RWANDA STANDARD

DRS 457

First edition

Approved by RSB 2021-mm-dd

Determination of bactericidal efficacy of disinfectants

ICS 71.100.35

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Reference number

DRS 457: 2021

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Foreword

Rwanda Standards are prepared by Technical Committees and approved by Rwanda Standards Board (RSB) Board of Directors in accordance with the procedures of RSB, in compliance with Annex 3 of the World Trade Organisation/Technical Barrier to Trade (WTO/TBT) agreement on the preparation, adoption and application of standards.

The main task of technical committees is to prepare national standards. Final Draft Rwanda Standards adopted by Technical committees are ratified by members of RSB Board of Directors for publication and gazettment as Rwanda Standards.

DRS 457 was prepared by Technical Committee RSB/TC 024, *Organic and Inorganic Chemicals*.

In the preparation of this standard, reference was made to the following standard:

US 1692: Determination of bactericidal efficacy of disinfectants/sanitizers

The assistance derived from the above source is hereby acknowledged with thanks.

Committee membership

The following organizations were represented on the Technical Committee on Organic and Inorganic Chemicals (RSB/TC 024) in the preparation of this standard.

Star Construction and Consultancy Ltd

Rwanda Inspectorate, Competition and Consumer Protection Authority

Rwanda Food and Drugs Authority

Rwanda Investigation Bureau

Rwanda Forensic Laboratory

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Determination of bactericidal efficacy of disinfectants

1 Scope

This Working Draft prescribes a method to determine the bactericidal efficacy of disinfectants using Kelsey Sykes test (modified).

This method is also applicable to detergent-disinfectants.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EAS 384, *Disinfectants — Glossary of terms*

ISO 6888-1, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species — Part 1: Technique using Baird-Parker agar medium*

ISO 7218, *Microbiology of food and anima feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 7251, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of presumptive Escherichia coli — Most probable number technique*

ISO 16266, *Water quality — Detection and enumeration of Pseudomonas aeruginosa — Method by membrane filtration*

3 Terms and definitions

For the purposes of this standard, the terms and definitions given in EAS 384 and the following apply.

3.1

bactericidal efficacy

ability of a disinfectant/sanitizer product to kill or prevent the growth of microorganisms, particularly bacteria, as required

4 Methods of microbiological testing

4.1 General

The test shall be undertaken by persons competent in microbiological techniques, using aseptic techniques. Media, reagents and equipment shall comply with the requirements and guidelines given in ISO 7218.

NOTE 1 In order to ensure accuracy of these tests, it is recommended that tests be repeated.

NOTE 2 Before these tests are carried out; the efficacy of the re-culture medium should be checked to ensure that it adequately inactivates the disinfectant to be tested.

NOTE 3 For the purpose of checking the resistance of the test organisms and other test conditions, it is advisable to include a reference standard. It is essential that it is a disinfectant based on the relative active ingredient, but, because a universal standard is difficult to select, each laboratory should make its own choice of material.

4.2 Accuracy

Except where otherwise specified, allow the following tolerances on:

a) Temperature: ± 2 °C;

b) Masses: ± 1.0%;

c) Volumes: ± 1.0%; and

d) pH value: ± 0.1 pH units.

4.3 Laboratory ware

4.3.1 Glassware, resistant to repeated heat sterilization with the glass free from inhibitory substances such as heavy metals and free alkali. Borosilicate glass with an expansion coefficient of less than 6 x 10^{-6} K^{-1} is recommended.

4.3.2 Universal container culture bottles, made of glass, fitted with standard screwed metal caps with rubber liners and that have nominal capacities of 30 ml and 100 ml. plastic containers or glass containers fitted with plastic tops shall not be used.

4.3.3 Culture tubes, rimless and cylindrical with hemispherical ends and nominal wall thickness of 1.5 mm, of internal diameter 16 mm and of length 160 mm.

4.3.4 Graduated pipettes, total delivery pipettes for bacteriological purposes only, that have an outflow opening of diameter 2 mm to 3 mm, are graduated in units of 0.1 ml and are of sizes to deliver 1.0 ml,5.0 ml and 10.0 ml.

4.3.5 Measuring cylinders, graduated with or without stoppers and of capacities 5 ml, 10 ml, 100 ml, 500 ml and 1 l.

4.3.6 **Culture flasks**, of capacities 250 ml, 500 ml and 1 l.

4.3.7 **Erlenmeyer flasks**, of capacities 250 ml, 2 l and 3 l.

4.3.8 **Petri dishes**, of diameter 90 mm and of height 20 mm and made of glass or of wettable polystyrene.

4.3.9 **Reagent bottles**, of capacities 50 ml and 100 ml that have polypropylene or other plastic stoppers of such design that the can be used to deliver drops of the reagent.

NOTE The analyst may use other volumes of laboratory ware, different from those stated above, as appropriate. However, the principle of the test method should be adhered to.

4.4 Test media

4.4.1 Reconstitution of media

Many of the media required are obtainable commercially in dehydrated form and for uniformity of results, the use of such media is recommended. If these are used, follow the manufacturer's instructions strictly regarding the reconstitution and sterilization. The water used in the preparation of media shall be distilled sterile water. Where sterilization by autoclaving is specified, autoclave the medium at 121 °C ± 3 °C for 15 min, unless otherwise stated. Unless otherwise specified, use a 0.1 M solution of hydrochloric acid or sodium hydroxide for pH adjustment as is relevant.

4.4.2 Standard hard water

4.4.2.1 Ingredients

Calcium chloride ($CaCl_2$):	0.304 g
Magnesium chloride ($MgCl_2.6H_2O$):	0.139 g
Water:	1000 ml

4.4.2.2 Preparation

Dissolve the ingredients in the water. Dispense 100 ml volumes into suitable containers and sterilize by autoclaving.

4.4.3 Nutrient agar

4.4.3.1 Ingredients

Agar:	15.0 g
Peptone:	5.0 g
Sodium chloride:	5.0 g

Yeast extract:	2.0 g
Beef extract:	1.0 g
Water:	1000 ml

4.4.3.2 Preparation

Dissolve the ingredients in the water and adjust the pH value to 7.1. Dispense in 10 ml and 15 ml volumes into suitable bottles and sterilize by autoclaving. Allow only the 10 ml volumes to solidify in a sloped position.

4.4.4 Nutrient broth

4.4.4.1 Ingredients

Peptone:	5.0 g
Sodium chloride:	5.0 g
Yeast extract:	2.0 g
Beef extract:	1.0 g
Water:	1000 ml

4.4.4.2 Preparation

Dissolve the ingredients in the water and adjust the pH value to 7.1. Dispense in 10 ml volumes into suitable bottles and sterilize by autoclaving.

4.4.5 Reculture medium

4.4.5.1 Ingredients

Beef extract:	10 g
Peptone:	10 g
Sodium chloride:	5 g
Polyoxyethylene sorbitan mono-oleate:	30 g
Water:	1000 ml

4.4.5.2 Preparation

Dissolve the ingredients in the water and adjust the pH to 7.5. Dispense in 1o ml volumes into tubes and sterilize by autoclaving.

4.4.6 Wright and Mundy medium (synthetic broth AOAC)

4.4.6.1 Part A

4.4.6.1.1 Ingredients

/-cystine:	0.05 g
d/-methionine:	0.37 g
/-arginine hydrochloride:	0.4 g
/-histidine hydrochloride:	0.3 g
/-lysine hydrochloride:	0.85 g
/-tyrosine:	0.21 g
d/-threonine:	0.5 g
d/-isoleucine:	0.44 g
glycine:	0.06 g
d/-serine:	0.61 g
d/-alanine:	0.43 g
/-glutamic acid hydrochloride:	1.3 g
/-aspartic acid:	0.45 g
d/-phenylalanine:	0.26 g
d/-tryptophan:	0.05 g
/-proline:	0.05 g
Water:	500 ml.

4.4.6.1.2 Preparation

Dissolve the ingredients in the water and add 18 ml of a sodium hydroxide [*c*(Na OH) = 1 mol/l] solution.

4.4.6.2 Part B

4.4.6.2.1 Ingredients

Sodium chloride:	3.0 g
Potassium chloride:	0.2 g
Magnesium sulphate:	0.05 g
Potassium phosphate:	1.5 g
Disodium phosphate:	4.0 g
Thiamine hydrochloride:	0.01 g
Nicotinamide:	0.01 g
Water:	500 ml

4.4.6.2.2 Preparation

Dissolve the ingredients in the water. Mix parts A and B, if necessary adjust the pH value to 7.1. Dispense half of the medium in 10 ml ± 0.2 ml volumes, and the other half in 6 ml ± 0.2 ml volumes into tubes, and sterilize by autoclaving. Before use, add to each tube in the two sets of tubes 0.1 ml and 0.06 ml (respectively), of a sterile 100 g/l (10%) solution of glucose.

4.4.7 Yeast suspension

4.4.7.1 Preparation of 20% (by mass) of moist yeast suspension

NOTE Whenever possible, the 20% (by mass) of moist yeast suspension should be sterilize on the day the yeast is received. If this is not feasible, the unopened yeast package should be stored at a temperature not higher than 5 °C for not longer than 48 h before use.

4.4.7.1.1 Crumble approximately 500 g of baker's yeast by hand into a previously tarred 1-l beaker and determine the mass of the moist yeast. Cream the yeast, adding a small volume of hard water (see 4.4.2) while stirring the mixture.

4.4.7.1.2 Carefully transfer the creamed portion to an Erlenmeyer flask of capacity 2 l and add a further small volume of hard water to any lumpy residue remaining in the beaker. Continue this process until all the yeast has been transferred from the beaker to the flask and the concentration of the yeast suspension in the flask has been reduced to approximately 40% (by mass) of moist yeast.

4.4.7.1.3 Shake the flask vigorously and remove large particles by passing the suspension through a sieve of aperture size 140 µm, supported in a funnel in an Erlenmeyer flask of capacity 3 l. Add enough hard water to reduce the concentration of the yeast to approximately 20 % (by mass) of moist yeast.

4.4.7.1.4 Shake thoroughly and while agitating, dispense 50ml volumes into bottles fitted with metal screw caps. Sterilize by autoclaving and store at 4 °C until required for use.

4.4.7.2 Determination of moisture content

Pipette 25 ml of the sterilized yeast suspension (see 4.4.7.1) into a dry tarred and dry to constant mass in a hot air oven maintained at 100 °C. Use this mass to determine the additional volume of hard water that shall be added to each bottle of sterilized yeast suspension to make a suspension that contains exactly 5% (by mass) of dry yeast.

4.4.7.3 Adjustment of pH value

Using a sodium hydroxide [*c*(Na OH) = 1 mol/l] solution, adjust the pH value of 50 ml of the 20% (by mass) of moist yeast suspension (see 4.4.7.1) to 7.0 and the volume of the sodium hydroxide solution required.

4.4.7.4 Preparation of 5% (by mass) of dry yeast suspension

Immediately before use, add to 50 ml of 20 % (by mass) moist yeast suspension the volume (see 4.4.7.2) of hard water necessary to make a suspension that contains exactly 5% (by mass) of dry yeast and enough sodium hydroxide [*c*(Na OH) = 1 mol/l] solution (see 4.4.7.3) to adjust the pH value to 7.0. Store the yeast suspension at 4 °C for not longer than seven days before use.

4.5 Test organisms

Use the following test organisms:

a) *Pseudomonas aeruginosa:* RS ISO 16266

b) *Escherchia coli:* RS ISO 7251; and

c) *Staphylococcus aureus:* RS ISO 6888-1.

Organisms that have survived the action of an antiseptic shall in no circumstances be used in a test.

NOTE 1 Additional organisms may be used if so desired.

NOTE 2 The extreme importance of using the standard strain is emphasized.

4.6 Maintenance of test organisms

4.6.1 From a newly opened freeze-dried culture or recently received agar culture, subculture the test organisms into bottles of 10 ml nutrients broth (see 4.4.4).

4.6.2 Incubate the bottles at 37 °C for 24 h. prepare subcultures from the cultures in the bottles onto slopes of nutrient agar (see 4.4.3). incubate the slopes at 37 °C for 24 h.

4.6.3 From each of these slope cultures, prepare four subcultures (stock cultures) of each test organism onto 10 ml nutrient agar slopes. Incubate the stock cultures at 37 °C for 24 h and then store in a refrigerator maintained at 4 °C, except for *Pseudomonas aeruginosa*, which is stored at room temperature.

4.6.4 Use the stock cultures to prepare further subcultures for the test (see 4.7), but do not make more than six serial subcultures from each stock culture. After the sixth serial subculture, resort to a new freeze-dried culture.

4.7 Preparation of cultures for test suspensions

4.7.1 For each of the test organisms, inoculate a tube that contains 10 ml of Wright and Mundy medium (see 4.4.6) from a slope subculture (see 4.6.4) and incubate at 30 °C for 24 h.

4.7.2 For the test, use a 24-h culture that has been sub-cultured for two successive days. After six subcultures, restart the process using a fresh culture (see 4.6.3).

NOTE The physiological condition of the test organisms is important and might influence inter-laboratory and intra-laboratory variations in test results.

4.8 Minimum inhibitory concentration test

4.8.1 Preparation of the test suspensions

Prepare a 1 in 10 dilution in Wright and Mundy medium (see 4.4.6) of a freshly grown subculture of each of the test organisms (see 4.7.2).

Before diluting a *Pseudomonas aeruginosa* culture, filter it though a coarse filter paper.

4.8.2 Preparation of the test sample dilutions

4.8.2.1 To 5 ml of test sample in a glass bottle of capacity 30 ml, and which has a standard screwed metal cap fitted with a rubber liner (do not use plastics containers or glass containers fitted with plastic tops), add 5 ml of Wright and Mundy medium (see 4.4.6). Mix well and transfer 5 ml of this dilution of test sample to a further 5 ml of Wright and Mundy medium. Repeat the procedure until 10 doubling dilutions of the test sample have been prepared. Discard 5 ml of the last dilution (so that each bottle will contain 5 ml of a dilution of the test sample).

4.8.2.2 Repeat 4.8.2.1 until three sets of 10 doubling dilutions of the test sample have been prepared.

4.8.3 Test procedure

4.8.3.1 To each of the 10 dilutions of the test sample (see 4.8.2.1) add 0.02 ml of the *Staphylococcus aureus* test suspension (see 4.8.1). Incubate the inoculated bottles at 30 °C for 72 h and examine the bottles for growth. The minimum inhibitory concentration is the highest dilution (minimum concentration) not showing growth.

4.8.3.2 Repeat the procedure given in 4.8.3.1 but using, successively, the *Escherichia coli* and *Pseudomonas aeruginosa* (see 4.8.1) test suspensions.

4.8.4 Interpretation of results

Determine which of the three test organisms is most resistant to the test sample, that is, the organism for which the minimum inhibitory concentration is the highest. Use this organism for the remainder pf the test (see 4.9)

4.9 Kelsey Sykes test

4.9.1 Test organism

Using the minimum inhibitory concentration test (see 4.8), determine which of the test organisms is the most resistant, and use it as the test organism for the determination.

4.9.2 Preparation of culture for test organism suspensions

On the day before the test is due to be carried out, inoculate a bottle that contains 10 ml of Wright and Mundy medium (see 4.4.6) from a daily subculture of the appropriate test organism which is at least a second and not more than a sixth subculture (see 4.6.4) and incubate the inoculated medium at 30 °C for 24 h.

4.9.3 Preparation of test organism suspension for the test under “clean” conditions

4.9.3.1 After incubation (see 4.9.2), centrifuge the culture of the test organism for 15 min at a resultant centrifugal force of 6 kN/kg. Using a sterile Pasteur pipette remove and discard the supernatant liquid and re-suspend the organism in 10 ml of hard water (see 4.4.2).

4.9.3.2 Transfer this suspension to a sterile glass bottle (see 4.3.2) of capacity 30 ml.

4.9.3.3 Vortex for 1 min.

NOTE If the test culture is a culture of *Pseudomonas aeruginosa*, filter it through sterile coarse filter paper before centrifuging.

4.9.4 Preparation of test organism suspension for the test under “dirty” conditions

Obtain a suspension that contains 2% (by mass) of dry yeast by adding 6 ml of the culture of test organism suspension (see 4.9.2) to 4 ml of 5% (by mass) of dry yeast suspension (see 4.4.7.4) contained in a sterile glass bottle (see 4.3.2) of capacity 30 ml. Vortex for 1 min.

4.9.5 Estimation of the number of viable organisms in the test organism suspension

4.9.5.1 From each of the test suspensions (see 4.9.3 and 4.9.4) prepare serial dilutions by pipetting 1 ml of each suspension into a bottle that contains 9 ml of sterile distilled water and making further dilutions (using a fresh pipette each time) by pipetting 1 ml of the first dilution into another bottle that contains 9 ml of diluent and repeating this procedure until eight successive dilutions have been prepared.

4.9.5.2 From each dilution of the suspension, starting at the sixth dilution, take two 1-ml volumes and transfer each volume to a Petri dish. For each plate one 15-ml volume of nutrient agar (see 4.4.3), melt, temper it to 45 °C, add it to the Petri dish, and mix. Avoid spilling any of the contents of the Petri dish during this procedure. Allow the agar to set and incubate the Petri dishes at 32°C ± 1 °C for 24 h ± 1 h. Ensure that the total period between the preparation of the dilutions of the suspension and the final plating does not exceed 30 min.

4.9.5.3 After incubation count the colonies on each plate and record the results given by plates that contain between 30 colonies and 300 colonies.

4.9.5.4 Multiply each of the recorded counts by the dilution factor involved (for example, for a dilution of 1 in 107, multiply the average plate count by 107), and take the average of the number of organisms in each millilitre of test suspension.

NOTE 1 If the number of viable organisms in a test suspension is less than 108 per ml or more than 1010 per ml, the results of a test (see 4.9.7 and 4.9.8) in which the suspension was used shall be considered invalid.

NOTE 2 Nephelometric or turbidimetric methods may be used instead of the procedure given in 4.9.5.1 to 4.9.5.4 to determine the number of viable organisms in the culture from which the test suspension is prepared if these can be shown to give satisfactory results.

4.9.6 Preparation of test solutions

Using hard water (see 4.4.2) and glass bottles of capacity 30 ml, and which have standard screwed metal caps fitted with rubber liners, prepare three different concentrations A, B and C of the test sample (disinfectant/sanitizer) that are such that

a) concentration B is that which is expected or claimed to pass the test,

b) concentration A is concentration B less 50%, and

c) concentration C is concentration B plus 50%.

For example, if a test sample (disinfectant/sanitizer) is expected to pass the test at a concentration of 1%, concentration A is a 0.5% concentration, B is a 1% concentration and C is a 1.5% concentration.

4.9.7 Test procedure under “clean” conditions

4.9.7.1 Dispense 3 ml of each concentration of the test sample (see 4.9.6) into glass bottles or test tubes of capacity 30 ml, and which have standard screwed metal caps fitted with rubber liners, and label these bottles A, B and C, as relevant.

4.9.7.2 Place these bottles and the bottle that contains the test organism suspension (see 4.9.3) for at least 30 min in a water bath maintained at 22 °C ± 2 °C. To maintain reproducibility of the test, adhere strictly to the temperature state.

4.9.7.3 Then, without removing the bottle that contains test concentration A from the water bath, add 1 ml of the test organism suspension (see 4.9.3) and start a stop-watch (zero time) simultaneously. Remove the bottle from the water bath, vortex well and immediately return it to the water bath.

4.9.7.4 One minute after zero time add, in the same way, 1 ml of the test suspension to the bottle that contains test concentration B.

4.9.7.5 Five minutes after zero time add, in the same way, 1ml of the test suspension to the bottle that contains test concentration C.

4.9.7.6 Eight minutes after zero time, transfer 0.02 ml from the bottle that contains test concentration A to each of five tubes of re-culture medium (see 4.4.5) each of which has been labelled A1.

4.9.7.7 Ten minutes after zero time add, in the same way as described in 4.9.7.3, a further 1 ml of test organism suspension (see 4.9.3) to the bottle that contains test concentration A.

4.9.7.8 Eighteen minutes after the zero time transfer 0.02 ml from the bottle that contains test concentration A to each of five tubes of re-culture medium each of which has been labelled A2.

4.9.7.9 Concurrently with 4.9.7.6 to 4.9.7.8, treat the test concentration B and C in the same way, but basing the time intervals on the times at which the first additions of test suspensions were made, and labelling the sets of tubes of re-culture medium B1 and B2, and C1 and C2 respectively. Thus, in the case of test concentration B addition and transference will be made 1 min later than the times given for test concentration A (that is, at 9 min, 11 min and 19 min after zero time) and in the case of test concentration C additions and transference will be made at 13 min, 15 min and 23 min after zero time.

NOTE In order to obviate errors in transference, a copy of the test timetable (see table 1) should be used during each test. Each step of the test should be kicked off on the timetable as it is carried out.

Table 1 – Kelsey Sykes test timetable

Time (min)	Test sample suspension A	Time (min)	Test sample suspension B	Time (min)	Test sample suspension C
0	1 ml test suspension to A	1	1 ml test suspension to B	5	1 ml test suspension to C
8	0.02 ml transferred from A to re-culture medium A1	9	0.02 ml transferred from B to re-culture medium B1	-	
10	1 ml test suspension to A	11	1 ml test suspension to B	13	0.02 ml transferred from C to re-culture medium C1
-		-		15	1 ml test suspension to C
18	0.02 ml transferred from A to re-culture medium A2	19	0.02 ml transferred from B to re-culture medium B2	23	0.02 ml transferred from C to re-culture medium C2

4.9.7.10 Incubate all the inoculated tubes of re-culture medium at 30 °C for 48 h.

4.9.7.11 After incubation examine the tubes of re-culture medium for growth and record the results as shown in the example given in table 2.

4.9.7.12 If test concentration B passes the test (see 4.9.9), repeat steps 4.9.7.1 to 4.9.7.11 on two subsequent days. If test concentration B fails the test and the minimum concentration that will pass the test is required, repeat the entire test on concentrations of greater strength until a value for concentration B is found that passes three successive tests.

4.9.8 Test procedure under “dirty” conditions

Carry out the procedure described in 4.9.7 but use the test organism suspension described in 4.9.4.

4.9.9 Interpretation of results

Deem the initial concentration of disinfectant/sanitizer (concentration B – see 4.9.6,a) to pass the test if there is no growth of the test organism in at least two of the five tubes of re-culture medium in sets inoculated at

a) the eighth, and

b) the eighteenth minute after the addition of the initial inoculum.

An example of a series of test results and their interpretation is given in table 2.

Table 2 – Test sample tested under "clean" conditions

Test No	Test sample concentration % (by volume)	Test suspension organisms (ml)	Re-culture medium		Result
			1	2	
1	0.8	6.9×10^8	+++++	+++++	Fail
	1.6	6.9×10^8	- -+++	- ++++	Fail
	2.4	6.9×10^8	- - - - -	- - - - -	Pass
Test solution at 1.6% (by volume) concentration has failed Test repeated at higher concentrations					
1	0.9	8.4×10^8	- ++++	++ - + -	Fail
	1.8	8.4×10^8	- - - - +	- - +++	Pass
	2.7	8.4×10^8	- - - - -	- - - - -	Pass
2	0.9	2.1×10^9	- ++++	+++++	Fail
	1.8	2.1×10^9	- - - - +	- - +++	Pass
	2.7	2.1×10^9	- - - - -	- - - - -	Pass
3	0.9	5.7×10^8	- - +++	+++++	Fail
	1.8	5.7×10^8	- - - - -	- - - ++	Pass
	2.7	5.7×10^8	- - - - -	- - - - -	Pass
Test organism: *Pseudomonas aeruginosa* NOTE: + refers to growth of the test organism; and - refers to no growth of the test organism					

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[1] ISO/IEC Directives, Part 2, *Rules for the structure and drafting of International Standards*, 2016

[2] ISO/IEC TR 10000-1, *Information technology — Framework and taxonomy of International Standardized Profiles — Part 1: General principles and documentation framework*

[3]

