

## Determination of minimum inhibitory concentrations

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**Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, and minimum bactericidal concentrations (MBCs) as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media. MICs are used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine the *in vitro* activity of new antimicrobials, and data from such studies have been used to determine MIC breakpoints. MBC determinations are undertaken less frequently and their major use has been reserved for isolates from the blood of patients with endocarditis. Standardized methods for determining MICs and MBCs are described in this paper. Like all standardized procedures, the method must be adhered to and may not be adapted by the user. The method gives information on the storage of standard antibiotic powder, preparation of stock antibiotic solutions, media, preparation of inocula, incubation conditions, and reading and interpretation of results. Tables giving expected MIC ranges for control NCTC and ATCC strains are also supplied.**

### Introduction

Minimum inhibitory concentrations (MICs) are considered the 'gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definitive answer when a borderline result is obtained by other methods of testing, or when disc diffusion methods are not appropriate, for example when determining the susceptibility of coagulase-negative staphylococci to teicoplanin.

The range of antibiotic concentrations used for determining MICs is universally accepted to be in doubling dilution steps up and down from 1 mg/L as required. The MIC is defined as the lowest concentration of a drug that will inhibit the visible growth of an organism after overnight incubation (this period is extended for organisms such as anaerobes, which require prolonged incubation for growth).

The method described below is an amended version of the procedure described in the BSAC Guide to Sensitivity Testing<sup>1</sup> and can be adapted for determining the minimum bactericidal concentration (MBC) of an antimicrobial for an organism by substituting IsoSensitest agar (ISA; Oxoid, Basingstoke, UK) with IsoSensitest broth (ISTB; Oxoid) and then subculturing to drug-free media or can be truncated for use as a 'breakpoint' method. However, if the

method is adapted, the control strains cited below may not act as adequate controls for the concentration of antibiotic contained within prepared plates.

### 1. Antibiotic stock solutions: general considerations

1.1 Obtain standard powder from the pharmaceutical company or a reputable supplier such as Sigma (Poole, Dorset, UK).

1.2 Obtain information from the supplier regarding expiry date, potency, solubility, stability as a powder and in solution, storage conditions and any relevant COSHH (Control of Substances Hazardous to Health) information.

1.3 Always prepare stock solutions following the manufacturer's recommendations.

1.4 Freeze and thaw stock solutions only once and then discard them. Table I shows the suppliers, solvent, diluents and storage conditions for antibiotics.

### 2. Preparation of antibiotic stock solutions

2.1 Choose a suitable range of antibiotic concentrations for the organisms to be tested (see suggested ranges in Table II).

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**Table I.** Preparation and storage of antibiotic solutions (stored solutions should contain  $\geq 1000$  mg/L)

Antibiotic	Solvent	Diluent	4°C	-20°C	-70°C	Storage of powder	Supplier <sup>a</sup>
14 hydroxylarithmeticromycin	methanol	water	-	-	-	+4°C; protect from light and moisture	Abbott Laboratories
Amikacin (base)	water	water	7 days	1 month	-	+4-25°C; protect from moisture and light	Bristol Myers Squibb
Amoxicillin (trihydrate)	DMSO or <sup>b</sup>	water	7 days	unstable	30 days	+4°C; protect from light and moisture	GlaxoSmithKline
Ampicillin (trihydrate)	<sup>b</sup>	water	7 days	unstable	30 days	+4°C; protect from light and moisture	GlaxoSmithKline
Azithromycin (dihydrate)	<sup>c</sup>	water	-	-	-	+4-25°C; protect from moisture and light	Pfizer
Aztreonam (anhydrous crystalline B form)	<sup>b</sup>	water	1 day	3 months	-	+4°C; protect from light and moisture	Bristol Myers Squibb
Carbencillin (disodium)	water	water	-	-	-	+4°C; protect from light and moisture	GlaxoSmithKline
Cefaclor	water	water	-	-	-	+4°C; protect from light and moisture	Eli Lilly & Co Ltd
Cefepime (dihydrochloride)	<sup>b</sup>	water	-	-	-	+4°C; protect from light and moisture	Bristol Myers Squibb
Cefixime	water	water	-	-	-	2-8°C; protect from moisture and light	Wyeth Laboratories
Cefotaxime (sodium)	water	water	10 days	6 months	6 months	+4-25°C; protect from moisture and light	Aventis Pharma
Cefoxitin (sodium)	water	water	-	6 months	-	+4-25°C; protect from moisture and light	Merck Sharpe & Dohme Ltd
Cefpirome (sulphate)	water	water	-	-	-	2-8°C; protect from moisture and light	Aventis Pharma
Cefpodoxime (sodium)	<sup>b</sup>	water	1 day	3 months	-	2-8°C; protect from moisture and light	Aventis Pharma
Ceftazidime (pentahydrate)	water	water	7 days	-	-	+4-25°C; protect from moisture and light	GlaxoSmithKline
Ceftizoxime (sodium)	water	water	-	-	-	+4-25°C; protect from moisture and light	GlaxoSmithKline
Ceftriaxone (disodium)	water	water	-	-	-	2-8°C; protect from moisture and light	Roche Products Ltd
Cefuroxime (sodium)	water	water	3 days	30 days	-	+4°C; protect from light and moisture	GlaxoSmithKline
Cephalexin (hydrate)	water	water	7 days	-	-	+4°C; protect from light and moisture	GlaxoSmithKline
Cephadrine	<sup>c</sup>	water	1 day	-	-	+4°C; protect from light and moisture	Bristol Myers Squibb
Chloramphenicol	water	water	2 weeks	3 months	-	+4°C; protect from light and moisture	Sigma
Ciprofloxacin (hydrochloride monohydrate)	water	water	2 weeks	3 months	3 months	+4-25°C; protect from moisture and light	Bayer
Clarithromycin	DMSO	water	-	-	-	15-30°C; protect from light and moisture	Abbott Laboratories
Clavulanate (acid)	<sup>d</sup>	water	1-5 days	unsuitable	4 weeks	2-8°C; protect from moisture and light	GlaxoSmithKline
Clindamycin (hydrochloride)	water	water	-	-	-	+4°C; protect from light and moisture	Sigma
Cloxacillin (sodium monohydrate)	water	water	-	-	-	15-30°C; protect from light and moisture	GlaxoSmithKline
Colistin (sulphate)	water	water	-	-	-	2-8°C; protect from moisture and light	Pharmax
Doxycycline (hydrochloride)	water	water	-	-	-	2-8°C; protect from moisture and light	Pfizer
Erythromycin (base)	<sup>c</sup>	water	1 week	-	-	+4°C; protect from light and moisture	Abbott Laboratories
Flucloxacillin (sodium)	water	water	-	-	-	2-8°C; protect from moisture and light	GlaxoSmithKline
Fosfomycin (calcium)	water	water	-	-	-	2-8°C; protect from moisture and light	Pharmax
Fusidic acid (sodium)	<sup>c</sup>	water	-	-	-	+4-25°C; protect from moisture and light	Leo Laboratories
Gatifloxacin	<sup>e</sup>	water	-	-	-	+4°C; protect from light and moisture	Grunenthal
Gemifloxacin (base)	methanol	water	-	-	-	+4°C; protect from light and moisture	GlaxoSmithKline
Gentamicin (sulphate)	water	water	6 months	NR	NR	+4-25°C; protect from moisture and light	Aventis Pharma
Grepafloxacin (hydrochloride)	<sup>e</sup>	water	-	-	-	+4°C; protect from light and moisture	GlaxoSmithKline

## Determination of MICs

	<i>f</i>	<i>g</i>							
Telithromycin									Aventis Pharma
Imipenem (monohydrate)									Merck Sharpe & Dohme Ltd
Kanamycin (monosulphate)	water	1 day	NR	1 month					Sanofi Winthrop
Levofloxacin (hemihydrate)	water								Aventis Pharma
Linezolid	water								Pharmacia & Upjohn Ltd
Mecillinam	water								Leo Laboratories
Meropenem (trihydrate)	water								Zeneca Pharma
Methicillin (sodium)	water								GlaxoSmithKline
Metronidazole	water								Aventis Pharma
Mezlocillin	water	1 week	1 month	4 months					Bayer
Moxifloxacin (hydrochloride)	water								Bayer
Mupirocin (lithium)	water								GlaxoSmithKline
Nalidixic acid	water								Sanofi Winthrop
Netilmicin (sulphate)	water	6 months	6 months	6 months					Schering Plough
Nitrofurantoin	DMF								Proctor & Gamble
Norfloxacin	water								Merck Sharpe & Dohme Ltd
Ofloxacin	water								Aventis Pharma
Oxacillin (sodium)	water								GlaxoSmithKline
Penicillin (benzyl)[potassium]	water		1 month	1 month					GlaxoSmithKline
Piperacillin (sodium)	water	2 days	1 month						Wyeth Laboratories
Quinupristin/dalfopristin	water		1 month						Aventis Pharma
Rifampicin (crystalline)	DMSO	1 month	1 month						Aventis Pharma
Roxithromycin	water								Aventis Pharma
Sparfloxacin	water								Aventis Pharma
Spectinomycin (dihydrochloride pentahydrate)	water								Pharmacia & Upjohn Ltd
Streptomycin (sulphate)	water								Medeva Pharma Ltd
Sulphamethoxazole (free acid)	water	1 month	6 months	2 years					GlaxoSmithKline
Tazobactam (sodium salt)	water								Wyeth Laboratories
Teicoplanin	water								Aventis Pharma
Tetracycline (hydrochloride)	water		NR <sup>i</sup>	NR <sup>i</sup>					Wyeth Laboratories
Ticarcillin (sodium)	water	1 week	1 month						GlaxoSmithKline
Tobramycin (sulphate)	water	1 week	3 months						Eli Lilly & Co Ltd
Trimethoprim (base)	water	1 month	6 months	2 years					GlaxoSmithKline
Vancomycin (hydrochloride)	water	1 week	3 months						Eli Lilly & Co Ltd

<sup>a</sup>Many agents are available from Sigma, Poole, UK.

<sup>b</sup>Saturated NaHCO<sub>3</sub> solution.

<sup>c</sup>Ethanol.

<sup>d</sup>Phosphate buffer (0.1 M, pH 6).

<sup>e</sup>Water and 0.1 M NaOH dropwise to dissolve.

<sup>f</sup>Water (1 mL) + 10 µL glacial acetic acid.

<sup>g</sup>Phosphate buffer (0.07 M, pH 8).

<sup>h</sup>1 M MOPS pH 6.8 buffer.

<sup>i</sup>Precipitation on freezing.

NR = not recommended; DMF = dimethylformamide; DMSO = dimethylsulphoxide. All solutions should be placed in glass containers.

**Table II.** Suggested ranges for MIC determinations (mg/L)

Antibiotic	Enterobacteriaceae	<i>Pseudomonas</i> spp.	<i>Haemophilus</i> spp.	<i>Neisseria</i> spp.	<i>B. fragilis</i>	Staphylococci	Haemolytic streptococci	Enterococci	Pneumococci
Amikacin	0.03–128	0.06–128	0.12–16	0.5–16	–	0.008–128	1–128	1–128	1–128
Amoxicillin	0.25–128	–	0.06–128	0.004–32	1–128	0.03–128	0.008–0.12	0.12–128	0.008–4
Ampicillin	0.25–128	–	0.06–128	0.004–32	1–128	0.03–128	0.008–0.12	0.12–128	0.008–4
Azithromycin	0.25–128	–	–	–	–	–	–	–	–
Azlocillin	0.25–128	0.5–512	0.03–2	0.004–8	1–16	0.06–128	–	–	–
Aztreonam	0.004–128	0.5–128	0.015–2	0.015–2	8–128	>128	–	–	–
Cefaclor	–	–	0.5–128	–	–	–	–	–	0.25–64
Cefixime	0.03–128	–	0.008–0.12	0.002–1	8–128	4–64	0.03–0.5	8–128	0.12–16
Cefotaxime	0.004–128	0.5–128	0.004–0.5	0.004–0.5	0.5–128	0.5–128	–	–	–
Cefoxitin	0.5–128	–	1–8	0.06–8	2–128	1–32	–	–	–
Cefpirome	0.008–32	0.25–128	0.008–0.5	0.001–0.12	4–128	0.06–128	0.004–0.12	1–128	0.008–1
Cefpodoxime	0.06–128	0.25–128	0.06–0.5	0.002–0.06	8–128	1–128	0.015–0.12	1–128	0.03–4
Ceftazidime	0.004–128	0.25–128	0.015–0.5	0.004–0.5	4–128	2–128	0.03–1	0.12–128	0.03–32
Ceftizoxime	0.004–128	–	0.008–0.25	0.004–0.015	0.5–128	1–128	–	–	–
Ceftriaxone	0.001–128	0.5–128	0.001–0.06	0.001–0.06	2–128	0.25–128	0.008–0.12	0.004–128	0.004–16
Cefuroxime	0.03–128	–	0.25–16	0.008–1	1–128	0.25–64	0.008–0.12	2–128	0.015–8
Cephalexin	0.25–128	–	1–128	–	4–128	0.5–128	–	–	–
Cephradine	0.25–128	–	1–128	–	1–128	0.25–128	–	–	–
Chloramphenicol	0.25–128	–	0.06–128	0.06–8	1–8	2–16	1–16	1–128	1–16
Ciprofloxacin	0.004–128	0.015–128	0.002–0.06	0.001–0.12	2–8	0.06–128	0.12–4	0.25–128	0.25–128
Clarithromycin	–	–	1–32	0.015–1	0.03–2	0.03–128	0.015–16	0.03–128	0.03–128
Co-amoxiclav <sup>a</sup>	0.5–128	–	0.03–128	0.004–32	0.5–128	0.008–16	0.008–0.12	0.12–16	0.008–4
Clindamycin	–	–	–	–	0.015–2	0.03–8	–	–	–
Colistin	0.5–128	0.5–64	–	–	–	–	–	–	–
Quinupristin/dalfopristin	–	–	–	–	4–32	0.12–16	0.12–1	0.25–8	0.12–32
Doxycycline	–	–	0.03–128	0.25–16	–	0.06–128	–	–	–
Erythromycin	–	–	0.25–128	0.03–0.5	0.25–128	0.06–128	0.06–8	0.25–128	0.06–128
Fusidic acid	–	–	–	–	–	0.03–128	–	–	–
Gatifloxacin	–	–	–	0.001–0.12	–	–	–	–	–
Gemifloxacin	–	–	–	0.001–0.12	–	–	–	–	–
Gentamicin	0.03–128	0.06–128	0.12–16	0.5–16	–	0.008–128	–	0.5–2048	–
Grepafloxacin	–	–	0.002–0.06	0.001–0.12	–	–	–	–	–
Telithromycin	–	–	0.25–8	0.002–0.5	0.03–8	0.03–128	0.001–0.25	0.015–4	0.004–1
Imipenem	0.06–4	0.06–16	0.25–4	0.004–0.25	0.015–4	0.03–128	0.002–0.25	0.25–128	0.002–0.25
Levofloxacin	–	–	–	0.001–0.12	–	–	–	–	0.5–32

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Linezolid	0.03-128	0.12-16	0.007-1	-	1-4	0.12-8	0.25-8	0.25-8	0.5-8
Mecillinam	0.03-128	-	-	-	-	-	-	-	-
Meropenem	0.015-4	0.015-16	0.015-0.25	0.002-0.03	0.03-32	0.015-128	0.002-0.06	0.004-128	0.002-16
Methicillin	-	-	-	-	-	0.12-128	-	-	-
Metronidazole	-	-	-	-	0.06-32	-	-	-	-
Mezlocillin	0.25-128	0.5-512	-	-	1-128	0.12-128	-	-	-
Moxifloxacin	-	-	-	0.001-0.12	-	-	-	-	-
Mupirocin	-	-	-	-	-	0.06-1024	-	-	-
Nalidixic acid	1-128	32-128	0.015-2	0.5-8	32-64	16-128	-	-	-
Netilmicin	0.03-128	0.06-128	0.12-16	0.5-16	-	0.008-128	-	-	-
Ofloxacin	0.06-128	0.25-8	0.015-2	0.001-0.06	1-8	0.12-128	-	1-128	1-128
Oxacillin	-	-	-	-	-	0.12-128	-	-	-
Penicillin	-	-	-	0.004-32	4-128	0.015-128	0.004-0.06	0.5-128	0.015-4
Piperacillin	0.25-128	0.5-512	0.004-128	0.015-32	0.25-128	0.25-128	-	-	-
Rifampicin	-	-	-	0.25-2	-	0.004-128	-	-	-
Roxithromycin	-	-	2-32	0.015-2	0.12-16	0.03-128	0.015-16	0.03-128	0.03-128
Sparfloxacin	0.008-128	0.12-16	0.004-0.03	0.001-0.12	0.12-1	0.06-0.25	0.12-1	0.25-128	0.12-128
Spectinomycin	-	-	-	4-64	-	-	-	-	-
Sulphamethoxazole	4-128	-	0.5-32	0.25-8	-	-	-	-	-
Teicoplanin	-	-	-	-	-	0.06-32	-	0.5-2048	-
Tetracycline	0.25-128	-	0.06-128	-	-	0.06-128	-	-	-
Ticarillin	0.25-128	0.5-512	0.06-128	-	4-128	0.5-128	-	-	-
Tobramycin	0.03-128	0.06-128	-	0.5-16	-	0.008-128	-	-	-
Trimethoprim	0.03-128	-	0.015-16	-	-	0.03-8	-	-	-
Vancomycin	-	-	-	-	-	0.06-32	0.12-1	0.12-128	0.12-1

\*Ratio of one part clavulanic acid:two parts amoxicillin.

## 2.2 Prepare stock solutions using the formula

$$\frac{1000}{P} \times V \times C = W$$

where P = potency given by the manufacturer ( $\mu\text{g}/\text{mg}$ ), V = volume required (mL), C = final concentration of solution (multiples of 1000) (mg/L), and W = weight of antibiotic (mg) to be dissolved in volume V (mL).

For example,  $\frac{1000}{980} \times 20 \times 10 = 204.08 \text{ mg}$

Powder (204.08 mg at a potency of 980  $\mu\text{g}/\text{mg}$ ) dissolved in 20 mL of solvent = 10 000 mg/L stock solution.

Microbial contamination of powder is extremely rare.<sup>2</sup> If broth methods are to be used, stock solution may be filter sterilized (0.2  $\mu\text{m}$  pore size cellulose acetate filters; Sartorius AG, Goettingen, Germany); however, it must be ascertained from the antibiotic manufacturer that the antibiotic does not bind to the surface of the filter.

For preparation of further stock solutions, from the initial 10 000 mg/L solution, prepare the following:

1 mL of 10 000 mg/L solution + 9 mL diluent\* = 1000 mg/L  
 100  $\mu\text{L}$  of 10 000 mg/L solution + 9.9 mL diluent\* = 100 mg/L

\*Consult Table I for appropriate sterile diluent.

## 3. Preparation of antibiotic dilution range

Example of dilution range: 0.25–128 mg/L.

Label 11 universal containers (containers and amounts of antibiotic and agar can be varied depending on the number of plates to be poured) as follows: 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 mg/L.

From the 10 000 mg/L stock, dispense the following amounts with a micropipette:

- 256  $\mu\text{L}$  into the container labelled 128
- 128  $\mu\text{L}$  into the container labelled 64
- 64  $\mu\text{L}$  into the container labelled 32
- 32  $\mu\text{L}$  into the container labelled 16

From the 1000 mg/L stock, dispense the following amounts:

- 160  $\mu\text{L}$  into the container labelled 8
- 80  $\mu\text{L}$  into the container labelled 4
- 40  $\mu\text{L}$  into the container labelled 2

From the 100 mg/L stock, dispense the following amounts:

- 200  $\mu\text{L}$  into the bottle labelled 1
- 100  $\mu\text{L}$  into the container labelled 0.5
- 50  $\mu\text{L}$  into the container labelled 0.25

No antibiotic is added to the bottle labelled 0 mg/L (antibiotic-free growth control). Other methods for preparing antibiotic dilutions can be used.<sup>2</sup>

## 4. Preparation of agar dilution plates

Prepare ISA or equivalent medium following the manufacturer's instructions. To prevent organisms such as *Proteus* species from swarming, media have been adapted by increasing the agar content or adding 50 mg/L *p*-nitrophenyl glycerol (PNPG) (BDH Merck, Lutterworth, Leicestershire, UK) or 350 mg/L Matexil (AstraZeneca, Cheshire, UK).<sup>3</sup> PNPG, Matexil and increased agar concentration can all alter MICs significantly with some agents. They must not be used unless essential and there is evidence that they do not affect antimicrobial action. Table III shows the appropriate medium for different organisms.

4.1 Add 20 mL of cooled molten agar (ensure that the medium is cooled to 50°C before adding to the antibiotic) to each container, including the antibiotic-free control. Mix well before pouring into 90 mm Petri dishes. Add agar, mix and pour each concentration in turn, so agents are kept at 50°C for minimum period of time.

4.2 Allow the agar to set and then dry the surface of the plates for c. 10 min in a fan-assisted drying cabinet (without ultraviolet light) or in a still incubator (the time needed will depend on the efficiency of the incubator).

4.3 Store the plates at 4–8°C and protected from light until inoculated. Ideally, the plates should be used on the day of preparation. If the plates are to be stored at 4–8°C before use, the stability of the drug must be determined by the individual laboratory as part of its routine quality control programme.

## 5. Preparation of inoculum

The inoculum should be adjusted so that 10<sup>4</sup> cfu/spot are applied to the plates. The following procedure describes a method for preparing the desired inoculum by comparison with a 0.5 McFarland standard.

### 5.1 Preparation of the McFarland standard

Add 0.5 mL of 0.048 M BaCl<sub>2</sub> (1.17% w/v BaCl<sub>2</sub>·2H<sub>2</sub>O) to 99.5 mL of 0.18 M H<sub>2</sub>SO<sub>4</sub> (1% v/v) with constant stirring. Distribute the standard into screw cap tubes of the same size and with the same volume as those used in growing the broth cultures. Seal the tubes tightly to prevent loss by evaporation. Store protected from light at room temperature. Vigorously agitate the turbidity standard on a vortex mixer before use. Standards may be stored for up to 6 months, after which time they should be discarded. Alternatively, prepared standards can be purchased (bio-Mérieux, Basingstoke, UK).

### 5.2 Preparation of inoculum

These suspensions should be used within 30 min of preparation.

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**Table III.** Appropriate media for different organisms

Organism	Medium to be used
Enterobacteriaceae	ISA
<i>Pseudomonas</i> spp.	ISA
Staphylococci	ISA
Enterococci	ISA
<i>Streptococcus pneumoniae</i>	ISA + 5% defibrinated horse blood
$\beta$ -Haemolytic streptococci	ISA + 5% defibrinated horse blood
<i>Moraxella catarrhalis</i>	ISA + 5% defibrinated horse blood
<i>Haemophilus</i> spp.	ISA + 5% whole horse blood + 20 mg/L NAD
<i>Neisseria meningitidis</i>	ISA + 5% defibrinated horse blood
<i>Neisseria gonorrhoeae</i>	ISA + 5% defibrinated horse blood
Anaerobes	Wilkins & Chalgren agar + 5% defibrinated horse blood

NAD = nicotinamide adenine dinucleotide.

### 5.2.1 Growth method

This method is used for non-fastidious organisms, e.g. Enterobacteriaceae, *Pseudomonas* spp. and staphylococci. Touch at least four morphologically similar colonies with a sterile loop. Transfer the growth into ISB or equivalent that has been shown not to affect the performance of the test, and incubate broth with shaking at 35–37°C until the visible turbidity is equal to or greater than the 0.5 McFarland standard. Alternatively, an overnight broth culture can be used.

### 5.2.2 Direct colony suspension method

The method of choice for fastidious organisms, e.g. *Haemophilus* spp., *Neisseria gonorrhoeae* and *Streptococcus pneumoniae*. Colonies are taken directly from the plate into ISB (or equivalent) or distilled water. The suspension should match or exceed the density of the 0.5 McFarland standard. With some organisms, the production of an even suspension of the required turbidity is difficult and growth in broth is a more satisfactory option.

### 5.2.3 Preparation of inoculum for testing anaerobes

#### 5.2.3.1 Anaerobes other than *Bacteroides*

Cultures should be grown on blood agar enriched with haemin and menadione. The colonies should not be >72 h old and should not remain in an aerobic atmosphere for >30 min before preparing a suspension. Prepare a suspension in Wilkins & Chalgren broth (Oxoid, Difco) to match a 0.5 McFarland standard. Anaerobic organisms have markedly different sizes and shapes, so using a turbidity standard as described has limitations. However, currently this is the only practical procedure for clinical laboratories.

#### 5.2.3.2 *Bacteroides*

In 1 mL of sterile distilled water, emulsify growth from a plate that has not been incubated for >24 h and prepare a

suspension to match or exceed a 0.5 McFarland standard. Mix using a vortex mixer.

### 5.3 Adjustment of the organism suspension to the density of the 0.5 McFarland standard

Adjust the density of the organism suspension to equal that of the 0.5 McFarland standard by adding sterile distilled water. To aid comparison, compare the test and standard against a white background with a contrasting black line. Suspensions should contain between  $10^7$  and  $10^8$  cfu/ml, depending on the genera.<sup>2</sup> Further dilution of suspension in sterile distilled water before inoculation is shown in Table IV.

## 6. Quality control

Appropriate controls, depending on genera, must be included with every batch of MIC determinations. Control

**Table IV.** Dilution of suspension (adjusted to 0.5 McFarland standard) in sterile distilled water before inoculation

1:10	No dilution
Haemolytic streptococci	<i>S. pneumoniae</i>
Enterobacteriaceae	<i>M. catarrhalis</i>
<i>Pseudomonas</i> spp.	<i>N. meningitidis</i>
<i>Acinetobacter</i> spp.	<i>N. gonorrhoeae</i>
<i>Haemophilus</i> spp.	anaerobes (not <i>Bacteroides</i> )
Enterococci	
Staphylococci	
<i>Bacteroides</i> spp.	

Organism suspensions should be used within 30 min of preparation.

**Table V.** Appropriate controls, depending on genera, that must be included with every batch of MIC determinations (strain number listed in the NCTC catalogue)

Organism	ATCC control strain	NCTC control strain
<i>Escherichia coli</i>	25922 (NCTC 12241)	10418
<i>Staphylococcus aureus</i>	25923 (NCTC 12981)	6571
<i>Pseudomonas aeruginosa</i>	27853 (NCTC 12934)	10662
<i>Enterococcus faecalis</i>	29212 (NCTC 12697)	
<i>Haemophilus influenzae</i>	49247 (NCTC 12699)	11931
<i>S. pneumoniae</i>	49619 (NCTC 12977)	
<i>N. gonorrhoeae</i>	49226 (NCTC 12700)	
<i>B. fragilis</i>		9343

strains available from national collections are shown in Table V.

## 7. Inoculation

Use a multipoint inoculator (Denley; Mast Diagnostics, Bootle, UK) to deliver 1–2 µL of suspension on to the surface of the agar. Allow the inoculum to be absorbed into the agar before incubation.

## 8. Incubation conditions

Conditions for incubation are shown in Table VI.

## 9. Reading and interpretation

9.1 After incubation, ensure that all of the organisms have grown on the antibiotic-free control plate.

9.2 The MIC is defined as the lowest concentration of antibiotic at which there is no visible growth of the organ-

ism. The growth of one or two colonies or a fine film of growth should be disregarded.

9.3 The MIC for the control strain should be within plus or minus one two-fold dilution of the expected MIC (see Table VII).

## 10. Broth dilution MICs

### 10.1 Macrodilution

10.1.1 Follow the steps in Sections 1–3.

10.1.2 Antibiotic ranges should be prepared one step higher than the final dilution range required, i.e. if a final dilution range of 0.5, 1, 2, 4, 8 and 16 mg/L is required then a range of 1, 2, 4, 8, 16 and 32 mg/L should be prepared to compensate for the addition of an equal volume of inoculum.

10.1.3 Substitute the broth equivalent for the media cited in Section 4. To improve the detection of visible growth when the medium is supplemented with blood, use lysed

**Table VI.** Conditions for incubation of MIC plates

Organism	Incubation conditions
Enterobacteriaceae	35–37°C in air for 18–20 h
<i>Pseudomonas</i> spp.	35–37°C in air for 18–20 h
Staphylococci (other than tests on methicillin/oxacillin)	35–37°C in air for 18–20 h
Staphylococci tests on methicillin/oxacillin	35–37°C in air for 18–20 h
<i>M. catarrhalis</i>	35–37°C in air for 18–20 h
β-Haemolytic streptococci	35–37°C in air for 18–20 h
Enterococci	35–37°C in air for 18–20 h
<i>Neisseria</i> spp.	35–37°C in 4–6% CO <sub>2</sub> in air for 18–20 h
<i>S. pneumoniae</i>	35–37°C in 4–6% CO <sub>2</sub> in air for 18–20 h
<i>Haemophilus</i> spp.	35–37°C in 4–6% CO <sub>2</sub> in air for 18–20 h
Anaerobes (anaerobic cabinet or jar)	10% CO <sub>2</sub> /10% H <sub>2</sub> /80% N <sub>2</sub> <sup>a</sup>

<sup>a</sup>Incubation time depends on individual organism requirements.

## Determination of MICs

**Table VIIa.** Target MICs (mg/L) for reference strains

Antibiotic	<i>H. influenzae</i> NCTC 11931	<i>H. influenzae</i> ATCC 49247	<i>E. faecalis</i> ATCC 29212	<i>S. pneumoniae</i> ATCC 49619	<i>B. fragilis</i> NCTC 9343	<i>N. gonorrhoeae</i> ATCC 49226
Amikacin	–	–	128	–	–	–
Gentamicin	–	–	8	–	128	–
Tobramycin	–	–	16	–	–	–
Azithromycin	2	2	–	0.12	–	–
Amoxycillin	0.5	4	0.5	0.06	32	0.5
Ampicillin	–	–	1	0.06	32	–
Azlocillin	–	–	–	–	4	–
Aztreonam	–	–	>128	–	2	–
Cefaclor	–	128	>32	2	>128	–
Cefamandole	–	–	–	–	8	–
Cefixime	0.03	0.25	–	1	64	–
Cefotaxime	–	0.25	32	0.06	4	–
Cefoxitin	–	–	–	–	4	–
Cefpirome	0.06	0.5	16	–	16	–
Cefpodoxime	0.12	0.5	>32	0.12	32	–
Ceftazidime	0.12	–	>32	–	8	–
Ceftriaxone	–	–	>32	0.06	4	–
Cefuroxime	2	16	>32	0.25	32	–
Cephadroxil	–	–	>32	–	32	–
Cephalexin	–	–	>32	–	64	–
Cephalothin	–	–	16	–	–	–
Co-amoxiclav	0.5	8	0.5	0.06	0.5	0.5
Faropenem	–	–	–	0.06	1	–
Flucloxacillin	–	–	–	–	16	–
Imipenem	–	–	0.5	–	0.06	–
Loracarbef	–	128	>32	2	>128	–
Mecillinam	–	–	>128	–	>128	–
Meropenem	–	–	2	–	0.06	–
Moxalactam	–	–	–	–	0.25	–
Oxacillin	–	–	–	1	–	–
Penicillin	–	4	2	0.5	16	–
Piperacillin	–	–	2	–	2	–
Ticarcillin	–	–	–	–	4	–
Co-trimoxazole	–	1	2	4	–	–
Trimethoprim	–	–	0.25	4	16	–
Teicoplanin	–	–	0.25	–	–	–
Vancomycin	–	–	2	0.25	16	–
ABT 773	2	1	0.008	0.015	–	0.03
Telithromycin	1	2	0.008	0.008	–	0.03
Clarithromycin	8	4	–	0.03	0.25	0.5
Clindamycin	–	–	8	0.12	0.5	–
Erythromycin	8	8	4	0.12	1	0.5
Linezolid	–	–	–	2	4	–
Roxithromycin	16	16	–	0.12	2	–
Chloramphenicol	–	–	4	4	4	–
Fusidic acid	–	–	2	–	–	–
Metronidazole	–	–	–	–	0.5	–
Nitrofurantoin	–	–	8	–	–	–
Rifampicin	–	–	2	0.03	–	–

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**Table VIIa.** (Continued)

Antibiotic	<i>H. influenzae</i> NCTC 11931	<i>H. influenzae</i> ATCC 49247	<i>E. faecalis</i> ATCC 29212	<i>S. pneumoniae</i> ATCC 49619	<i>B. fragilis</i> NCTC 9343	<i>N. gonorrhoeae</i> ATCC 49226
Quinupristin/ dalfopristin	–	–	1	0.5	16	–
Ciprofloxacin	0.008	0.008	1	1	2	0.004
Enoxacin	–	–	–	–	1	–
Fleroxacin	–	–	–	–	4	–
Gatifloxacin	–	–	–	–	0.5	–
Grepafoxacin	–	0.004	–	0.25	–	–
Levofloxacin	–	0.015	–	0.5	0.5	–
Moxifloxacin	0.03	0.03	0.25	0.5	–	0.004
Nalidixic acid	–	1	–	>128	64	–
Norfloracin	–	–	2	–	16	–
Ofloxacin	–	–	2	–	1	–
Pefloxacin	–	–	–	–	1	–
Rufloxacin	–	–	–	–	16	–
Sparfloxacin	–	0.002	–	0.25	1	–
Trovafoxacin	0.008	0.002	0.06	0.12	0.12	–
Tetracycline	–	16	16	0.12	0.5	–

**Table VIIb.** Target MICs (mg/L) for reference strains

Antibiotic	<i>E. coli</i> NCTC 10418	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> NCTC 10662	<i>P. aeruginosa</i> ATCC 27853	<i>S. aureus</i> NCTC 6571	<i>S. aureus</i> ATCC 25923	<i>S. aureus</i> ATCC 29213
Amikacin	0.5	1	2	2	1	–	2
Gentamicin	0.25	0.5	1	1	0.12	0.25	0.25
Kanamycin	1	–	1	–	2	–	–
Neomycin	–	–	32	–	0.12	–	–
Netilmicin	0.5	–	1	–	0.25	–	–
Tobramycin	0.25	0.5	0.5	0.5	0.12	–	0.5
Azithromycin	–	–	–	–	0.12	0.12	0.12
Amoxycillin	2	4	>128	>128	0.12	0.25	–
Ampicillin	2	4	>128	>128	0.06	–	–
Azlocillin	4	–	4	–	0.25	–	–
Aztreonam	0.03	0.25	4	2	>128	–	>128
Carbenicillin	2	–	32	–	0.5	–	–
Cefaclor	1	2	>128	>128	1	–	1
Cefamandole	0.25	–	>128	>128	0.25	–	–
Cefixime	0.06	0.25	16	–	8	8	16
Cefotaxime	0.03	0.06	8	8	0.5	–	1
Cefotetan	0.06	–	>128	>128	4	–	–
Cefoxitin	4	–	>128	>128	2	–	–
Cefpirome	0.03	0.03	4	1	0.25	–	0.5
Cefpodoxime	0.25	0.25	128	>128	1	4	2
Ceftazidime	0.06	0.25	1	1	4	–	8
Ceftizoxime	0.008	–	–	–	2	–	–

### Determination of MICs

**Table VIIIb.** (Continued)

Antibiotic	<i>E. coli</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. aureus</i>	<i>S. aureus</i>
	NCTC 10418	ATCC 25922	NCTC 10662	ATCC 27853	NCTC 6571	ATCC 25923	ATCC 29213
Ceftriaxone	0.03	0.06	8	8	1	–	2
Cefuroxime	2	4	>128	>128	0.5	1	1
Cephadroxil	8	8	>128	>128	1	–	2
Cephalexin	4	8	>128	>128	1	–	4
Cephaloridine	–	–	>128	>128	0.06	–	–
Cephalothin	4	8	>128	>128	0.5	–	0.25
Cephradine	–	–	>128	>128	2	–	–
Co-amoxiclav	2	4	>128	128	0.12	0.12	0.25
Faropenem	0.25	–	>128	>128	0.12	–	–
Imipenem	0.06	0.12	2	1	0.015	–	0.015
Meropenem	0.015	0.008	2	0.25	0.03	–	0.06
Trimethoprim	0.12	0.25	32	–	0.25	–	0.5
Teicoplanin	–	–	–	–	0.25	0.5	0.5
Vancomycin	–	–	–	–	0.5	0.5	1
Telithromycin	–	–	–	–	0.03	0.06	0.06
Clarithromycin	–	–	–	–	0.12	0.12	0.12
Clindamycin	–	–	–	–	0.06	0.12	0.06
Dirythromycin	–	–	–	–	1	–	1
Erythromycin	–	–	–	–	0.12	0.5	0.25
Linezolid	–	–	–	–	0.5	1	–
Roxithromycin	–	–	–	–	0.25	0.5	0.5
Chloramphenicol	2	4	128	–	2	–	2
Colistin	0.5	–	2	–	128	–	–
Fosfomycin	4	–	>128	>128	8	–	–
Fusidic acid	>128	–	–	–	0.06	0.12	0.06
Mupirocin	–	–	–	–	0.25	0.25	0.12
Nitrofurantoin	4	8	–	–	8	–	16
Rifampicin	16	–	–	–	0.004	0.015	0.004
Quinupristin/ dalbopristin	–	–	–	–	0.12	0.25	0.25
Ciprofloxacin	0.015	0.015	0.25	0.25	0.12	0.5	0.5
Enoxacin	0.25	–	1	–	0.5	–	–
Fleroxacin	0.06	0.12	1	–	0.5	–	–
Flumequine	2	–	>128	>128	–	–	–
Gatifloxacin	0.015	–	1	–	0.03	–	–
Grepafloxacin	0.03	0.03	0.5	–	0.03	–	–
Levofloxacin	0.03	0.03	0.5	0.5	0.12	0.25	0.25
Lomefloxacin	–	–	–	–	0.5	–	–
Moxifloxacin	0.03	0.03	2	2	0.06	0.06	–
Nalidixic acid	2	4	>128	>128	>128	128	128
Norfloxacin	0.06	0.06	1	1	0.25	–	1
Ofloxacin	0.06	0.03	1	1	0.25	–	0.5
Pefloxacin	0.06	–	0.5	–	0.25	–	–
Rufloxacin	0.5	–	8	–	1	–	–
Sparfloxacin	0.015	0.015	0.5	0.5	0.03	–	–
Trovafloxacin	0.015	0.015	0.5	0.5	0.015	0.03	0.03
Tetracycline	1	2	–	32	0.06	–	0.5

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horse blood. The performance of lysed blood used for MIC testing may vary, therefore individual laboratories should confirm, as part of their QC programme, that the lysed blood used supports the growth of the organism being tested.

10.1.4 Arrange sufficient 75 × 12 mm sterile capped tubes in two rows for each antibiotic to cover the range of antibiotic dilutions chosen in duplicate.

10.1.5 Transfer 1 mL volumes of each antibiotic dilution in broth to the tubes.

10.1.6 Prepare inocula following the procedures cited in Section 5. A final inoculum of 10<sup>5</sup> cfu/mL is required and therefore suspensions should be diluted 1:100 in broth medium used for preparing the antibiotic dilutions for the following organisms: haemolytic streptococci, staphylococci, Enterobacteriaceae, *S. pneumoniae*, *Pseudomonas* spp., *Moxarella catarrhalis*, *Acinetobacter* spp., *Neisseria meningitidis*, *Haemophilus* spp., *N. gonorrhoeae* and enterococci.

10.1.7 Add 1 mL aliquots of test organism to one set of tubes and 1 mL of control organism to the other. Mix the contents of the tubes thoroughly.

10.1.8 Include inoculated and uninoculated tubes of antibiotic-free broth (the first tube controls the adequacy of the broth to support the growth of the organism, the second is a check of sterility). Incubate at 35–37°C for 18–20 h in air.

### 10.2 Microdilution

10.2.1 Follow steps 1 to 3 as for broth macrodilution.

10.2.2 Label a 96-well sterile microtitre tray with the appropriate antibiotic dilutions.

10.2.3 Add 75 µL of each antibiotic dilution to two rows of wells.

10.2.4 Prepare organism suspension as for broth macrodilution.

10.2.5 Dispense 75 µL of test organism suspension into one row and 75 µL of control organism suspension into the second row of wells.

10.2.6 Include inoculated and uninoculated wells of antibiotic-free broth (the first controls the adequacy of the broth to support the growth of the organism, the second is a check of sterility).

10.2.7 Cover with a lid or plate sealing tape and incubate at 35–37°C for 18–20 h in air.

### 10.3 Reading and interpretation

10.3.1 Read the MIC endpoint as the lowest concentration of antibiotic at which there is no visible growth.

10.3.2 The MIC for the control strain should be within one two-fold dilution of the expected MIC (see Table VII).

## References

1. Report of the Working Party on Antibiotic Sensitivity Testing of the British Society of Antimicrobial Chemotherapy. (1991). A guide to sensitivity testing. *Journal of Antimicrobial Chemotherapy* **27**, Suppl. D, 1–50.
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