Designation: E2871 – 13

Standard Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method[1]

This standard is issued under the fixed designation E2871; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method specifies the operational parameters required to perform a quantitative liquid disinfectant efficacy test against biofilm bacteria.

1.2 The test method was developed using a *Pseudomonas aeruginosa* biofilm grown in the CDC Biofilm Reactor (Test Method E2562), modified to include borosilicate glass coupons as a hard nonporous surface and *P. aeruginosa* ATCC 15442.

1.3 Disinfectant preparation and contact time are used in the assessment according to the manufacturer's instructions for use.

1.4 The test method uses a closed system to treat biofilm. A coupon is placed in a single tube for the treatment, neutralization, and sampling steps to prevent the loss of cells.

1.5 Verification of disinfectant neutralization is determined prior to conducting the test method.

1.6 This test method describes how to sample and analyze treated and untreated control biofilms for viable cells. Biofilm population density is recorded as $\log_{10}$ colony-forming units per coupon. Efficacy is reported as a $\log_{10}$ reduction of viable cells.

1.7 Basic microbiology training is required to perform this assay.

1.8 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.9 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E1054 Test Methods for Evaluation of Inactivators of Antimicrobial Agents

E2562 Test Method for Quantification of ***Pseudomonas aeruginosa*** Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor

2.2 *Other Standards:*

Method 9050 C.1.a Buffered Dilution Water Preparation according to Eaton et al **(1)**[3]

3. Terminology

3.1 *Definitions:*

3.1.1 *biofilm, n*—microorganisms living in a self-organized community attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, while exhibiting altered phenotypes with respect to growth rate and gene transcription.

3.1.1.1 *Discussion*—Biofilm may be comprised of bacteria, fungi, algae, protozoa, viruses, or infinite combinations of these microorganisms. The qualitative characteristics of a biofilm including, but not limited to, population density, taxonomic diversity, thickness, chemical gradients, chemical composition, consistency, and other materials in the matrix that are not produced by the biofilm microorganisms, are controlled by the physicochemical environment in which it exists.

3.1.2 *contact time, n*—predetermined time that the biofilm is exposed to the activity of a disinfectant.

3.1.3 *coupon, n*—biofilm growth surface.

3.1.4 *disinfectant, n*—a chemical that destroys vegetative forms of microorganisms, but does not ordinarily kill bacterial spores.

3.2 *Acronyms:*

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The boldface numbers in parentheses refer to a list of references at the end of this standard.

3.2.1 *ATCC*—American Type Culture Collection.

3.2.2 *CDC*—Centers for Disease Control and Prevention.

3.2.3 *CFU*—colony-forming unit.

4. Summary of Test Method

4.1 This test method describes the use of the single tube method to evaluate the efficacy of a liquid disinfectant against a *Pseudomonas aeruginosa* biofilm on a hard nonporous surface grown in the CDC Biofilm Reactor. The test method consists of adding a disinfectant (treated) or a control buffer (untreated) to individual coupons held in 50-mL conical tubes. Three coupons are treated with disinfectant and three coupons receive buffered dilution water. Neutralizer is added to the tubes after the appropriate contact time. A combination of vortexing and sonication are used to remove the biofilm from the coupon and disaggregate the clumps. The cell suspension is serially diluted and plated on agar medium. Viable plate counts from treated and untreated control coupons are used to calculate the $\log_{10}$ reduction of viable cells.

5. Significance and Use

5.1 Vegetative biofilm bacteria are phenotypically different from suspended planktonic cells of the same genotype. Biofilm growth reactors are engineered to produce biofilms with specific characteristics (**2**). Altering either the engineered system or operating conditions will modify those characteristics as well as the physicochemical environment. The goal in biofilm research and efficacy testing is to choose the growth reactor and operating conditions that generate the most relevant biofilm for the particular study.

5.2 The test method was developed using *Pseudomonas aeruginosa* ATCC 15442 biofilm grown on borosilicate glass coupons in the CDC Biofilm Reactor and liquid disinfectants. Efficacy data developed using other bacteria, different shear, different coupons, or other standardized biofilm reactor systems, and/or other forms of disinfectants may result in different $\log_{10}$ reduction (LR) values and repeatability and reproducibility standard deviations.

5.3 The efficacy test was designed to determine the $\log_{10}$ reduction in bacteria after exposure to a disinfectant in a closed system.

5.4 The test method was developed using 50-mL conical tubes. The conical geometry allows for disinfectant exposure to biofilm on all surfaces of the coupon.

5.5 Each efficacy test includes a single contact time and temperature for three untreated control coupons (exposed to buffered dilution water) and three treated coupons (per disinfectant/concentration combination).

6. Apparatus

6.1 *Conical centrifuge tubes,* sterile, any with 50-mL volume capacity and secure leakproof lids.

6.2 *Ultrasonic water bath,* any capable of maintaining a homogeneous sound distribution at 45 kHz with a variable power setting and a volume large enough to accommodate 50-mL conical tubes in a wet environment.

6.3 *Test tube rack,* any capable of holding 50-mL conical centrifuge tubes.

6.4 *Micropipettes,* continuously adjustable pipettes with volume capacity of 100 µL and 1000 µL.

6.5 *Sterile pipette tips,* 100-µL and 1000-µL volumes.

6.6 *Bunsen burner,* used to flame-sterilize Allen wrench and plate spreader.

6.7 *95 % Ethanol,* used to flame-sterilize Allen wrench and plate spreader.

6.8 *Small Allen wrench,* for loosening set screws and pushing coupons out of reactor rods.

6.9 *Timer,* any that can display time in seconds.

6.10 *Vortex mixer,* any vortex that will ensure proper agitation and mixing of centrifuge tubes.

6.11 *Serological pipettes,* sterile single-use pipettes with volume capacity of 1, 5, 10, 25, and 50 mL.

6.12 *Plate spreader,* for spreading serial dilutions on agar plates.

6.13 *Water bath,* any capable of maintaining a constant temperature of 20 ± 1°C.

6.14 *Sterilizer,* any steam sterilizer capable of producing the conditions of sterilization.

6.15 *Colony counter,* any one of several types may be used. A hand tally for recording of the bacterial count is recommended if manual counting is done.

6.16 *Environmental incubator,* any capable of maintaining a temperature of 36 ± 2°C.

6.17 *Appropriate glassware/plasticware,* as required to make media and agar plates.

6.18 *Volumetric flasks,* used for preparing disinfectants.

6.19 *Magnetic stir bars,* sterile, for mixing prepared disinfectant.

6.20 *Magnetic stir plate,* any capable of mixing.

7. Reagents and Materials

7.1 *Purity of Water*—all references to water as diluent or reagent shall mean distilled water or water of equal purity.

7.2 *Bacterial Plating Medium*—R2A agar is recommended.

7.3 *Buffered Water*—0.0425 g KH_2PO_4/L distilled water, filter-sterilized and 0.405 g $MgCl{\cdot}6H_2O$/L distilled water; filter-sterilized (prepared according to Method 9050 C.1.a Buffered Dilution Water Preparation (**1**)).

7.4 *Disinfectant*—product to be tested.

7.5 *Neutralizer*—Dey/Engley Neutralization Broth or one specific to the disinfectant being evaluated as determined for effectiveness and toxicity according to Test Method E1054.

8. Culture/Inoculum Preparation

8.1 Borosilicate glass coupons with mature *Pseudomonas aeruginosa* ATCC 15442 biofilm grown according to Test Method E2562 through step 10.2.4.

9. Procedure

9.1 The test is conducted with three treated and three untreated control coupons.

9.2 An overview of the procedure is shown in Fig. 1.

9.3 *Prepare Disinfectant:*

9.3.1 Prepare disinfectant according to manufacturer's specifications in sterile volumetric glassware. Ensure that the disinfectant is adequately mixed. Use within 3 h of preparation or as specified in the manufacturer's instructions.

9.3.2 Place prepared disinfectant in water bath equilibrated to 20 ± 1°C for 10 to 15 min.

9.4 *Remove Coupons from the CDC Biofilm Reactor:*

9.4.1 Prepare sampling materials: treatment tubes, rinse tubes, small flame-sterilized Allen wrench, and serological pipettes.

9.4.2 Aseptically remove a randomly selected rod containing coupons with biofilm from the CDC Biofilm Reactor by pulling it straight up firmly.

9.4.3 Rinse the coupons to remove planktonic cells. Orient the rod in a vertical position directly over a 50-mL conical centrifuge tube that contains 30-mL sterile buffered water. Immerse the rod with a continuous motion into the buffered water with minimal to no splashing, then immediately remove. A new 50-mL conical tube containing 30-mL sterile buffered water is used for each rod.

9.4.4 Hold the rod with one of the randomly selected coupons centered over an empty, sterile 50-mL conical tube. Loosen the set screw and allow the coupon to drop directly to the bottom of the tube. If the coupon does not freely drop, press directly in the center of the coupon with the Allen wrench used to loosen the set screw.

NOTE 1—The use of 50-mL conical tubes allows for uniform disinfectant contact around the entire coupon surface.

9.4.5 Repeat coupon removal twice more for a total of three tubes each containing a coupon.

NOTE 2—All biofilm on the coupon must be exposed to disinfectant. Do not lose any biofilm from the coupon by allowing it to touch the top or the inner sides of the 50-mL conical tube as it falls to the bottom of the tube. To ensure that the maximum biofilm surface area is in contact with the disinfectant, the coupon should be at an angle in the bottom of the tube. Discard any tubes where the coupon touched the inner side of the tube and/or held coupons that were not angled and replace them with new tubes and coupons.

9.5 *Conduct Efficacy Evaluation:*

9.5.1 Slowly pipette 4 mL previously prepared and equilibrated disinfectant (treatment) or equilibrated buffered dilution water (untreated control) into the tubes containing the coupons, being careful to completely cover the coupon. Note and record the time.

NOTE 3—The order of application of disinfectant or buffered dilution water is randomly selected.

NOTE 4—For a 10-min contact time, a 1-min interval between coupons is recommended.

9.5.2 Tap each tube to release any air bubbles trapped below the coupon. Do not shake the tubes.

9.5.3 Incubate the tubes at 20 ± 1°C for the specified contact time.

9.5.4 At the end of the contact time, add appropriate volume of neutralizer to each tube. Replace the cap and mix thoroughly by vigorously shaking the tube several times. Allow the coupon to remain in the neutralized disinfectant at room temperature until step 9.6.

NOTE 5—It is recommended that a neutralization study be conducted prior to running the efficacy test (Test Method E1054) to determine the appropriate neutralizer formulation, concentration, volume, and neutralization time. For each efficacy test, the concentration, volume, and neutralization time should be the same for the control and treated coupons. Thirty-six (36) mL of neutralizer was used in the collaborative study.

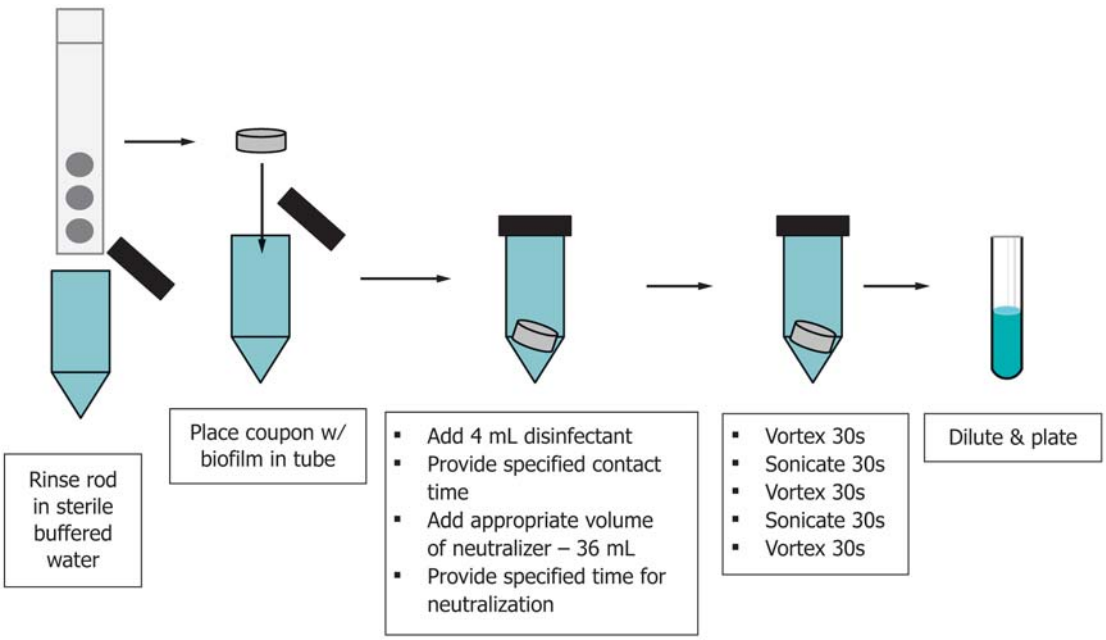

FIG. 1 Single Tube Method Overview

9.5.5 Obtain a set of three coupons for the remaining treatment(s) or control(s) as described in steps 9.4.2 through 9.4.5.

9.5.6 Repeat steps 9.5.1 through 9.5.4 with randomly selected treatment(s) or control(s).

9.6 *Remove and Disaggregate Biofilm:*

9.6.1 Vortex each tube on the highest setting for 30 ± 5 s.

9.6.2 Place all tubes into a test tube rack and suspend the rack in the ultrasonic water bath so that the liquid level in the tubes is even with the water level in the tank of the bath.

9.6.3 Sonicate the tubes at 45 kHz for 30 ± 5 s.

9.6.4 Vortex the tubes as described in 9.6.1.

9.6.5 Sonicate the tubes as described in 9.6.2 and 9.6.3.

9.6.6 Vortex the tubes as described in 9.6.1. These tubes are the 10^0 dilution.

NOTE 6—The results from an interlaboratory study of this test method demonstrated the importance of following the disaggregation and removal protocol exactly as written including using the recommended ultrasonic water bath and vortex mixer.

9.7 *Dilute and Plate Disaggregated Biofilm Samples:*

9.7.1 Serially dilute the sample in buffered water.

9.7.2 Culture each dilution in duplicate for colony growth using an accepted plating technique such as spread- or pour-plating.

9.7.3 Incubate plates at 36 ± 2°C for 24 to 28 h.

9.7.4 Count the appropriate number of colonies according to the plating method used.

10. Data Analysis

10.1 Calculate biofilm density.

10.1.1 Calculate *X*, the arithmetic mean CFU from the replicate samples plated (**3**).

10.1.2 The $\log_{10}$ density for each coupon is calculated as follows:

$$\mathrm{Log_{10}\,(CFU/coupon)} = \mathrm{Log_{10}}\left[(X/B)(V/D)\right] \quad (1)$$

where:

X = average CFU of the replicate sample plates,
B = volume plated,
V = volume of disinfectant or buffered water plus neutralizer,
D = 10^{-k}, and
k = dilution.

10.2 Calculate the mean $\log_{10}$ density for each set of treated and control coupons as follows:

$$\mathrm{Mean\ LD} = \left[\mathrm{Log_{10}\,(Coupon\ A)} + \mathrm{Log_{10}\,(Coupon\ B)} + \mathrm{Log_{10}\,(Coupon\ C)}\right]/3 \quad (2)$$

10.3 Calculate the $\log_{10}$ reduction for each disinfectant as follows (**4**) :

$$\mathrm{LR} = \mathrm{Mean\ Log_{10}\ Untreated\ Coupons} - \mathrm{Mean\ Log_{10}\ Treated\ Coupons} \quad (3)$$

10.4 Calculate the standard error of the mean LR as follows:

$$\mathrm{SE\ of\ mean\ LR} = \frac{\mathrm{SD}}{\sqrt{m}} \quad (4)$$

where:

SD = standard deviation of the LR, and
m = number of experiments performed in a single laboratory.

10.5 Calculate the percent kill as follows:

$$\%\ \mathrm{Kill} = \left(1 - 10^{-LR}\right) \times 100 \quad (5)$$

11. Precision and Bias

11.1 *Precision:*

11.1.1 Two statements concerning the precision of this method can be made, one for the mean log density of the untreated control coupons per test, and one for the log reductions.

11.1.2 An interlaboratory study RR:E35-1008[4] of this method tested a control (buffered water) and three chemical disinfectants (sodium hypochlorite (NaOCl), phenolic, and quaternary alcohol) at two concentrations (high and low) against a *Pseudomonas aeruginosa* ATCC 15442 biofilm grown on borosilicate glass coupons in the CDC Biofilm Reactor. Each chemical disinfectant x concentration condition was tested in three repeated experiments using triplicate coupons for every experiment. The precision statements are based upon data collected from six laboratories. An ANOVA model was fit to the data to determine the repeatability and reproducibility of the log density of organisms associated with the mean of the untreated control coupons per test, and the repeatability and reproducibility of the log reductions of organisms associated with the disinfectant chemicals.

11.1.3 The repeatability standard deviation for the untreated control coupons was 0.22 $\log_{10}$ (CFU/cm^2); and the reproducibility standard deviation was 0.24 $\log_{10}$ (CFU/cm^2). Contributions to variance were 18% due to among-lab sources, 0.00% due to among-technician sources, 78% due to among-experiment sources, and 3% due to within-experiment sources.

11.1.4 The repeatability and reproducibility standard deviations of the log reductions for each chemical disinfectant are summarized in the Table 1 and shown graphically in Fig. 2.

11.2 *Bias:*

11.2.1 Since an accepted reference value is not available, randomization is used whenever possible to reduce the potential for systematic bias.

11.2.2 Several potential biases exist for this method.

11.2.3 A bias may occur between treated and control coupons due to increased fixation or washoff effects of particular disinfectants. Untreated control coupons receive buffered dilution water only and are, therefore, not exposed to the inactive ingredients found in disinfectants. These inactive ingredients may enhance removal of surface-associated bacteria due to surfactant properties or inhibit removal due to increased pH.

11.2.4 For treated coupons, some disinfectants cause oxidative injury to cell membranes. Cells that have compromised membranes may be completely killed by sonication (**5**). Untreated control coupons are exposed to buffered dilution water only. Since the surface-associated bacteria are removed from

[4] Supporting data have been filed at ASTM International Headquarters and may be obtained by requesting Research Report RR:E35-1008. Contact ASTM Customer Service at service@astm.org.

TABLE 1 Repeatability and Reproducibility Standard Deviations for Log Reductions for the Three Chemical Disinfectants and Two Concentrations Tested.

Chemical	Concentration	Mean LR	Within lab %	Among lab %	Repeatability SD	Reproducibility SD
NaOCl	HIGH	3.89	86%	14%	1.306	1.404
	LOW	2.32	71%	29%	0.751	0.891
Phenol	HIGH	3.78	18%	82%	0.580	1.349
	LOW	3.32	100%	0%	1.345	1.345
Quatalcohol	HIGH	4.58	75%	25%	1.448	1.668
	LOW	2.71	63%	37%	0.731	0.922

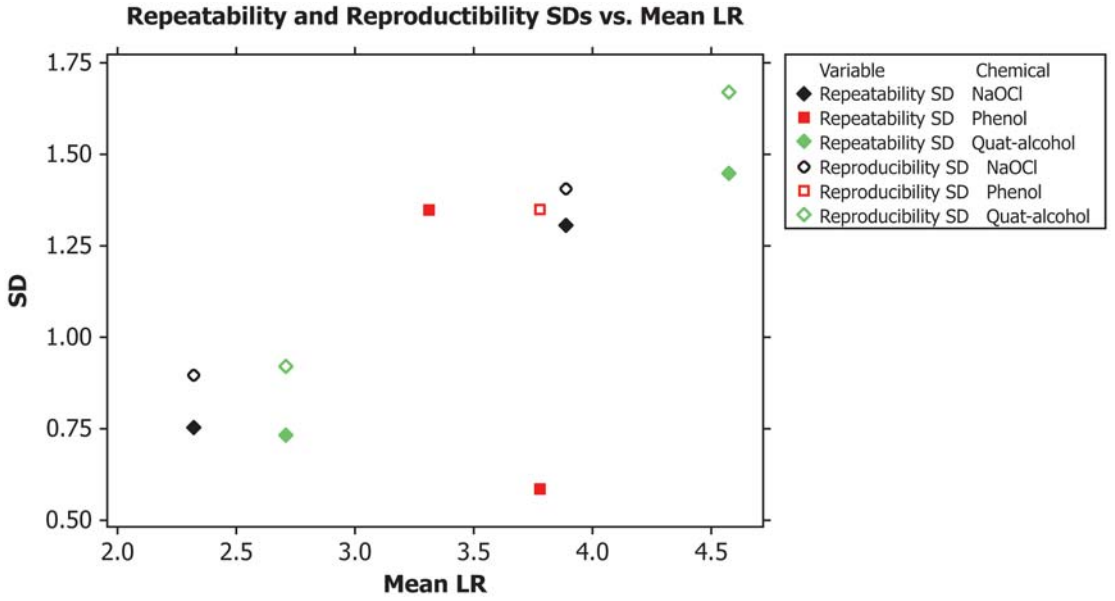

FIG. 2 Repeatability and Reproducibility Standard Deviations as a function of the Mean Log Reduction for each Chemical Disinfectant

the coupons with vortexing and sonication, it is not possible to determine if ultrasonic waves exhibit an efficacy of their own without performing bias checks prior to the efficacy testing using planktonic cultures.

11.2.5 Bias checks can be performed by viewing coupon surfaces microscopically for biofilm removal. Cell suspensions can be filtered onto membranes, stained and examined microscopically for clumps of cells.

11.3 *Responsiveness:*

11.3.1 The method was statistically significantly responsive to the increasing efficacy level for the NaOCl (the mean LR changed by 1.57, p-value = 0.002) and quat-alcohol (the mean LR changed by 1.87, p-value = 0.013) disinfectants, but not phenol (the mean LR changed by 0.45, p-value = 0.170) as shown in Fig. 3.

12. Keywords

12.1 antimicrobial; biofilm; CDC Biofilm Reactor; disinfectant; efficacy testing; log reduction; *Pseudomonas aeruginosa*; sampling

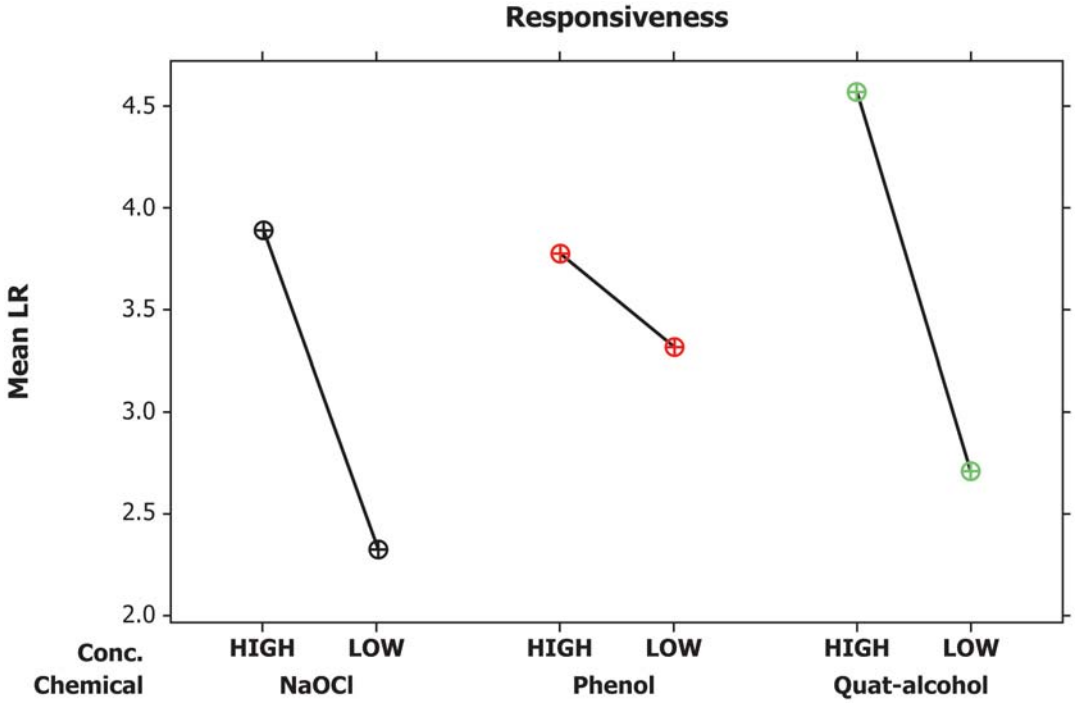

FIG. 3 Method was Statistically Responsive to a Decrease in the NaOCl and Quat-alcohol Concentration

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