

The Single-Plate Protocol

Proposed modification of the Alderman & Smith (2001) disc diffusion assay protocol to include internal controls

1. Media preparation

1.1. Media type

Disc diffusion assays should be performed using Mueller–Hinton agar purchased from a reputable commercial source. Only Mueller–Hinton agar formulations that comply with the acceptance limits described in NCCLS document M6-P (Protocols for Evaluating Dehydrated Mueller–Hinton Agar: Approved Standard) should be used.

1.2. Modification to standard media

Whenever possible un-modified Mueller-Hinton agar should be used. However for some bacterial groups modifications are necessary (see Appendix 1).

1.3 Media preparation

Mueller–Hinton agar should be prepared according to the manufacturer’s instructions. The pH of each preparation of Mueller–Hinton agar should be checked and must lie between 7.2 and 7.4. (NCCLS M31-T p 19 provides acceptable methods for checking the pH of agar media). (In practice, if the medium is purchased from a reputable manufacturer, this has rarely been found to be necessary.)

Before use each batch of media should be checked for its ability to support the growth of the test isolates and control strains.

1.4. Water quality

Media should be prepared with distilled or freshly deionised water. The conductivity of such water should be checked regularly and should not exceed 10 µS/cm. (Variations in water quality have much less impact when the single-plate method is being used. So this may not be a critical issue)

1.5. Preparation of plates

Before pouring the agar medium should be cooled to 40–50°C and plates should be poured to a depth of 4 mm on a level surface.

1.6. Storage of media

Unpoured Mueller–Hinton agar may be stored in air-tight screw capped bottles under the conditions and for the times specified by the manufacturers.

1.7. Moisture levels of media

Immediately prior to inoculation media should be moist but free of droplets, which should not be present on either the agar surface nor on the Petri-dish lids. If necessary, plates may be dried by incubation at 30–37°C or in a laminar flow cabinet for a maximum of 30 min.

1.8. Sterility

A representative sample of each batch of plates should be examined for sterility by incubation at 22°C for 72 h.

2. Inoculum

2.1. Starting culture

The inocula should be prepared from cultures of the test organism and the control strain streaked for single colonies on an agar plate. Where possible, the agar plates should be Mueller–Hinton and the plates should be fresh. If the inoculum is prepared from colonies growing on plates other than Mueller–Hinton, this must be recorded. Except in the case of slow growing strains, the plates should be no more than 48 h old.

2.2. Inoculum preparation

The inoculum should be prepared by emulsifying a minimum of three colonies from the starting culture in sterile 0.9% saline. The inoculum should be standardised to have a concentration of $1-2 \times 10^8$ CFU/ml. Inocula of the appropriate density can be prepared using a McFarland 0.5 standard (see Appendix 2).

2.3. Preparation of inoculation swab

Within 15 min of its preparation, a sterile cotton swab should be dipped into the standardised inoculum suspension. To remove surplus moisture, the swab should be rotated several times whilst pressed firmly against the walls of the tube at a level above that of the liquid.

2.4. Inoculation of test media

One half of the agar surface should be inoculated using a swab containing the control strain. It is good practice to rotate the swab while rubbing the semi circle and care should be taken that the whole area is inoculated. The other half should then be inoculated in the same way using a second swab containing the test strain.

2.5. Moisture of test media

If the plates have been correctly prepared, then no excess moisture should be present after their inoculation by this swabbing procedure. If such moisture is present, plates may be left at room temperature with their lids ajar for a maximum of 15 min.

3. Diffusion discs

3.1. Source of diffusion discs

Paper discs supplied by a reputable manufacturer should be used. The diameter of these discs should approximate to 6 mm.

3.2. Storage of discs

Discs in their original manufacturers packaging should be stored at 2–8°C. Once opened, a package should be stored in a tightly sealed container with a desiccant.

3.3. Age of discs

Discs must be disposed of when they have reached the manufacturer's expiry data.

3.4. Application of discs

Discs may be applied using sterile forceps. A gentle downward pressure should be applied to each disc before incubation to ensure complete contact between the disc and the agar surface. Discs must not be relocated once they have made contact with the agar surface.

3.5. Content of discs

The agent content of discs has a major impact on inhibition zone sizes and the lack of consistency in the issue of disc contents was probably the most significant factor hindering the exchange of information between laboratories.

It is strongly recommended that discs with a standard content should be used. Table 1 below provides a list of disc contents that should be used. This list has been developed from a consideration of the recommendations of CLSI, M42-A and Alderman and Smith (2001).

Table 1 Recommended contents of discs.

| Agent | Content (µg) |
|-------------------------------|--------------|
| Amoxicillin | 25 |
| Ampicillin | 10 |
| Chloramphenicol | 5 |
| Clindamycin | 2 |
| Doxycycline | 30 |
| Enrofloxacin | 5 |
| Erythromycin | 15 |
| Florfenicol | 30 |
| Flumequine | 30 |
| Fosfomycine | 200 |
| Furazolidone | 100 |
| Gentamicin | 10 |
| Kanamycin | 30 |
| Lincomycin | 2 |
| Minocycline | 30 |
| Nalidixic acid | 30 |
| Nitrofurantoin | 300 |
| Ormetoprim-sulfadimethoxine | 1.25 / 23.75 |
| Oxolinic acid | 2 |
| Oxytetracycline | 30 |
| Rifampin | 5 |
| Sulfamethoxazole | 200 |
| Tetracycline | 30 |
| Tiamulin | 30 |
| Trimethoprim | 5 |
| Trimethoprim-sulfamethoxazole | 1.25 / 23.75 |

In studies of any agent not included in the list, laboratories are free to adopt any disc content that provides useful data.

4. Incubation

4.1. Avoidance of pre-incubation and pre-diffusion

Where the room temperature is more than 5°C above or below the incubation temperature, plates must be incubated within 10 min of the application of the discs. Where room temperature lies within 5°C of the incubation, temperature plates should be incubated as soon as possible after the application of the discs.

4.2 Incubation temperatures

Plates should be incubated aerobically at either $22 \pm 2^\circ\text{C}$ or at $28 \pm 2^\circ\text{C}$. The choice of incubation temperature will depend on the bacterial group being investigated. In exceptional cases other incubation temperatures may be used (see Appendix 3).

Plates should be incubated in an inverted position, in stacks of no more than five plates. If stacks higher than five plates are employed an expanded metal separator should be used to facilitate air circulation. Care should be taken to ensure adequate humidity.

4.3. Standard incubation times

For the majority of bacteria encountered in aquaculture inhibition zones will be readable after one or two days incubation. Laboratories should adopt either 24–28 h or 44–48 h as their standard incubation time. The time adopted should be the shortest incubation after which the zone sizes can be reliably read.

5. Rejection criteria

5.1. Inoculum density

Plates on which the growth of the test or control strain produces isolated colonies (less than semi-confluent growth) should not be read.

5.2. Overlapping zones

If zones of inhibition produced by adjacent discs overlap to the extent that two measurements at right angles cannot be made, the zones around these discs should not be recorded. Equally zones demonstrating significant distortion from semi-circular should not be reported.

6. Reading plates

6.1. Definition of zones

In measuring inhibition zones the important issue is that the same criteria are used to judge the edge of the zone for both the test and control zones on any plate. It is also very helpful if each laboratory uses the same criteria in all the determinations of zone size they make.

The limit of the zone of inhibition should be taken as the point at which no growth is visible to the unaided eye. The way the plates are illuminated make a difference to the sizes of the zones that are observed. Laboratories should set a standard method of plate illumination and keep to it as part of their standard protocol.

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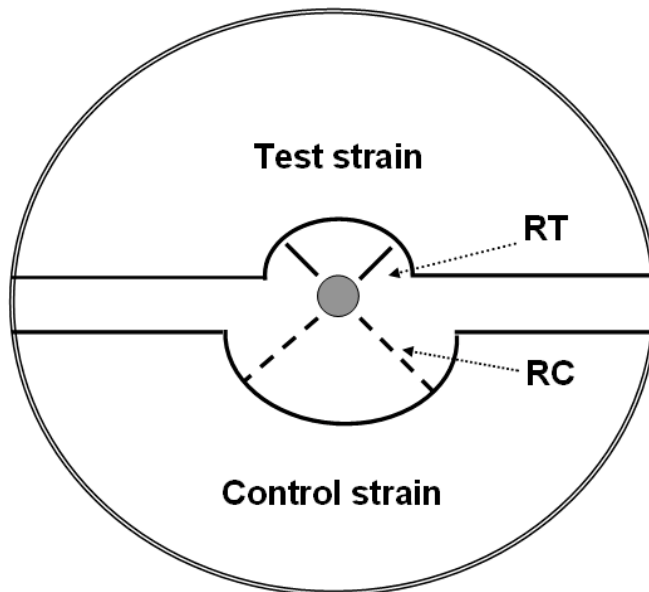
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If 'fuzzy' zones are these should be reported. When 'fuzzy zones' are encountered two measurements should be taken, one using the point at which normal growth ceases and one from the point of no growth.

When interpreting results using sulfonamide disks the inhibitory zone border may be indistinct, with weak growth approaching the region of heavy growth. Measure up to the margin of heavy growth (80 to 100% of the growth density outside the zone of inhibition) and disregard the slight growth (20% or less) region.

The presence of individual colonies within zones of inhibition should be recorded. These colonies may indicate that the test strain suspension used is not pure. If many colonies of this type are observed within an inhibition zone, representative colonies should be re-isolated and re-tested. Pin-head colonies occurring within zones around discs containing quinolone agents are regularly observed and normally need no further investigation.

6.2. Reading zones



The radii of the zones of inhibition (RT & RC) should be read preferably with digital or mechanical calipers, although a ruler graduated to 0.5 mm can be used. Each radius should be read twice (at right angles) and the average result recorded to the nearest millimetre (averages of 0.5 mm should be rounded up).

Some operators have reported that measuring from the edge of the zone of

inhibition (see 5.1) to the centre of the disc resulted in some imprecision. They found it easier to estimate the RC & RT radii by measuring the distance from the edge of the inhibition zone to the far side of the disc and then to subtract the radius of the disc (normally 3 mm) from their measurement.

6.3 Recording zone data

The Table below provides a suggested method for recording data obtained using this protocol.

Table 2 Recording data

| Strain | Mean RC (mm) | Mean RT (mm) | Difference (RC – RT) | Classification |
|----------|--------------|---------------|----------------------|----------------|
| PS 1-09 | 17 | 19 | -2 | WT |
| PS 2-09 | 18 | 17 | +1 | WT |
| PS 3-09 | 19 | 10 | +9 | NWT |
| PS 4-09* | 18 | No zone (< 3) | <+15 | NWT |

* The data in this row assume that the diameter of the disc was 6 mm.

6.4 Interpreting zone data

Note the key column in Table 2 is the Difference in mm between mean RT and mean RC. The current recommendations for the interpretation of the data in the Difference column are;

If the difference is + 3 or less then the clinical isolate (test strain) should be classified as WT.

If the difference is + 4 or greater then the clinical isolate (test strain) should be classified as NWT.

Appendix 1 Modification to Mueller-Hinton agar

Two considerations should guide the selection of the media to be used in SPP susceptibility testing.

The first is that whenever it is possible preference should be given to the use of unmodified Mueller-Hinton agar (MHA).

The second is that when unmodified MHA proves inadequate preference should be given to the alternatives suggested in CLSI, M42-A or in subsequent versions of this guideline.

The current version of M42-A recommends;

The addition of 1% NaCl to MHA for obligate halophilic Vibrios.

The use of dilute (1:7) MHA for gliding bacteria such as Flavobacteria.

The addition of 5% sheep blood to MHA for Streptococci and other Gram-positive cocci

Appendix 2 Inocula standardisation

Obtaining a McFarland 0.5 standard

Details of how to make up a McFarland 0.5 standard are given in Wikipedia (search term; McFarland standards). Having made one, it is essential that the optical density be checked. It should be OD_{625} of 0.08–0.1 (1 cm light path). In my experience this is more difficult than it looks and I recommend purchasing a commercial standard. Most commercial McFarland standards are latex bead suspensions and are, therefore, more stable than the chemical versions.

Using a McFarland 0.5 standard.

ALWAYS SHAKE THE MCFARLAND STANDARD VIGOROUSLY BEFORE USE

Place your McFarland 0.5 standard in a container identical to the one in which you intend to prepare the saline suspensions of your inocula. Use a thick marker pen to draw a black line on a piece of paper. Compare the density of your saline suspension with the McFarland 0.5 standard by placing both in front of the black line.

Appendix 3 Non-standard incubation temperatures

The CLSI guideline M42-A recommends incubation (for prolonged times) at 15°C for;

Vibrio salmonicida,

Moritella viscosa,

Flavobacterium psychrophilum

Flavobacterium branchiophilum

Renibacterium salmoninarum

The CLSI guideline M42-A recommends incubation at 35°C for;

Erysipelothrix rhusiopathiae

