGTCAAGGACGGATTG-3'</td>
<td>60°C</td>
<td>950 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pncA-R</td>
<td>5'-'ATCGCGATGGAACGTTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>katG</td>
<td>Isoniazid</td>
<td>katG-1 (katG-F)</td>
<td>5'-'TGGCCGCAGCGGCCGTCAATT-3'</td>
<td>60°C</td>
<td>330 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>katG-2 (katG-R)</td>
<td>5'-'CCAGCAGGCTCTTCGTCAG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primer sequences for antibiotic gene resistance

b) Amplification mix preparation
- 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

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentration</th>
<th>Quantity (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Buffer</td>
<td>1 X</td>
<td>5.0</td>
</tr>
<tr>
<td>DMSO</td>
<td>10 %</td>
<td>5.0</td>
</tr>
<tr>
<td>MgCl₂ 50 mM</td>
<td>1.5 mM</td>
<td>1.5</td>
</tr>
<tr>
<td>Sens primer 10 µM</td>
<td>0.3 µM</td>
<td>1.5</td>
</tr>
<tr>
<td>Antisens primer 10 µM</td>
<td>0.3 µM</td>
<td>1.5</td>
</tr>
<tr>
<td>dNTP 25 mM each</td>
<td>200 µM</td>
<td>0.4</td>
</tr>
<tr>
<td>Biotaq™ 5U/µL</td>
<td>2 U</td>
<td>0.4</td>
</tr>
<tr>
<td>H₂O</td>
<td>29.7</td>
<td></td>
</tr>
<tr>
<td><strong>Final mix volume</strong></td>
<td></td>
<td>45.0</td>
</tr>
</tbody>
</table>

**PCR mix for antibiotic gene resistance sequencing**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final concentration</th>
<th>Quantity (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Buffer</td>
<td>1X</td>
<td>25</td>
</tr>
<tr>
<td>Sens primer 10 µM</td>
<td>0.4 µM</td>
<td>2</td>
</tr>
<tr>
<td>Antisens primer 10 µM</td>
<td>0.4 µM</td>
<td>2</td>
</tr>
<tr>
<td>dNTP 2,5 mM each</td>
<td>400 µM</td>
<td>8</td>
</tr>
<tr>
<td>LA Taq™ 5U/µL</td>
<td>2 U</td>
<td>0.4</td>
</tr>
<tr>
<td>H₂O</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td><strong>Final mix volume</strong></td>
<td></td>
<td>45.0</td>
</tr>
</tbody>
</table>
- 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:
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:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Organism</th>
<th>Accession</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>gyrA</strong></td>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>Rv0006</td>
<td><strong>2517 bp</strong>&lt;br&gt;DNA GYRASE (SUBUNIT A) GYRA (DNA TOPOISOMERASE (ATP-HYDROLYSING)) (DNA TOPOISOMERASE II) (TYPE II DNA TOPOISOMERASE)</td>
</tr>
<tr>
<td><strong>rrs</strong></td>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>MTB000019</td>
<td><strong>1537 bp</strong>&lt;br&gt;ribosomal RNA 16S</td>
</tr>
<tr>
<td><strong>rpoB</strong></td>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>Rv0667</td>
<td><strong>3519 bp</strong>&lt;br&gt;DNA-DIRECTED RNA POLYMERASE (BETA CHAIN) RPOB (TRANSCRIPTASE BETA CHAIN) (RNA POLYMERASE BETA SUBUNIT)</td>
</tr>
<tr>
<td><strong>pncA</strong></td>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>Rv2043</td>
<td><strong>561 bp</strong>&lt;br&gt;PYRAZINAMIDASE/NICOTINAMIDAS PNCA (PZase)</td>
</tr>
<tr>
<td><strong>katG</strong></td>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>Rv1908</td>
<td><strong>2223 bp</strong>&lt;br&gt;CATALASE-PEROXIDASE-PEROXYNITRITASE T KATG</td>
</tr>
</tbody>
</table>

Compare to databases (11) [http://tuberculist.epfl.ch](http://tuberculist.epfl.ch)
6. Bibliographic references


# Annexe

Table 1
Conversion table between the two co-existing numbering systems for the most frequent rolA mutations associated with rifampicin resistance among Mycobacterium tuberculosis complex, *Mycobacterium leprae* and *Mycobacterium kansasi*

<table>
<thead>
<tr>
<th>WT nucleotide sequence (amino acid)</th>
<th>Codon position (conversion from <em>E. coli</em> numbering)</th>
<th>WT nucleotide sequence (amino acid)</th>
<th>Codon position (conversion from <em>E. coli</em> numbering)</th>
<th>WT nucleotide sequence (amino acid)</th>
<th>Codon position (conversion from <em>E. coli</em> numbering)</th>
<th>WT nucleotide sequence (amino acid)</th>
<th>Codon position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTT (Val)</td>
<td>170 (+24)</td>
<td>GTC (Val)</td>
<td>146</td>
<td>GTG (Val)</td>
<td>907</td>
<td>GTC (Val)</td>
<td>511</td>
</tr>
<tr>
<td>GGC (Gly)</td>
<td>426 (+22)</td>
<td>CAG (Glu)</td>
<td>432 (+22)</td>
<td>CAG (Glu)</td>
<td>442 (+22)</td>
<td>CAG (Glu)</td>
<td>513</td>
</tr>
<tr>
<td>CTG (Leu)</td>
<td>430 (+21)</td>
<td>CTG (Leu)</td>
<td>430 (+21)</td>
<td>CTG (Leu)</td>
<td>430 (+21)</td>
<td>CTG (Leu)</td>
<td>516</td>
</tr>
<tr>
<td>GAA (Lys)</td>
<td>432 (+21)</td>
<td>GAA (Lys)</td>
<td>432 (+21)</td>
<td>GAA (Lys)</td>
<td>432 (+21)</td>
<td>GAA (Lys)</td>
<td>519</td>
</tr>
<tr>
<td>GAG (Glu)</td>
<td>435 (+21)</td>
<td>GAG (Glu)</td>
<td>435 (+21)</td>
<td>GAG (Glu)</td>
<td>435 (+21)</td>
<td>GAG (Glu)</td>
<td>526</td>
</tr>
<tr>
<td>CAC (His)</td>
<td>444 (+20)</td>
<td>CAC (His)</td>
<td>444 (+20)</td>
<td>CAC (His)</td>
<td>444 (+20)</td>
<td>CAC (His)</td>
<td>526</td>
</tr>
<tr>
<td>TCG (Ser)</td>
<td>450 (+19)</td>
<td>TCG (Ser)</td>
<td>450 (+19)</td>
<td>TCG (Ser)</td>
<td>450 (+19)</td>
<td>TCG (Ser)</td>
<td>531</td>
</tr>
<tr>
<td>CTT (Leu)</td>
<td>452 (+18)</td>
<td>CTT (Leu)</td>
<td>452 (+18)</td>
<td>CTT (Leu)</td>
<td>452 (+18)</td>
<td>CTT (Leu)</td>
<td>533</td>
</tr>
<tr>
<td>ATC (Ile)</td>
<td>455 (+18)</td>
<td>ATC (Ile)</td>
<td>455 (+18)</td>
<td>ATC (Ile)</td>
<td>455 (+18)</td>
<td>ATC (Ile)</td>
<td>572</td>
</tr>
</tbody>
</table>

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.