

# Procedure Manual

## Nitrate Reductase Assay (NRA)

*Drug susceptibility testing for Mycobacterium tuberculosis*

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## Drug Susceptibility Testing using the Nitrate Reductase Assay (NRA)

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### Procedure for the NRA test for first-line drugs and ofloxacin resistance detection

#### 1. Principle of the test

Nitrate Reduction Assay is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, which is routinely used for biochemical identification of mycobacterial species. The presence of nitrite can easily be detected with specific reagents, which produce a color change. Nitrate reduction assay uses the detection of nitrite as indication of growth when it is used as a drug susceptibility test.

#### 2. Reagents and materials

- Löwenstein Jensen medium (to test first-line drugs), fresh eggs
- All material needed to prepare a LJ media (2 L Erlenmeyer with stopper; glass beads (ϕ10mm); glass funnel; 1L measuring cylinder; 25 ml measuring cylinder; sterile gauze; crepe paper; 1L burette and inspissator)
- 7H11 medium (to test ofloxacin) (500 gr Difco ref. 0838-17)
- KNO<sub>3</sub> (500 gr Sigma ref.P-8394)
- Incubator 37°C
- Analytic balance
- Glycerol (ref. 356350 Merck 500 ml)
- hydrochloric acid (HCl)
- 0.2 % (wt/vol) sulphanilamide (100 gr Merck ref. 1.11799.0100)
- 0.1% (wt/vol) *n*-1-naphthylethylenediamine dihydrochloride (5 gr Merck ref.1.06237.0005)
- Drugs used in the following critical concentration:

Drugs	Critical Concentration (µg/ml)	Medium	References of drugs	Conservation of drugs powder
Isoniazid (INH)	0.2	LJ	Sigma I-3377 (5 g)	+4°C
Rifampicin (RMP)	40	LJ	Sigma R7382 (5 g)	-20°C
Streptomycin (SM)	4	LJ	Sigma D-5155 (25 g)	+4°C
Ethambutol (EMB)	2	LJ	Sigma E-4630 (25 g)	+4°C
Ofloxacin (OFLO)	2	7H11	Sigma 0-8757 (1 g)	+4°C

### 3. Preparation of the media

#### 3.1 Preparation of egg-based media Löwenstein-Jensen (LJ) medium

Preparation of 1 L of LJ

##### **Ingredients**

###### *Mineral salt solution*

Potassium dihydrogen phosphate anhydrous (KH <sub>2</sub> PO <sub>4</sub> )	2.4g
Magnesium sulphate (MgSO <sub>4</sub> . 7H <sub>2</sub> O)	0.24g
Magnesium citrate	0.6g
Asparagine	3.6g
Glycerol (reagent grade)	12ml
Distilled water	600ml

Dissolve the ingredients *in order* in the distilled water by heating. Autoclave at 121°C for 30 minutes to sterilise. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator.

###### *Malachite green solution, 2%*

Malachite green dye	2.0g
Sterile distilled water	100ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will *not* store indefinitely and may precipitate or change to a less-deeply coloured solution. In either case discard and prepare a fresh solution.

###### *Homogenised whole eggs*

Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.

##### **Preparation of complete medium**

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

Mineral salt solution	600ml
Malachite green solution	20ml
Homogenised eggs (20-25 eggs, depending on size)	1000ml

The complete egg medium is distributed in 6-8ml volumes in sterile 14ml or 28ml McCartney bottles or in 20ml volumes in 20 x 150mm screw-capped test tubes, and the

tops are securely fastened. Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

### Preparation KNO<sub>3</sub> stock

Stock solution: 1gr KNO<sub>3</sub> / 5 ml distilled water

Add 1 ml stock KNO<sub>3</sub> to 200 ml LJ

### Preparation of drugs

drugs	Working solution1			Working solution 2			For 200 ml of LJ medium			
concentration stock solution (µg/ml)	volume stock solution (ml)	Sterile distilled water (ml)	Final concentration (µg/ml)	Working solution (ml)	Sterile distilled water (ml)	Final concentration (µg/ml)	Quantity ml	Final concentration (µg/ml)	Working solution to used	Quantity (ml)
INH (10 000)	0,5	4,5	1000	0,5	4,5	100	200	0,2	2	0,4
EMB (10 000)	0,5	4,5	1000	0,5	4,5	100	200	2,0	2	4,0
SM (10 000)	0,5	4,5	1000	2	2	500	200	4,0	2	1,6
RMP (4 000)	Work on the stock solution						200	40,0	Stock solution	2,0

### Coagulation of medium

Before loading, heat the inspissator to 80°C to quicken the build-up of the temperature. Place the bottles in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80°-85°C (since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilise it). Heating for a second or third time has a detrimental effect on the quality of the medium. The quality of egg media deteriorates when coagulation is done at too high a temperature or for too long. Discolouration of the coagulated medium may be due to excessive temperature. The appearance of little holes or bubbles on the surface of the medium also indicates faulty coagulation procedures. Poor quality media should be discarded.

***Sterility check***

After inspissation, the whole media batch or a representative sample of culture bottles should be incubated at 35°-37°C for 24 hours as a check of sterility.

***Storage***

The LJ medium should be dated and stored in the refrigerator and can keep for several weeks if the caps are tightly closed to prevent drying out of the medium. For optimal isolation from specimens, LJ medium should not be older than 4 weeks.

**3.2 7H11 media**

7H11 supplemented with 10% OADC, 0.5% glycerol with 1 mg/ml of KNO<sub>3</sub>

***For 200 ml of 7H11 medium***

Suspend 4.2 grams in 900 ml distilled water containing 1 ml glycerol and boil to dissolve completely. Sterilize at 122-124°C for 15 minutes. Aseptically add 100 ml of OADC enrichment to the medium at 50-55°C. Add the drug ofloxacin at a final concentration of 2 µg/ml.

**Stock solution: OFLO** should be dissolved in **0.1 N NaOH**

drugs	Working solution1			Working solution 2			For 200 ml of 7H11 medium			
concentration stock solution (µg/ml)	volume stock solution (ml)	0.1 N NaOH (ml)	Final concentration	Working solution (ml)	Sterile distilled water (ml)	Final concentration	Quantity ml)	Final concentration (µg/ml)	Working solution to used	Quantity (ml)
OFLO (10 000)	0,5	4,5	1000	2	2	500	200	4,0	2	1,6

**4. Preparation of inoculum****4.1 Inoculum from growth on solid medium**

It is very important to have fresh growth on a solid medium (21-28 days old). Older cultures may result in unreliable susceptibility test results.

Prepare a bacterial suspension of 1mg/ml as follow:

#### 4.1.1 Procedure A

- Weigh a sterile vial with glass beads (W1)
- Take a full loop of bacterial growth with a sterile loop and put it in the sterile vial with glass beads (try not to take any medium when removing growth)
- Weigh the sterile vial again (W2)
- Calculate the weight of bacteria you have in your vial:  $W3=W2-W1$
- To obtain a bacterial suspension of 1 mg/ml add an amount of sterile distilled water equal to 1000 x bacterial weight  
Example: add 3.7 ml of distilled water if  $W3=3.7$  mg
- Vortex the suspension for at least 1 minute to obtain a homogenous suspension and to break the clumps

#### 4.1.2 Procedure B - McFarland 1.0 standard

McFarland Standard	1% BaCl <sub>2</sub> (ml)	1% H <sub>2</sub> SO <sub>4</sub> (ml)	Approximate bacterial suspension per ml
0.5	0.05	9.95	$1.5 \times 10^8$
1.0	0.1	9.9	$3.0 \times 10^8$
2.0	0.2	9.8	$6.0 \times 10^8$
3.0	0.3	9.7	$9.0 \times 10^8$
4.0	0.4	9.6	$1.2 \times 10^9$
5.0	0.5	9.5	$1.5 \times 10^9$
6.0	0.6	9.4	$1.8 \times 10^9$
7.0	0.7	9.3	$2.1 \times 10^9$
8.0	0.8	9.2	$2.4 \times 10^9$
9.0	0.9	9.1	$2.7 \times 10^9$
10.0	1.0	9.0	$3.0 \times 10^9$

McFarland Standards are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension with the McFarland Standard. A McFarland Standard is a chemical solution of barium chloride (BaCl<sub>2</sub>) and sulfuric acid (H<sub>2</sub>SO<sub>4</sub>); the reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. When shaken well, the turbidity of a McFarland Standard is visually comparable to a bacterial suspension of known concentration as indicated in the table.

- Take a full loop of bacterial growth with a sterile loop and put it in a sterile vial with glass beads just covering the bottom of the vial with 2.5 ml of 7H9-S broth (try not to take any medium when removing growth).
- Vortex the vial for at least 1 minute to break the clumps until a fairly turbid suspension is obtained.
- Transfer the supernatant to a new sterile vial and leave it to sediment for 15 minutes.
- Compare the turbidity of the suspension using the scale McFarland 1.0 standard.

McFarland Standards should be stored in standing position at 4°C to 8°C and protected from light during 12 weeks.

#### **4.2 Inoculum from a liquid medium**

- Transfer 2.5 ml of the culture to a sterile vial containing glass beads.
- Shake it in the vortex for at least 1 minute until a fairly turbid suspension is obtained.
- Transfer the suspension to a new plastic tube and leave it to sediment for 15 minutes.
- Compare the turbidity of the suspension with that of a McFarland standard tube 1.

#### **4.3 Dilution of the inoculum**

The inoculum turbidity is adjusted to a McFarland tube no. 1 and further diluted 1:10 in PBS.

### **5. Preparation of antibiotics**

#### **5.1 Drug potencies**

The true potency of the drug is the number of micrograms of active drug per milligram total weight of the product. Not all antimicrobial drugs have been isolated in pure form, and a portion of their weight may be due to impurities or to the sulphate or another radical component of the molecule. Each lot of drugs could be varied from previous ones, and the potency of one lot may not be the same as that of another lot.

To calculate the weight of drug necessary according to the potency use the following formula:

To prepare 10 ml of a 10000 µg/ml (1 mg/ml):

$$\frac{10000}{\text{Potency (in mg/g)}} \times 10 = \text{milligrams to weigh}$$

- Example: potency of EMB: 740 mg of active EMB per gr:

$$\frac{10000}{740} \times 10 = 135$$

Dissolve 135 mg in 10 ml of distilled water for 10000 µg EMB per ml.

## 6. Inoculation of the NRA tubes

0.2 ml of the 1:10 dilution is inoculated in three drugs free tubes and 0.2 ml of the undiluted suspension (McFarland tube no. 1) will be inoculated into the drug containing tube.

## 7. Incubation

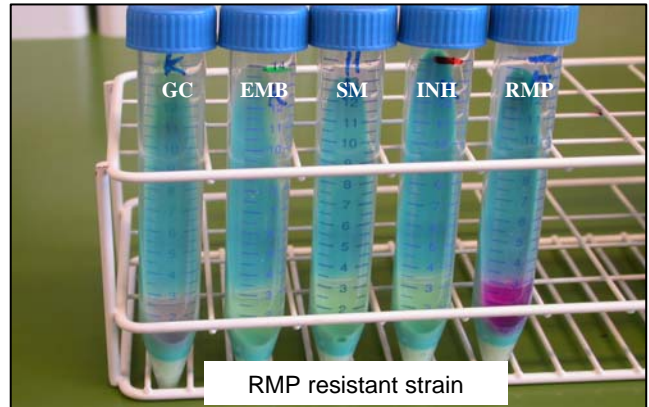
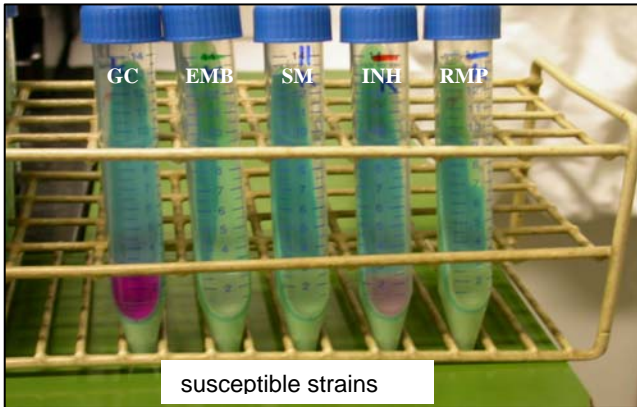
The tubes will be incubated at 37 °C until seventh day.

## 8. Reading

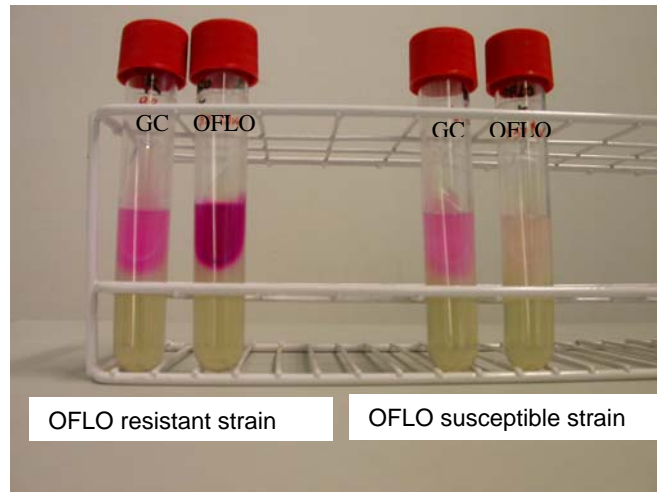
Reagents mix: 1 part 50 % (vo/lvol) concentrated hydrochloric acid (HCl), 2 parts 0.2 % (wt/vol) sulfanilamide, and 2 parts 0.1% (wt/vol) n-1-naphthylethylenediamine dihydrochloride. They will be mixed shortly to will be used.

**After** 7 days of incubation , 0.5 ml of a reagent mixture will be added to one free drug tube. If any color occurs, the corresponding antibiotic containing tube will be developed by the reagent mixture. If not color change occurs, the tube will be discarded and the others tubes will be reincubated. The procedure will be repeat at day 10 using the second growth control tube, and if is necessary, also at day 14 using the third growth control tube.

**Results interpretation:** A strain will be considered resistant if a color change in the drug containing tube is greater or the same than 1:10-diluted growth control tube.



GC: growth control



**References:**

- 1. Ängeby, K. A., Klintz, L. and S. E. Hoffner.** 2002. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. *J. Clin. Microbiol.* 40, 553-555.
- 2. Lemus, D., Martin, A., Montoro, E., Portaels, F., Palomino, JC.** 2004. Rapid alternative methods for detection of rifampicin resistance in *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* 54:130-133.
- 3. Montoro E, Lemus D, Echemendia M, Martin A, Portaels F, Palomino JC.** Comparative evaluation of the nitrate reduction assay, the MTT test, and the resazurin microtitre assay for drug susceptibility testing of clinical isolates of *Mycobacterium tuberculosis*. *J Antimicrob Chemother.* 2005 Apr;55(4):500-5
- 4. Martin A, Palomino JC, Portaels F.** Rapid detection of ofloxacin resistance in *Mycobacterium tuberculosis* by two low-cost colorimetric methods: resazurin and nitrate reductase assays. *J Clin Microbiol* 2005; 43: 1612-6.
- 5. Panaiotov S, Kantardjiev T.** Nitrate reductase assay for drug susceptibility testing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2002; 40(10): author reply 3881-3882.  
Ängeby KA, Klintz L, Hoffner SE. Rapid and inexpensive drug susceptibility testing of *Mycobacterium tuberculosis* with a nitrate reductase assay. *J Clin Microbio.* 2002; 40: 553-555.
- 6. Martin A, Montoro E, Lemus D, Simboli N, Morcillo N, Velasco M, Chauca J, Barrera L, Ritacco V, Portaels F, Palomino JC.** Multicenter evaluation of the nitrate reductase assay for drug resistance detection of *Mycobacterium tuberculosis*. *J Microbiol Methods* 2005; 63: 145-50.
- 7. Martin A, Panaiotov S, Portaels F, Hoffner S, Palomino JC, Angeby K.** The nitrate reductase assay for the rapid detection of multidrug resistant *M. tuberculosis*: a systematic review and meta-analysis. *J Antimicrob Chemother.* 2008, 62: 56-65