Sample inoculum preparation: a UK ring trial of the Inoclic device

Preparation of a correct inoculum is vitally important when performing antibiotic susceptibility testing. The Inoclic sample preparation device is intended to provide a standardised, reproducible EUCAST-compliant confluent growth. Here, Anne Grayson and Charlotte Duncan present the results of a UK-wide comparative trial.

Disk diffusion has been a much used method for antimicrobial susceptibility testing (AST) in most clinical microbiological laboratories since Bauer *et al*. first described this technique in the 1960s.[1] In the UK this method has been further supported by the British Society for Antimicrobial Chemotherapy (BSAC) guidelines, which were first published in 2001.

However, in 2016, BSAC announced that it would cease active support, maintenance and development of the BSAC method, and instead would focus on helping laboratories in the UK to move over to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method. There are several differences between each method, including confluent growth as opposed to the semi-confluent growth, different media and antibiotic concentrations.[2]

The EUCAST disk method was developed in 2009. It is a robust and standardised method, providing evidence-based guidance on performing susceptibility tests for organism/antibiotic combinations via extensive studies. These studies are performed in accordance with the international standard method for testing antimicrobial susceptibility (ISO20776-1:2006). The EUCAST disk-diffusion method is now the most commonly used standardised method in most European countries and increasingly those outside Europe.

The disk-diffusion susceptibility method[3–6] is simple and easy to fit into a routine laboratory workflow. Testing should be performed using a bacterial inoculum of 0.5 McFarland (for the majority of bacteria). This is used to inoculate either a Mueller-Hinton, Mueller-Hinton with blood agar or rapid Mueller-Hinton plate within 15 minutes of preparing the inoculate, and spreading across the surface using a cotton swab to achieve confluent growth.

Antibiotic disks of fixed concentration are placed on the inoculated agar surface within 15 minutes, and plates are incubated within a further 15 minutes, and read subsequently at a specified time. The zones of growth inhibition around each antibiotic disk are then measured to the nearest millimetre. The diameter of the zone indicates the susceptibility of the isolate to the antimicrobial agent as it diffuses through the medium.

The zone diameters of each antibiotic/organism combination are interpreted using breakpoint criteria published by EUCAST. Interpreted results are reported as sensitive, intermediate or resistant. If a quantitative result is required, a minimum inhibitory concentration (MIC) test should be performed.

There are several advantages to using the disk-diffusion method: the test is very

Table 1. Materials provided.

Product code	Product
PL.041003	Inoclic Pack 500
PL.040001	Inoclic Short x250
PL.041001	SIRscan Swab x100
PL.050020	Physiological Saline (0.7 mL) x 100
PL.CR.0325	Inoclic Rack
PL.DEN.050102P	Pro-Den Densitometer
SD.2300	0.5 McFarland

Table 2. Recommended strains used for routine quality control.

Quality control organism (Procult, Pro-Lab)	Type strain	Repeats	Product code
Escherichia coli	NCTC12241/ATCC25922	5	PLD02
Pseudomonas aeruginosa	NCTC12903/ATCC27853	5	PLD10
Staphylococcus aureus	NCTC12973/ATCC29213	5	PLD14
Enterococcus faecalis	NCTC12697/ATCC29212	5	PLD18
Haemophilus influenzae	NCTC12975/ATCC49766	5	PLD37
Streptococcus pneumoniae	NCTC12977/ATCC49619	5	PLD95

Table 3. Results obtained with a wide range of antibiotics.

Escherichia coli NCTC12241 ATCC25922	*Pseudomonas aeruginosa* NCTC12903 ATCC27853	*Staphylococcus aureus* NCTC12973 ATCC29213	*Enterococcus faecalis* NCTC12697 ATCC29212	*Haemophilus influenzae* NCTC12975 ATCC49766	*Streptococcus pneumoniae* NCTC12977 ATCC49619
Ampicillin 10	Amikacin 30	Erythromycin 15	Ampicillin 2	Ampicillin 2	Erythromycin 15
Trimethoprim 5	Ceftazidime 10	Clindamycin 2	Linezolid 10	Augmentin 3	Tetracycline 30
Nitrofurantoin 100	Ciprofloxacin 5	Tetracycline 30	Tigecycline 15	Tetracycline 30	Levofloxacin 5
Augmentin 30	Gentamicin 10	Rifampicin 5	Vancomycin 5	Ciprofloxacin 5	Chloramphenicol 30
Cefalexin 30	Pip-tazobactam 36	Fusidic acid 10	Teicoplanin 30	Chloramphenicol 30	Oxacillin 1
Cefpodoxime 10	Meropenem 10	Cefoxitin 30	Gentamicin 30	Cefuroxime 30	Clindamycin 2
Gentamicin 10	Tobramycin 10	Penicillin 1	Nitrofurantoin 100	Ceftriaxone 30	Penicillin 1
Ciprofloxacin 5	Aztreonam 30	Gentamicin 10	Co-trimoxazole 25	Levofloxacin 5	Co-trimoxazole 25
Pip-tazobactam 36	Imipenem 10	Linezolid 10	Trimethoprim 5	Co-trimoxazole 25	Norfloxacin 10
Cefotaxime 5		Trimethoprim 5	Levofloxacin 5	Penicillin 1	Teicoplanin 30
Meropenem 10		Teicoplanin	Ciprofloxacin 5	Erythromycin 15	Cefaclor 30
Ceftazidime 10		Mupirocin 200		Nalidixic acid 30	Optichin
Ertapenem 10		Ciprofloxacin 5		Cefotaxime 5	Linezolid 10
Co-trimoxazole 25		Nitrofurantoin 100			
Aztreonam 30		Ampicillin 2			
Amikacin 30		Co-trimoxazole 25			
Cefoxitin 30		Chloramphenicol 30			
Chloramphenicol 30					

simple to perform, it does not require specialised equipment, results are easily interpreted by all clinicians, and it provides flexibility in the selection of disks for testing. It is the least costly of all susceptibility methods available, and is suitable for all bacteria, including fastidious bacteria.[6]

Use of a standardised method for disk diffusion such as EUCAST is essential for any laboratory working towards United Kingdom Accreditation Service (UKAS) ISO15189 compliance.

Inoclic sample preparation

The Inoclic sample preparation system is a rapid, simple-to-use method for inoculum standardisation for antimicrobial sensitivity testing (AST). This unique method is based on traditional inoculation preparation. A chosen colony is picked using the Inoclic rod by inserting the rod through the colony to the bottom of the agar plate. The user guidance also suggests touching a further two colonies to account for any colonial variation. The colony-forming units (CFUs) on the rod are then emulsified into the vial of physiological saline (0.7 mL) provided.

This easy process will create a sample dilution to obtain confluent growth required both for the Clinical and Laboratory Standards Institute (CLSI) and EUCAST methods. Inoclic is also recommended for any other inoculating operations traditionally performed using

Table 4. Combined data showing high correlation between Inoclic and the manual method.

Quality control organism (Procult, Pro-Lab)	Type strain	Manual *vs.* Inoclic within 2 mm	Concordance (%)
Escherichia coli	NCTC12241/ATCC25922	1075/1104	98
Pseudomonas aeruginosa	NCTC12903/ATCC27853	998/1001	99.7
Staphylococcus aureus	NCTC12973/ATCC29213	1144/1172	97.6
Enterococcus faecalis	NCTC12697/ATCC29212	809/812	99.6
Haemophilus influenzae	NCTC12975/ATCC49766	648/670	96.7
Streptococcus pneumoniae	NCTC12977/ATCC49619	622/650	96
TOTAL		5241/5354	98%

Table 5. Results consolidated to show the frequency with which either method fell outside the EUCAST reference range for all organisms.

Outside target reference range (mm)		1	2	3	>3	Total out of range
Escherichia coli NCTC12241/ATCC25922	Inoclic	10	8	1	0	19
	Manual	8	2	2	1	13
Pseudomonas aeruginosa NCTC12903/ATCC27853	Inoclic	17	6	0	0	23
	Manual	15	7	1	0	23
Staphylococcus aureus NCTC12973/ATCC29213	Inoclic	8	1	0	0	9
	Manual	16	4	6	1	27
Enterococcus faecalis NCTC12697/ATCC29212	Inoclic	4	2	0	0	6
	Manual	3	0	0	0	3
Haemophilus influenzae NCTC12975/ATCC49766	Inoclic	5	1	0	0	6
	Manual	6	2	0	0	8
Streptococcus pneumoniae NCTC12977/ATCC49619	Inoclic	3	3	3	0	9
	Manual	10	3	5	0	18
TOTAL	Inoclic	47	21	4	0	72
	Manual	58	18	14	2	92

a loop (eg inoculation of agar culture, holding media in test tubes by deep central injection or the suspension of bacteria in a liquid medium). Another application is preparation of bacterial spotting for matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) testing.

Inoclic study

The study reported below was designed to provide independent verification of Inoclic's performance, and to determine any additional advantages or disadvantages of using the system compared to the manual method. It was coordinated by Pro-Lab Diagnostics to compare the performance of the Inoclic sample preparation system against the current laboratory manual method for AST using the EUCAST method. A ring trial was conducted across the UK in which 18 laboratories were invited to take part. Sample throughput and staffing levels were not selection criteria.

Materials and methods

Each laboratory in the ring trial used various media suppliers, antibiotic disk suppliers and antibiotic disk sets. Inoclic is intended to provide confluent growth in accordance with EUCAST guidance and therefore these variations would not affect the results.

Each site was provided with a 30-test Inoclic pack (Table 1) and were asked to perform routine disk quality control (QC) in parallel with their current manual method (Table 2). The manual method involves directly adding a colony to a known volume of saline to achieve a 0.5 McFarland inoculum, the density of which is then checked using a calibrated densitometer to allow direct comparison with the Inoclic method.

Zone diameters were read by a minimum of two trained staff to ensure standardised reading and to generate an average value for comparison. Ninewells Hospital, Dundee, and Victoria Hospital, Kirkcaldy, both use the SIRscan 2000 i2a (Pro-Lab Diagnostics) automated zone reader to read their sensitivity plates. The SIRscan system is calibrated to 0.1 mm using a calibration standard in accordance with ISO17025, therefore only one reading for each zone was required for these sites. Quality control strains were also cultured in accordance with EUCAST guidance.

Inoclic procedure

- Aseptically remove the Inoclic rod from the packet.
- Hold the rod at the opposite end to the one intended for contact with the colony.
- Holding the rod straight at 90˚ to the agar surface, push the rod through the colony to the bottom of the agar plate. (Fig 1a)
- Remove the rod from the agar, maintaining an angle at 90˚ to avoid excessive load.
- Touch the surface of one or two additional colonies to account for possible phenotypic colonial variance.
- Aseptically remove the cap from a tube of physiological saline (0.7 mL) and carefully emulsify the colony collected with the rod in the meniscus of the fluid (Fig 1b)
- Discard the rod, observing biohazard precautions.
- Reseal the tube of physiological saline and vortex-mix (3–5 sec) to ensure homogenisation of the suspension. The resultant suspension conforms to EUCAST requirements.
- Aseptically remove the cap from a tube of physiological saline and dip the SIRscan swab into the saline and remove. Take care not to allow contact with the side of the tube.
- Streak the swab over the surface of the selected agar plate using standard technique, and incubate as required to obtain confluent growth.

To avoid possible contamination, the rods should not be handled by the end which contacts the colony. Inoclic rods must not be placed in a flame before use. Colonies should not be pricked twice with the same rod, nor should the Inoclic come into contact with the wet agar surface as this will affect bacterial adherence to the rod. Mucoid colonies which do not adhere to the standard loop will not adhere correctly to Inoclic rods. These colonies should not be sampled using Inoclic rods.

Table 6. Analysis of zones where one method fell outside the EUCAST target range for one or both methods but showed correlation between the manual and Inoclic methods.

Difference in Inoclic and manual method (mm)	0	1	2	3	>3
Escherichia coli NCTC12241/ATCC25922	15	21	23	16	13
Pseudomonas aeruginosa NCTC12903/ATCC27853	5	48	24	1	2
Staphylococcus aureus NCTC12973/ATCC29213	5	12	11	15	13
Enterococcus faecalis NCTC12697/ATCC29212	7	12	2	1	2
Haemophilus influenzae NCTC12975/ATCC49766	5	18	10	7	15
Streptococcus pneumoniae NCTC12977/ATCC49619	20	20	15	10	18
TOTAL	57	131	85	50	63

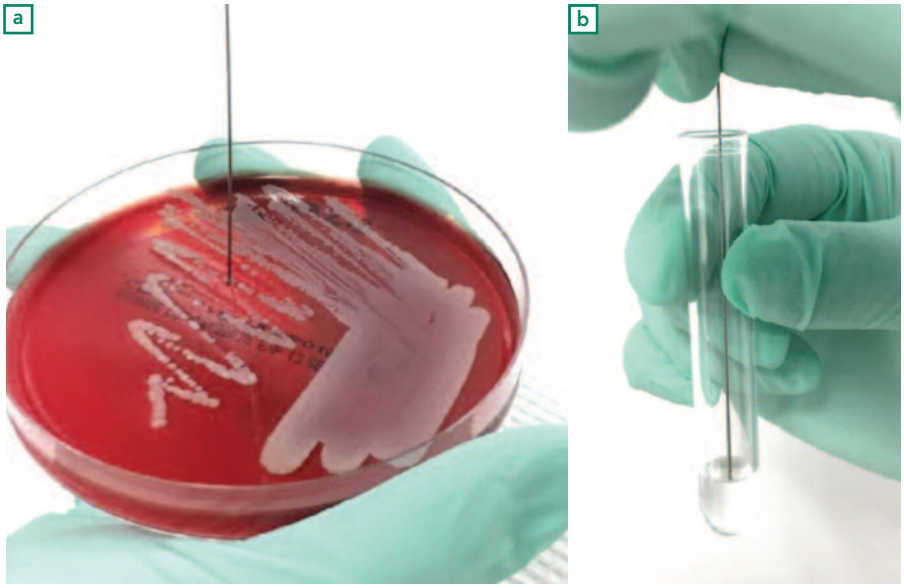


Fig 1. a) Holding the rod at 90° to the surface, it is pushed through the colony to the bottom of the agar plate. **b)** The colony collected on the rod is emulsified in the meniscus of the physiological saline.

Inoclic rods are not recommended for small colonies that present a diameter which is equal to, or smaller than, the rod itself (eg pneumococci). However, for the purpose of this study, *Streptococcus pneumoniae* NCTC12977/ATCC49619 (x5 repeats) was also included for testing. The method for *S. pneumoniae* may be adapted to sample double the recommended number of colonies, and to sample the saline inoculum three times between inoculating the same plate, thus allowing for valid growth on the AST plate.[7]

Manual procedure

The manual procedure adopted was in accordance with the current laboratory method and compliant with EUCAST guidance. A densitometer was used on all inocula to ensure 0.5 McFarland was achieved.

Results

Results were submitted for either Oxoid or E&O media and across a wide range of antibiotics from three different suppliers (Table 3).

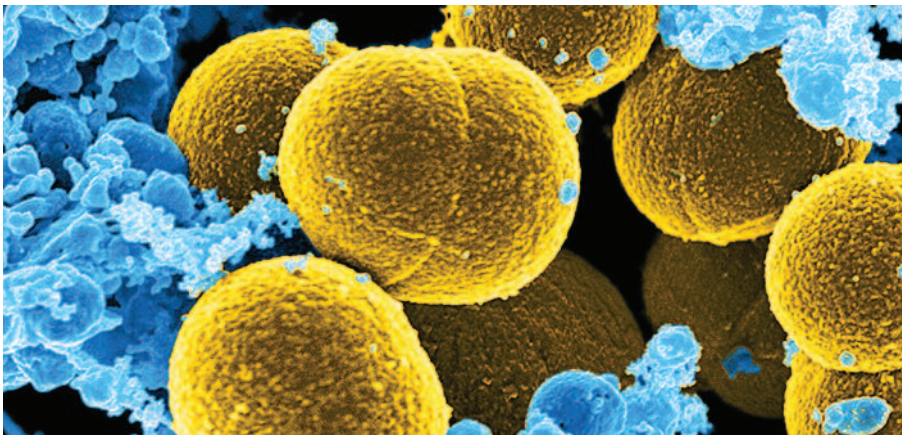
The results out of range both by Inoclic and manual methods were removed from the data set for general analysis and are discussed separately. In this subset of out-of-range results there was consistency between both techniques, and therefore these discrepant results are likely to be due to other factor such as media, disks, organism or incubation.

When all data were combined, a high correlation between Inoclic and the manual method was observed, as shown in the Table 4. These results can be further consolidated to show the frequency in which either method fell outside the EUCAST reference range for all organisms (Table 5). This shows correlation and precision across both methods.

The Inoclic was shown to be better for *Staphylococcus aureus* and *Haemophilus influenzae*, whereas the manual method had a slightly higher success rate with *Escherichia coli* and *Enterococcus faecalis*, with results for *Pseudomonas aeruginosa* being identical between the two methods (Tables 6 and 7).

Improvements achieved using Inoclic

With use of Inoclic, some of the trial sites reported that an increased number of inhibition zones fell between the acceptable recommended reference ranges compared to manual preparation using a densitometer (Table 8). This occurred across various antibiotic/organism combinations.



Frank DeLeo, National Institute of Allergy and Infectious Diseases (NIAID)

Inoclic was shown to be better than the manual method with organisms such as *Staphylococcus aureus*.

One site was completely in range both with the manual and Inoclic methods across all testing. This site operates the SIRscan 2000 automated zone reader using i2a disks. The other SIRscan 2000 automated zone reader user only found that eight results out of the 265 tests performed fell out of range, five of which were *Pseudomonas aeruginosa* ATCC27853/NCTC12903 against piperacillin/tazobactam.

In terms of time comparison, a clear

Table 7. Analysis of zones where both methods fell inside the EUCAST target range and showed correlation between the manual and Inoclic methods.

Difference in Inoclic and manual method (mm)	0	1	2	3	>3
Escherichia coli NCTC12241/ATCC25922	189	193	60	20	9
Pseudomonas aeruginosa NCTC12903/ATCC27853	132	145	100	31	19
Staphylococcus aureus NCTC12973/ATCC29213	190	199	93	36	20
Enterococcus faecalis NCTC12697/ATCC29212	141	163	63	23	11
Haemophilus influenzae NCTC12975/ATCC49766	54	106	70	34	31
Streptococcus pneumoniae NCTC12977/ATCC49619	53	84	53	23	17
TOTAL	759	890	439	167	107

Table 8. Improvements noted using the Inoclic device.

Organism	Sites in range for both methods	Number of sites showing improvement using Inoclic	Number of zones brought within range using Inoclic
Escherichia coli NCTC12241/ATCC25922	6/17	3	11
Pseudomonas aeruginosa NCTC12903/ATCC27853	5/17	4	16
Staphylococcus aureus NCTC12973/ATCC29213	8/17	7	22
Enterococcus faecalis NCTC12697/ATCC29212	11/17	2	3
Haemophilus influenzae NCTC12975/ATCC49766	3/12	4	7
Streptococcus pneumoniae NCTC12977/ATCC49619	3/12	4	14
TOTAL		24	73

Table 9. Time savings achieved.

Escherichia coli NCTC12241 / ATCC2592	**Repeat 1**	**Repeat 2**	**Repeat 3**	**Repeat 4**	**Repeat 5**	**Average (sec)**
Inoclic	39.5	30	32	29	34	33
Manual	39.5	49	54 (x2)	36	42.5	44
Pseudomonas aeruginosa NCTC12903 / ATCC27853	**Repeat 1**	**Repeat 2**	**Repeat 3**	**Repeat 4**	**Repeat 5**	**Average (sec)**
Inoclic	33.5	32	30	32	36	33
Manual	58 (x2)	57.5 (x2)	102 (x2)	39	45	60
Staphylococcus aureus NCTC12973 / ATCC29213	**Repeat 1**	**Repeat 2**	**Repeat 3**	**Repeat 4**	**Repeat 5**	**Average (sec)**
Inoclic	37	31	29	31	32	32
Manual	55 (x2)	119 (x3)	54 (x2)	39	58 (x2)	65
Enterococcus faecalis NCTC12697 / ATCC29212	**Repeat 1**	**Repeat 2**	**Repeat 3**	**Repeat 4**	**Repeat 5**	**Average (sec)**
Inoclic	21	22	20	24	25	22
Manual	56 (x2)	92 (x4)	45 (x2)	86 (x3)	46 (x2)	81
TOTAL (sec)						**TOTAL (sec)**
Inoclic	131	115	111	116	127	600
Manual	208.5	317.5	255	200	191.5	1172.5

time saving with Inoclic was noted in comparison to the manual method (Table 9).

Discussion

Advantages of Inoclic have clearly been highlighted. There is an obvious time saving and no need for repeat testing. This provides potential cost savings in terms of consumables as well as staff time. It was noted by some laboratories that Inoclic could potentially permit the use of lower banded staff to prepare the inoculum for antimicrobial sensitivity testing, which would allow AST plates to be prepared centrally and free biomedical scientists staff to do other work.

The use of Inoclic standardises the preparation of inocula required for the EUCAST disk-diffusion method. It provides equivalent results to the standard 0.5 McFarland method for susceptibility testing of various QC organism strains, regardless of the type of medium used and the antibiotic disk manufacturer. Inoclic is easy and quick to use, and results show that it could be a useful addition to any laboratory.

There is a high correlation between the manual method and Inoclic, with greater than 98% concordance between the two methods (within 2 mm) for all organisms and antibiotics. Inoclic also offers the further advantage of time saving, which is invaluable in a busy laboratory.

It is a CE/IVD-marked product used routinely in a large number of laboratories in the UK, France and globally. It also offers the added benefit that all necessary consumables are included in the kit, with no further equipment required, eliminating the need for a densitometer.

The i2a swab, which is included in the kit for plating out, also offers a smooth inoculation due to its flexibility, providing significant improvements in growth, particularly on blood agar plates.

Further work

The data obtained during the ring trial indicated there was potential for Inoclic to work with *Streptococcus pneumoniae*, as indicated in the study by Ferguson and Chaudhry.[7] Therefore, it was decided that a site would be chosen to focus primarily on *S. pneumoniae*. Several methods were tested using clinical and QC strains (Procult; Table 10), as follows:

- 'Stab, dab, dab emulsify, stab, dab, dab emulsify' and then plate out, streaking normally but re-entering saline before each one-third turn. Noticeably light growth was achieved which was sufficient for reading controls but not clinical samples.
- 'Stab, dab, dab emulsify, stab, dab, dab emulsify' and then plate out one-quarter, entering saline four times. Light growth was obtained.
- 'Stab, dab, dab emulsify, stab, dab, dab emulsify' and then plate a full plate (x3), re-entering saline each time (ie like a rotary plater). This method gave the best results and was selected for use.

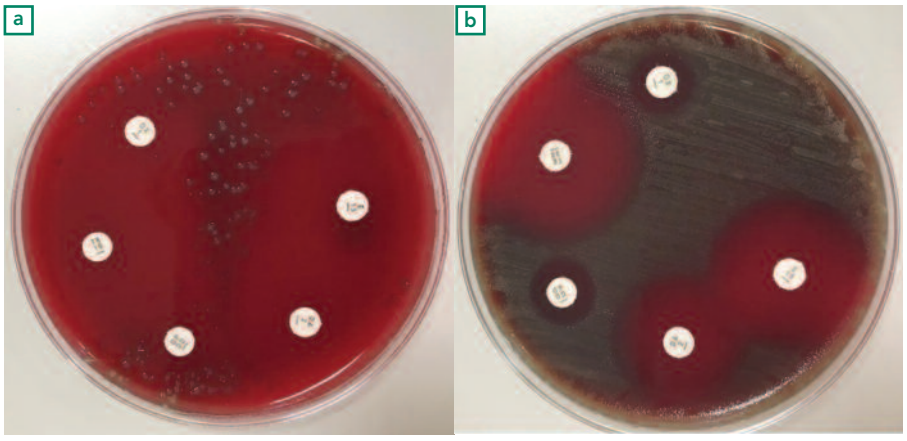


Fig 2. a) An agar plate set up using Method 3. **b)** A duplicate plate set up with a fresh control strain. The only difference is that plate A used a control that was a day older than the control on plate B. This difference in growth was observed several times using all three methods.

Table 10. Results for Method 3 using Procult.

Antibiotic No	1	2	3	4	5
Antibiotic	**Erythromycin 15**	**Clindamycin 2**	**Optochin**	**Tetracycline 30**	**Oxacillin 1**
EUCAST target	29	25		31	11
EUCAST target range	26–32	22–28	12–35	28–34	8–14
Inoculum method	**Inoclic**	**Inoclic**	**Inoclic**	**Inoclic**	**Inoclic**
Diameter 1	29.8	25	13.6	31.8	12.4
Diameter 2	30	25	13.8	33	12.5
Diameter 3	29.3	25.9	14	33	12.2
Diameter 4	28	26	15	32	12
Diameter 5	29	24.5	14.7	31.1	11.8
Diameter 6	30	26	13.7	33	12.2
Diameter 7	30.3	25.7	14.4	33.9	12
Diameter 8	29.66	24.8	14	33	13.4
Diameter 9	30.9	26	13.6	34	12.9
Diameter 10	30	25.7	14.5	33.5	12.5
Diameter 11	27.51	23.73	14.3	29.86	11.52
Diameter 12	27.31	23.34	13.27	29.67	12.12
Diameter 13	26.82	23.27	12.98	30.32	12.26
Diameter 14	27.56	23.44	13.02	29.65	11.6
Diameter 15	27.44	24.15	14.36	29.82	11.99
Diameter 16	28.46	24.34	13.9	30.41	11.63
Diameter 17	30.56	25.43	14.13	30.71	12.83
Diameter 18	28.42	25.54	14.48	30.36	11.53
Diameter 19	30.44	25.66	14.89	31.34	12.96
Diameter 20	30.45	24.26	13.7	30.33	12.74

Older control strains were also tested but did not grow using any of the methods. This is a likely cause for some of the failures seen in the ring trial. The plate shown in Figure 2a was set up using Method 3 above, in duplicate with the fresh control strain shown in Figure 2b, the only difference being that the former used a control that was a day older. This difference in growth was observed several times using all three methods.

Recommendations

- The method for *S. pneumoniae* may be adapted to sample double the recommended number of colonies, and to sample the saline inoculum three times between inoculating the same plate, thus permitting valid growth on the AST plate.
- When using Inoclic, the method is 'stab, dab, dab, emulsify, stab, dab, dab, emulsify', vortex-mix and then inoculate the plate completely from top to bottom (x3), re-entering the saline each time and turning the plate each time.
- Fresh cultures must be used.
- This adapted method must be validated by users in accordance with current laboratory standards.

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Anne Grayson is Field Applications Specialist and Charlotte Duncan is Technical Manager at Pro-Lab Diagnostics.

For further information, please contact a local Territory Manager or email Pro-Lab Diagnostics (uksupport@pro-lab.com).

Pro-Lab Diagnostics
3 Bassendale Road, Bromborough
Wirral, Merseyside CH62 3QL, UK
Tel: +44 (0)151 353 1613
Web: www.pro-lab.co.uk