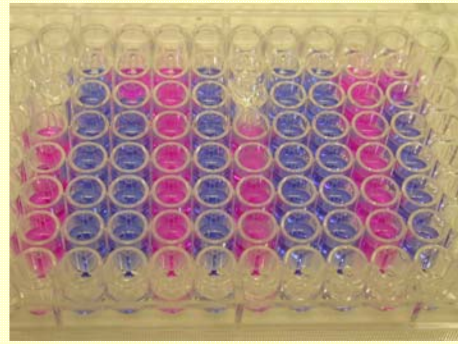

Procedure Manual

Resazurin Microtiter Assay (REMA)

Colorimetric redox indicator (CRI)

Drug susceptibility testing for Mycobacterium tuberculosis

By

Anandi MARTIN, PhD amartin@itg.be

&

Juan Carlos PALOMINO, PhD palomino@itg.be

Institute of Tropical Medicine, Mycobacteriology Unit Antwerp, Belgium

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Anandi MARTIN, PhD
&
Juan Carlos PALOMINO, PhD

Institute of Tropical Medicine, Mycobacteriology Unit
Antwerp, Belgium

With the collaboration of

Dihadenys LEMUS, PhD
&
Ernesto MONTORO, MD, PhD

Institute of Tropical Medicine 'Pedro Kouri'
La Habana, Cuba

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Resazurin Microtiter Assay (REMA) - Colorimetric Assay

Procedure for the REMA plate for first- and second-line drugs

1. Principle of the test

Colorimetric methods for detecting drug resistance in *M. tuberculosis* are based on the reduction of an oxidation-reduction indicator added to a liquid culture medium after *M. tuberculosis* has been exposed *in vitro* to different antibiotics. Resistance is detected by a change in colour of the oxidation-reduction indicator, which is directly proportional to the number of viable mycobacteria in the medium.

2. Reagents and materials

- 7H9 broth: Middlebrook 7H9 (ref. 271310-500 g- Becton Dickinson)
- Casitone: Bacto Casitone (ref. 225930-500 g- Becton Dickinson)
- OADC: Oleic Acid Dextrose Catalase (ref. 211886-10 x 20 ml-Becton Dickinson)
- Glycerol: (ref. 356350 Merck 500 ml)
- 96-well plate with lid: (ref. 353072, box of 50 plate-Becton Dickinson)
- Filter 0.2 µm: Acrodisc Syringe filter (ref. 4652- 50 filters-Pall Life Sciences)
- Syringes for filter: 2 or 5 ml
- Distilled water
- 37°C incubator
- Micropipettes: 1000 µl / 200 µl
- Tips + tips with filter (1000 µl / 200 µl)
- Multichannel pipette
- Resazurin: Resazurin sodium salt (ref. 189900010-1 g-Acros Organics)
- Analytic balance
- Incubator 37°C
- Autoclave
- Drugs:

Drugs	Name	References	Conservation
INH	Isoniazid	Sigma I-3377 (5 g)	+4°C
RMP	Rifampicin	Sigma R7382 (5 g)	-20°C
SM	Streptomycin	Sigma D-5155 (25 g)	+4°C
EMB	Ethambutol	Sigma E-4630 (25 g)	+4°C
PAS	para-aminosalycylic acid	Acros 22753-0055 (5 g)	+4°C
CAP	Capreomycin	Sigma C-4142 (1 g)	-20°C
ETH	Ethionamide	Sigma E-6005 (5 g)	+4°C
KAN	Kanamycin	ICN-biomedicals 194531 (5 g)	+4°C
OFLO	Ofloxacin	Sigma 0-8757 (1 g)	+4°C

3. Preparation of the Middlebrook 7H9-S broth

7H9-Supplemented (7H9-S) = 7H9 broth + 10% OADC+ 0.5 % glycerol
+ 0.1 % casitone

For 200 ml of 7H9-S media:

- Weigh 0.94 g of 7H9 powder and dissolve in 180 ml of distilled water; mix until complete solubilization.
- Weigh 0.2 g of casitone and add to the previous solution until complete solubilization. Warm the solution if necessary.
- Autoclave the broth in a 250 ml flask.
- After autoclaving and cooling, add 20 ml of OADC (oleic acid dextrose catalase) enrichment and 1 ml of sterile glycerol. Mix well.
- Check in the incubator for sterility (leave one night in the incubator and check the day after if there is no turbidity and if the media is still transparent).
- Store the medium protected from direct light at 4°C.

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

- Weight 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% $BaCl_2$ (ml)	1% H_2SO_4 (ml)	Approximate bacterial suspension per ml
0.5	0.05	9.95	$1.5x10^8$
1.0	0.1	9.9	$3.0x10^8$
2.0	0.2	9.8	$6.0x10^8$
3.0	0.3	9.7	$9.0x10^8$
4.0	0.4	9.6	$1.2x10^9$
5.0	0.5	9.5	$1.5x10^9$
6.0	0.6	9.4	$1.8x10^9$
7.0	0.7	9.3	$2.1x10^9$
8.0	0.8	9.2	$2.4x10^9$
9.0	0.9	9.1	$2.7x10^9$
10.0	1.0	9.0	$3.0x10^9$

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 ($BaCl_2$) and sulfuric acid (H_2SO_4); 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

- Make a 1:20 dilution when testing first-line drugs and 1:10 dilution for second-line drugs with 7H9-S broth. If first and second-line drugs are testing together do only one dilution 1:10 for both drugs.

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.

5.2 Preparation of antibiotics stock solutions

Drug / Weight	Solvent / Volume	Drug Stock (µg/ml)	Drug Dilution		Working Solution (4X)	Range (µg/ml)
			Drug stock (µl)	7H9-S (µl)		
INH / 2 mg	distilled water / 2 ml	1 000	10	2 490	4 µg/ml	1 - 0.0312
RMP / 20 mg	methanol / 2 ml	(I) 10 000	(I) 250	2 250		
		(II) 1 000	(II) 20	2 480	8 µg/ml	2 - 0.0625
EMB / 2 mg	distilled water / 2 ml	1 000	160	2 340	64 µg/ml	16 - 0.5
SM / 2 mg	distilled water / 2 ml	1 000	40	2 460	16 µg/ml	4 - 0.125
OFLO / 2 mg	NaOH 0.1N / 2 ml	1 000	80	2 420	32 µg/ml	8 - 0.25
KAN / 2 mg	distilled water / 2 ml	1 000	200	2 300	80 µg/ml	20 - 0.62
ETH / 2 mg	DMSO / 2 ml	1 000	200	2 300	80 µg/ml	20 - 0.62
CAP / 2 mg	distilled water / 2 ml	1 000	100	2 400	40 µg/ml	10 - 0.3
PAS / 2mg	distilled water / 2 ml	1 000	80	2 420	32 µg/ml	8 - 0.25

- **Isoniazid (INH):** Weigh 2 milligrams of isoniazid powder and dissolve in 2,0 ml of distilled water, making a stock solution of **1 mg/ml**. Sterilize by filtration with a 0,22 µm syringe filter; aliquot and store frozen (-20°C) until use.

- **Rifampicin (RMP):** Weigh 20 milligrams of rifampicin powder, dissolve with a small volume of absolute methanol and complete to 2,0 ml with distilled water, making a stock solution of **10 mg/ml**. Sterilize by filtration with a 0,22 µm syringe filter; aliquot and store frozen (-20°C) until use.

- **Ethambutol (EMB):** Weigh 2 milligrams of ethambutol powder and dissolve in 2,0 ml of distilled water, making a stock solution of **1 mg/ml**. Sterilize by filtration with a 0,22 µm syringe filter; aliquot and store frozen (-20°C) until use.

- **Streptomycin (SM):** Weigh 2 milligrams of streptomycin powder and dissolve in 2.0 ml of distilled water, making a stock solution of **1 mg/ml**. Sterilize by filtration with a 0.22 µm syringe filter; aliquot and store frozen (-20°C) until use.

- **Ofloxacin (OFLO):** Weigh 2 milligrams of ofloxacin powder and dissolve in 2.0 ml of 0.1 N NaOH, making a stock solution of **1 mg/ml**. Sterilize by filtration with a 0.22 µm syringe filter; aliquot and store frozen (-20°C) until use.

- **Kanamycin (KAN):** Weigh 2 milligrams of kanamycin powder and dissolve in 2.0 ml of distilled water, making a stock solution of **1 mg/ml**. Sterilize by

filtration with a 0.22 µm syringe filter; aliquot and store frozen (-20°C) until use.

- **Ethionamide (ETH):** Weigh 2 milligrams of ethionamide powder and dissolve in 2.0 ml of DMSO, making a stock solution of **1 mg/ml**. Sterilize by filtration with a 0.22 µm syringe filter; aliquot and store frozen (-20°C) until use.

- **Capreomycin (CAP):** Weigh 2 milligrams of capreomycin powder and dissolve in 2.0 ml of distilled water, making a stock solution of **1 mg/ml**. Sterilize by filtration with a 0.22 µm syringe filter; aliquot and store frozen (-20°C) until use.

- **Para-amino salycilic acid (PAS):** Weigh 2 milligrams of para-amino salycilic acid powder and dissolve in 2.0 ml of distilled water, making a stock solution of **1 mg/ml**. Sterilize by filtration with a 0.22 µm syringe filter; aliquot and store frozen (-20°C) until use.

5.3 Preparation of antibiotic working solutions

Each antibiotic working solution is prepared at 4 times the final concentration to be tested in the plate.

- **INH**: add 10 µl of stock solution (1 mg/ml) to 2490 µl of 7H9-S broth to make a 4 µg/ml INH solution.

- **RMP:** make two dilutions! add 250 µl of the stock solution (10 mg/ml) to 2250 µl of 7H9-S and from this concentration, add 20 µl to 2480 µl of 7H9-S broth to make a 8 µg/ml RIF solution.

- **EMB:** add 160 µl of the stock solution (1 mg/ml) to 2340 µl of 7H9-S broth to make a 64 µg/ml EMB solution.

- **SM:** add 40 µl of the stock solution (1 mg/ml) to 2460 µl of 7H9-S broth to make a 16 µg/ml SM solution.

- **OFLO:** add 80 µl stock solution (1mg/ml) to 2420 µl 7H9-S broth to make a 32 µg/ml OFLO solution

- **KAN:** add 200 µl stock solution (1mg/ml) to 2300 µl 7H9-S broth to make a 80 µg/ml KAN solution

- **ETH:** add 200 µl stock solution (1mg/ml) to 2300 µl 7H9-S broth to make a 80 µg/ml ETH solution

- **CAP:** add 100 µl stock solution (1mg/ml) to 2400 µl 7H9-S broth to make a 40 µg/ml CAP solution.

- **PAS:** add 80 µl stock solution (1mg/ml) to 2420 µl 7H9-S broth to make a 32 µg/ml PAS solution.

6. Storage of drug stock solutions

Drug solutions may be frozen in aliquots at -20ºC and stored for up to 3 months. Once thawed, discard the leftover and do not store or refreeze.

7. Preparation of the REMA plate

7.1 For first-line drugs: INH-RMP-EMB-SM

See the diagram of the microtiter below.

In a 96-well flat-bottom microtiter plate there is enough space to test two isolates against the four drugs in six two-fold dilutions of each drug.

- Add 100 µl of 7H9-S broth to columns 2-11from rows B to G
- Add 100 µl of the working solution of INH to well B2 and B6
- Add 100 µl of the working solution of RMP to well B3 and B7
- Add 100 µl of the working solution of EMB to well B4 and B8
- Add 100 µl of the working solution of SM to well B5 and B9

  - With a multi-channel pipette make dilutions from rows B to G (columns 2-5 and 6-9) discarding the last 100 µl after mixing in row G.

  - Add 100 µl of 7H9-S broth to wells B11 and C11; these will represent the negative and sterility controls of the test.

  - Add 200 µl of sterile distilled water to all the outer wells left without broth; these will prevent evaporation during incubation of the plate.

7.2 For second-line drugs: PAS-ETH-KAN-OFLO-CAP

See the diagram of the microtiter below.

In a 96-well flat-bottom microtiter plate there is enough space to test two isolates against the five drugs in six two-fold dilutions of each drug.

- Add 100 µl of 7H9-S broth to columns 2-11 from rows B to G
- Add 100 µl of the working solution of PAS to well B2 and B7
- Add 100 µl of the working solution of ETH to well B3 and B8
- Add 100 µl of the working solution of KAN to well B4 and B9
- Add 100 µl of the working solution of OFLO to well B5 and B10
- Add 100 µl of the working solution of CAP to well B6 and B11

  - With a multi-channel pipette make dilutions from rows B to G (columns 2-6 and 7-11) discarding the last 100 µl after mixing in row G.

  - Add 200 µl of 7H9-S broth to wells B1 and B12; these will represent the negative and sterility controls of the test.

- Add 100 µl of 7H9-S broth to wells C2-C12; these will serve for the growth control in duplicate of the two isolates to be tested on the plate.

- Add 200 µl of sterile distilled water to all the outer wells left without broth; these will prevent evaporation during incubation of the plate.

8. Inoculation of the plates

Using tips with filters inoculate the plates with 100 µl of the strain A (or strain B) (dilution 1:20 or 1:10) to all wells included GC (+) well but not in the well containing the negative control.

9. Incubation of the plates

After inoculation, seal the plates in plastic bags and incubate at 37 °C for 7 days.

10. Preparation of the resazurin solution

Prepare a resazurin solution at 0.01 % or 0.02% in distilled water, sterilized by filtration through a 0.2 µm and keep it at 4°C for 1-2 weeks and protected from the light.

11. Color development and interpretation of results

After 7 days incubation, the plates are taken from the incubator and 30 µl of a the resazurin at 0.01 % or 0.02% is added to all the wells and the plate again sealed and incubated overnight for color development.

A change in color from blue to pink means a growth of the isolate at that concentration of the drug. For better interpretation of the results, the color must be compared to the color present in the growth control well.

- **The minimal inhibitory concentration (MIC)** of each drug is interpreted as the lowest concentration of the antibiotic that prevents a change in color of the resazurin. MIC values are scored for each isolate for comparison with the results obtained with the proportion method.

- **Positive and negative controls**: the positive control should show positive growth and the negative control should show no growth within the incubation protocol period. If negative control shows a growth, investigate procedures, could be a cross-manipulation and check for all reagents for possible source of contamination.

11.1 Breakpoint concentration

Drugs	Cut-off point by REMA
INH	0.25 µg/ml
RMP	0.5 µg/ml
EMB	4µg/ml
SM	1 µg/ml
ETH	2.5 µg/ml
CAP	2.5 µg/ml
OFLO	2 µg/ml
KAN	2.5 µg/ml
PAS	2 µg/ml

When the MIC is > to the cut-off point the strain is considered resistant.
In any case, a strain giving a MIC equal to the cut-off point should be repeated.

12. Detection of contamination

The incidence of contamination varies from laboratory to laboratory depending of several factors. The recommendation is that up to 5% contamination rate is acceptable. Liquid media are more susceptible to contamination than solid media. It is extremely important to take care during the manipulation and to work with sterile material.

-Any well with a turbid appearance is suspected of contamination and result of this well is not valid.
-At the moment that you add the resazurin if you observe directly a change of color the well could be contaminated and has to be discarded.

13. Precautions

-All work should be carried out in a proper biological safety cabinet (class II).
-All materials should be sterilized by autoclaving prior to disposal.
-Plate should be kept in the incubator together in a box to avoid that someone moved the plate by accident.
-Plate should be closed in both sides by a small tape for security.

14. Quality control (QC)

It is important to perform a quality control of drug susceptibility testing for first- and second-line drugs testing. Add the *M. tuberculosis* H37Rv (ATCC -American Type Culture Collection-number 27294) as a QC strain which is susceptible to all anti-tuberculosis drugs. A susceptible or resistant strain from other culture collections can be also used for quality control. If the susceptible H3Rv shows some resistance, then all the results obtained within during the experiment become invalid and the test should be repeated.

15. Diagram of the plate

Two diagrams of the plate are shown below as an example.

The diagram of the plate can be change, it depends of which drugs has to be tested. Some of the first-line drugs can be tested together with some of the second line drugs. Any diagram can be tested.

15.1 Diagram 96-well plate for first-line drugs

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		I 1µg/ml	R 2µg/ml	E 16µg/ml	S 4µg/ml	I 1µg/ml	R 2µg/ml	E 16µg/ml	S 4µg/ml	GC (+) strain A	GC (-) strain A	
C		0,5	1	8	2	0,5	1	8	2	GC (+) strain B	GC (-) strain B	
D		0,25	0,5	4	1	0,25	0,5	4	1			
E		0,125	0,25	2	0,5	0,125	0,25	2	0,5			
F		0,0625	0,125	1	0,25	0,0625	0,125	1	0,25			
G		0,0312	0,0625	0,5	0,125	0,0312	0,0625	0,5	0,125			
H												

Strain A (columns 2–5) Strain B (columns 6–9)

Inoculum: McFarland 1.0 dilution 1/20

Preparation of antibiotics:

Stock solution	Working solution	Dilution in 7H9-S	Final concentration
INH 1 mg/ml	4 µg/ml	-> 10 µl stock + 2490 µl 7H9-S	1 µg/ml
RMP 10 mg/ml	8 µg/ml	-> 250 µl stock + 2250 µl 7H9-S (i) ->20µl in 2480 µl 7H9-S (ii)	2 µg/ml
EMB 1 mg/ml	64 µg/ml	-> 160 µl stock + 2340 µl 7H9-S	16 µg/ml
SM 1 mg/ml	16 µg/ml	-> 40 µl stock + 2460 µl 7H9-S	4 µg/ml

Incubation: 37°C, 7 days

15.2 Diagram 96-well plate for second-line drugs

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	GC-	PAS 8 µg/ml	ETH 20µg/ml	KAN 20µg/ml	OFL 8 µg/ml	CAP 10µg/ml	PAS 8 µg/ml	ETH 20µg/ml	KAN 20µg/ml	OFL 8 µg/ml	CAP 10µg/ml	GC -
C	GC + strain A	4	10	10	4	5	4	10	10	4	5	GC + strain B
D		2	5	5	2	2,50	2	5	5	2	2,50	
E		1,00	2,50	2,50	1,00	1,25	1,00	2,50	2,50	1,00	1,25	
F		0,5	1,25	1,25	0,5	0,62	0,5	1,25	1,25	0,5	0,62	
G		0,25	0,62	0,62	0,25	0,3	0,25	0,62	0,62	0,25	0,3	
H												

Strain A (columns 1–6) Strain B (columns 7–12)

Inoculum: McFarland 1.0 dilution 1/10

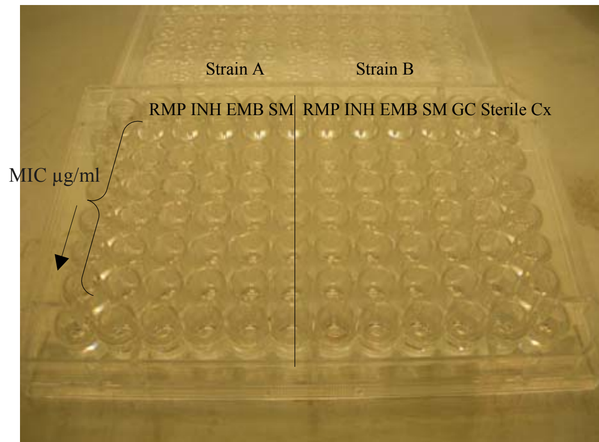
Preparation of antibiotics:

Stock solution	Working solution	Dilution in 7H9-S	Final concentration
PAS 1 mg/ml	32 µg/ml	-> 80 µl stock + 2420 µl 7H9-S	8 µg/ml
ETH 1 mg/ml	80 µg/ml	-> 20 µl stock + 2300 µl 7H9-S	20 µg/ml
KAN 1 mg/ml	80 µg/ml	-> 20 µl stock + 2300 µl 7H9-S	20 µg/ml
OFLO 1 mg/ml	32 µg/ml	-> 80 µl stock + 2420 µl 7H9-S	8 µg/ml
CAP 1mg/ml	40 µg/ml	-> 100 µl stock + 2400 µl 7H9-S	10 µg/ml

Incubation: 37°C, 7 days

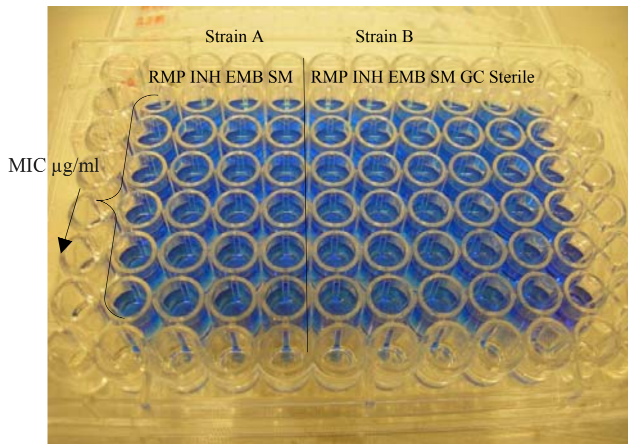
15.3 Resazurin microtiter assay in pictures

1. Preparation and inoculation of plate



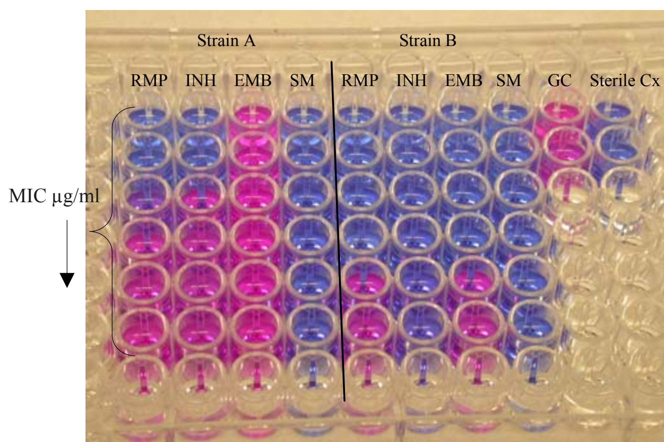
- Serial dilutions of the drug are prepared directly in a sterile 96-well flat bottom microtiter plate using a volume of 100 µl of 7H9-S medium.
- Add 100 µl of the inoculum McFarland 1.0 dilution 1/10 or 1/20 of strain A or strain B in the corresponding well.
- Incubation at 37°C for 7 days

2. Addition of the indicator after 7 days



- After 7 days of incubation add in each well 30 µl resazurin 0.01% or 0.02%.
- Re-incubation overnight for color development.

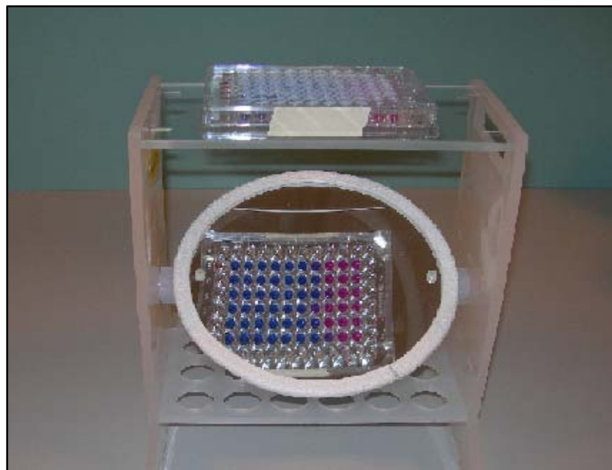
3. *Reading the plate after 24-48 hours*



- A change to pink indicates reduction of resazurin and therefore bacterial growth.
- The MIC is defined as the lowest drug concentration that prevented this color change.

15.4 Mirror for reading

For reading an amplified mirror can be used.



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