Designation: E2196 – 17

Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with Medium Shear and Continuous Flow Using Rotating Disk Reactor[1]

This standard is issued under the fixed designation E2196; 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 is used for growing a reproducible (1)[2] *Pseudomonas aeruginosa* biofilm in a continuously stirred tank reactor (CSTR) under medium shear conditions. In addition, the test method describes how to sample and analyze biofilm for viable cells.

1.2 Although this test method was created to mimic conditions within a toilet bowl, it can be adapted for the growth and characterization of varying species of biofilm (rotating disk reactor—repeatability and relevance (2)).

1.3 This test method describes how to sample and analyze biofilm for viable cells. Biofilm population density is recorded as $\log_{10}$ colony forming units per surface area (rotating disk reactor—efficacy test method (3)).

1.4 Basic microbiology training is required to perform this test method.

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

1.6 *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.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D5465 Practices for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

2.2 *Other Standards:*

Method 9050 C.1.a Buffered Dilution Water Preparation (4)

3. Terminology

3.1 *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 *Discussion*—Biofilms 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 physiochemical environment in which it exists.

3.2 *coupon, n*—biofilm sample surface.

4. Summary of Test Method

4.1 This test method is used for growing a reproducible *Pseudomonas aeruginosa* biofilm in a rotating disk reactor. The biofilm is established by operating the reactor in batch mode (no flow) for 24 h. Steady state growth (attachment is equal to detachment) is reached while the reactor operates for an additional 24 h with continuous flow of the nutrients. The residence time of the nutrients in the reactor is set to select for biofilm growth, and is species and reactor parameter specific. During the entire 48 h, the biofilm is exposed to continuous fluid shear from the rotation of the disk. At the end of the 48 h, biofilm accumulation is quantified by removing coupons from

[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.

Current edition approved April 1, 2017. Published May 2017. Originally approved in 2002. Last previous edition approved in 2012 as E2196 – 12. DOI: 10.1520/E2196-17.

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

[3] 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.

the disk, harvesting the biofilm from the coupon surface, disaggregating the clumps, then diluting and plating for viable cell enumeration.

5. Significance and Use

5.1 Bacteria that exist in a biofilm are phenotypically different from suspended cells of the same genotype. The study of biofilm in the laboratory requires protocols that account for this difference. Laboratory biofilms are engineered in growth reactors designed to produce a specific biofilm type. Altering system parameters will correspondingly result in a change in the biofilm. The purpose of this method is to direct a user in the laboratory study of biofilms by clearly defining each system parameter. This method will enable a person to grow, sample, and analyze a laboratory biofilm. The method was originally developed to study toilet bowl biofilms, but may also be utilized for research that requires a biofilm grown under moderate fluid shear.

6. Apparatus

6.1 *Wooden Applicator Sticks,* sterile.

6.2 *Inoculating Loop.*

6.3 *Petri Dish,* 100 by 15 mm, plastic, sterile and empty to hold rotor while sampling.

6.4 *Culture Tubes and Culture Tube Closures,* any with a volume capacity of 10 mL and minimum diameter of 16 mm. Recommended size is 16 by 125 mm borosilicate glass with threaded opening.

6.5 *Pipette(s),* continuously adjustable pipette(s) with volume capacity of 1 mL.

6.6 *Micropipette(s),* continuously adjustable pipette(s) with a volume capacity of 10 – 250 µL.

6.7 *Vortex,* any vortex that will ensure proper agitation and mixing of culture tubes.

6.8 *Homogenizer,* any capable of mixing at 20 500 ± 5000 r/min in a 5 to 10 mL volume.

6.9 *Homogenizer Probe,* any capable of mixing at 20 500 ± 5000 r/min in a 5 to 10 mL volume that can withstand autoclaving or other means of sterilization.

6.10 *Sonicating Bath,* any cavitating sonicating bath that operates at 45 to 60 kHz for cleaning the coupons.

6.11 *Bunsen Burner,* used to flame inoculating loop and other instruments.

6.12 *Stainless Steel Dissecting Tools,* for removing the coupons.

NOTE 1—Alternatively, a coupon manipulation tool[4] may be used.

6.13 *Stainless Steel Hemostat Clamp,* with curved tip.

6.14 *Environmental Shaker,* capable of maintaining temperature of 36 ± 2°C.

6.15 *Analytical Balance,* sensitive to 0.01 g.

6.16 *Sterilizer,* any steam sterilizer capable of producing the conditions of sterilization is acceptable.

6.17 *Colony Counter,* any one of several types may be used, such as the Quebec, Buck, and Wolfhuegel. A hand tally for the recording of the bacterial count is recommended if manual counting is done.

6.18 *Peristaltic Pump,* pump head capable of holding tubing with inner diameter of 3.1 mm and outer diameter of 3.2 mm.

6.19 *Digital Magnetic Stir Plate,* top plate 10.16 by 10.16 cm, capable of rotating at 200 ± 5 r/min.

6.20 *Silicone Tubing,* two sizes of tubing: one with inner diameter of 3.1 mm and outer diameter of 3.2 mm, and the other with inner diameter of 7.9 mm and outer diameter of 9.5 mm. Both sizes must withstand sterilization.

6.21 *Norprene*[5] Tubing, inner diameter of 3.1 mm and outer diameter of 3.2 mm.

6.22 *Glass Flow Break,* any that will connect with tubing of inner diameter 3.1 mm and withstands sterilization.

6.23 *Clamp,* used to hold flow break, extension clamp with 0.5 cm minimum grip size.

6.24 *Clamp Stand,* height no less than 76.2 cm, used with clamp to suspend glass flow break vertically and stabilize tubing above reactor.

6.25 *Reactor Components*[6]:

6.25.1 *Berzelius Borosilicate Glass Beaker,* 1000 mL without pour spout, 9.5 ± 0.5 cm diameter. Borosilicate barbed outlet spout added at 250 ± 15 mL mark at 30 to 45° angle, spout should accommodate silicone tubing with an inner diameter of 8 to 11 mm.

NOTE 2—The rotor, described in 6.25.3, will displace approximately 50 mL of liquid. Therefore, an outlet spout at the 250 mL mark will result in an operating volume of approximately 200 mL. Before use, the user should confirm the actual liquid volume in the reactor, after the rotor is in place and the stir plate is turned on. The measured operating volume is used to calculate an exact pump flow rate.

6.25.2 *Reactor Top,* size 15 rubber or machined stopper, with three holes bored through top to accommodate 6 cm pieces of stainless steel tubing or other suitable rigid autoclavable tubing with an outside diameter of 4 to 6 mm. One port accommodates tubing for media, the second port is fitted with a short piece of silicone tubing that holds a bacterial air vent, and the third is an inoculum port as shown in Fig. 1.

6.25.3 *Rotor or Disk,* nominal 1.6 mm thick PTFE sheet cut into a disk with a diameter of 7.0 ± 0.2 cm containing six evenly spaced holes with a diameter of 1.27 ± 0.1 cm. The

[4] The sole source of supply of the apparatus (coupon manipulation tool) known to the committee at this time is BioSurface Technologies, Corp., www.biofilms.biz. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend. The user may also build the holder,

[5] Trademarked by the Saint-Gobain Performance Plastics Corporation.

[6] The sole source of supply of the apparatus (rotating disk reactor) known to the committee at this time is BioSurface Technologies, Corp., www.biofilms.biz. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarter. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend. The user may also build the reactor.

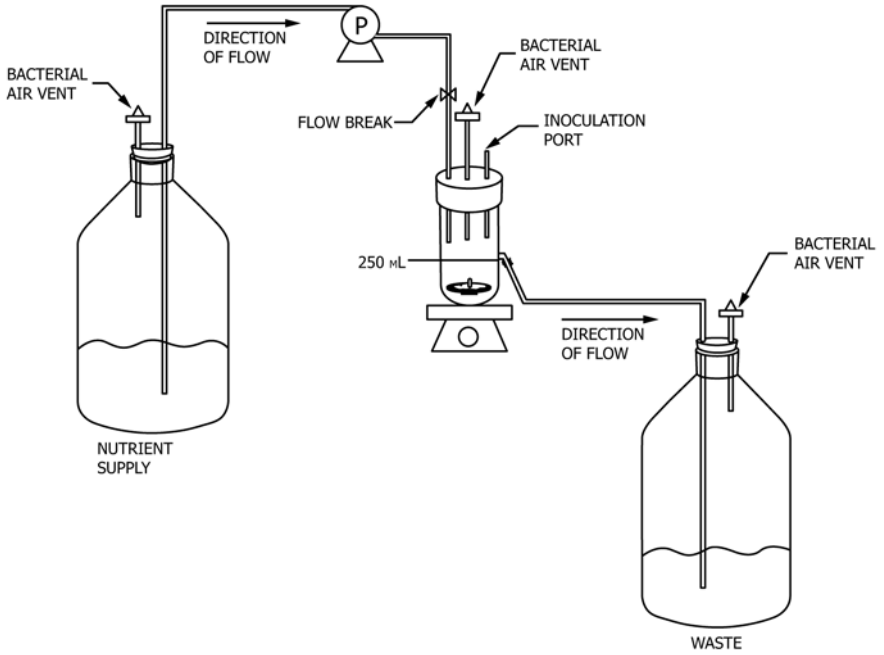

FIG. 1 Rotating Disk Reactor System

center of each hole is located 2.55 ± 0.03 cm from the center of the disk. 4.5 to 7.0 mm thick rubber sheet, or other suitable autoclavable material, cut into a disk with a diameter of 7.0 ± 0.2 cm containing six evenly spaced holes with a diameter of 1.27 ± 0.15 cm (the holes in the rubber are aligned with the holes in the PTFE) and a small hole in the center to house the disk retrieving port. PTFE washer with disk retrieving port. Four nylon screws. PTFE-coated 4.0 by 1.4 cm star-head magnetic stir bar, set flush against PTFE disk, with holes drilled for assembly using nylon screws. The PTFE ridges on one side of the magnet may be shaved to provide a flush mounting surface. Assemble the pieces conforming to the general details shown in Fig. 2.

NOTE 3—Nominal implies that the manufacturer's tolerance is acceptable.

6.25.4 *Six Cylindrical Polycarbonate Coupons,* with a diameter of 1.27 ± 0.013 cm and a height of 1.5 to 4.0 mm.

6.26 *Carboys,* two 20 L autoclavable carboys, to be used for waste and nutrients.

6.26.1 *Carboy Lids,* two: one carboy lid with at least 2 barbed fittings to accommodate tubing ID 3.1 mm (one for nutrient line and one for bacterial air vent), one carboy lid with at least two 1 cm holes bored in the same fashion (one for effluent waste and one for bacterial air vent).

NOTE 4—Carboy tops can be purchased with fittings.

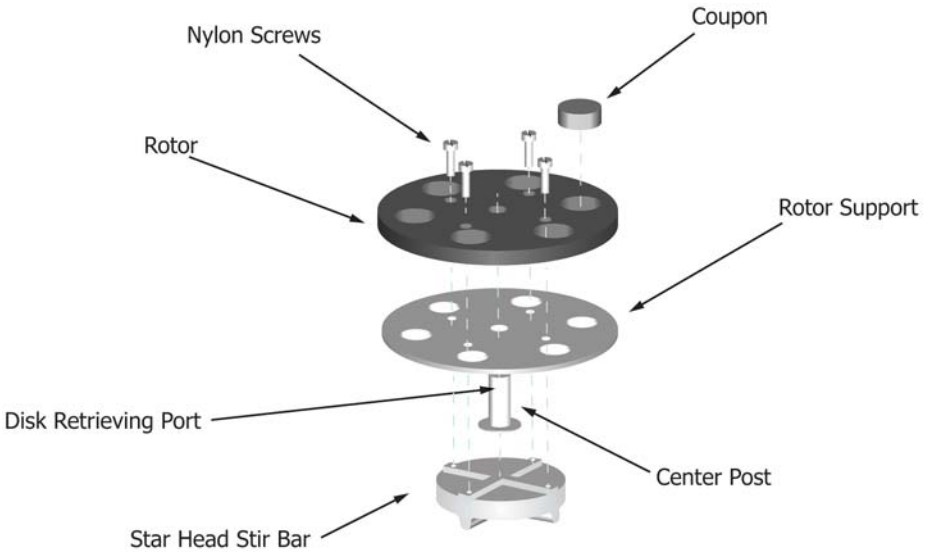

FIG. 2 Rotor Assembly

6.26.2 *Bacterial Air Vent,* autoclavable 0.2 µm pore size, to be spliced into tubing on waste carboy, nutrient carboy, and reactor top (37 mm and 25mm diameter recommended).

7. Reagents and Materials

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

7.2 *Culture Media:*

7.2.1 *Bacterial Liquid Growth Broth*—Tryptic Soy Broth (TSB) is recommended.

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

NOTE 5—Two different concentrations of TSB are used in the protocol, 300 mg/L for the inoculum and batch reactor operation and 30 mg/L for the continuous flow reactor operation.

7.3 *Buffered Water*—0.0425 g/L KH_2PO_4 distilled water, filter sterilized, and 0.405 g/L $MgCl \cdot 6H_2O$ distilled water, filter sterilized, prepared according to Method 9050 C.1.a(**4**).

8. Culture Preparation

8.1 *Pseudomonas aeruginosa* ATCC 700888 is the organism used in this test. Aseptically remove an isolated colony from an R2A plate and place into 100 mL of sterile TSB (300 mg/L). Incubate bacterial suspension in an environmental shaker at 36 ± 2°C for 22 ± 2 h. Viable bacterial density should be about 10^8 CFU/mL, which may be checked by serial dilution and plating.

9. Reactor Preparation

9.1 *Preparation of Polycarbonate Coupons:*

NOTE 6—Coupons can be used once and discarded or used repeatedly with proper cleaning and sterilization between each use. Check each coupon for scratching, chipping, other damage, or accumulated debris before each use by screening under a dissecting microscope at a magnification of at least 20×. Discard those with visible damage to surface topography.

NOTE 7—Coupons may be made out of alternative materials such as glass or stainless steel. The user should adjust the cleaning procedure so that it is appropriate for the coupon material being used.

9.1.1 Sonicate coupons for 30 s in a 1+99 dilution of laboratory soap and tap water. The soapy water must completely cover the coupons.

9.1.2 Rinse coupons with reagent grade water and sonicate for 30 s in reagent grade water.

9.1.3 Repeat rinsing and sonication with reagent grade water until, upon visual inspection, no soap is left on the coupons.

NOTE 8—Wear gloves to prevent oils and other residues from soiling the coupon.

9.1.4 Place a coupon into each hole in the rotor, leaving the top of the coupon flush with the rubber rotor surface.

9.1.5 Place the rotor with the coupons facing up inside of the beaker.

9.2 *Preparation of Reactor Stopper Top:*

9.2.1 Use a small section of appropriately-sized tubing to connect the bacterial air vent to the stainless steel tubing on the reactor stopper top.

9.2.2 The glass flow break is spliced into the nutrient tubing line near the reactor stopper top.

NOTE 9—The other ports on the reactor top may be adapted for anaerobic use, dilution water, or treatment solutions as necessary.

9.3 *Sterilization of Reactor System:*

9.3.1 Assemble the reactor, with the overflow (waste) line clamped and the reactor top securely fastened to the beaker before sterilization.

9.3.2 Cover the end of the nutrient tubing that connects to the nutrient carboy and the end of the overflow (waste) tubing with aluminum foil. Cover any extra openings on the reactor top with aluminum foil.

9.3.3 Sterilize the reactor system.

10. Procedure

10.1 *Batch Phase:*

10.1.1 Prepare batch nutrient broth by dissolving TSB (300 mg/L) in 250 mL reagent grade water, sterilize.

10.1.2 Remove the top of the sterile reactor and aseptically pour the sterile batch nutrients into the reactor. Secure the reactor top and place the reactor onto a stir plate.

10.1.3 Inoculate the reactor with 1 mL of bacteria from the culture prepared previously (see 8.1): Aseptically pipette the inoculum into the beaker through the inoculation port.

10.1.4 Turn the magnetic stir bar on to allow the rotor to spin freely. The rotational speed should equal approximately 200 ± 5 r/min. The reactor system is allowed to incubate in batch mode at room temperature (21 ± 2°C) for 24 h.

NOTE 10—The rotor rotational speed is a critical factor that must be controlled. The rotational speed of the rotor directly determines the amount of shear stress that the biofilm experiences, and therefore the biofilm accumulation on the coupons. If a digital stir plate is not available, use a strobe light to confirm rotational speed.

10.2 *Continuous Flow Operation (CSTR Mode):*

10.2.1 Aseptically connect the nutrient tubing line to the carboy containing the continuous flow nutrient broth.

10.2.2 Prepare continuous flow nutrient broth by dissolving bacterial liquid growth medium (30 mg/L) in 20 L sterile reagent grade water. Dissolve and sterilize the broth in a smaller volume to prevent caramelization. Aseptically pour the concentrated broth into the carboy of sterile water to make a total of 20 L.

10.2.3 Pump a continuous flow of nutrients into the reactor through a pump set to a flow rate of 6.7 ± 0.2 mL/min. Attach tubing from the drain spout to a waste carboy and remove clamp. The drain spout at the 250 mL mark on the beaker allows effluent overflow to occur, maintaining a constant bacterial liquid growth broth concentration of 30 mg/L in the reactor during CSTR mode.

NOTE 11—Flow rate is calculated by dividing the reactor volume by the residence time. The residence time is 30 min. The reactor volume is approximately 200 mL (see Note 2). The operator should set an exact flow rate based upon the measured fluid volume in the reactor when the rotor is in place to achieve an exact 30 min residence time. The reactor residence time is a critical parameter that is specific to the bacterial species used during the experiment. To select for biofilm growth in the reactor, the residence time must be less than the doubling time for the suspended cells. This will result in the suspended cells washing out of the reactor, leaving only biofilm.

10.2.4 Operate the reactor in CSTR mode for 24 h.

10.3 *Sampling the Biofilm:*

10.3.1 Prepare sampling materials: vortex, homogenizer, culture tubes, pipettes, empty sterile petri dish, wooden applicator sticks, flame-sterilized stainless steel dissecting tools, stainless steel hemostat or coupon holder.

10.3.2 Open the reactor and retrieve the rotor with a stainless steel dissecting tool via the disk retrieving port (see Fig. 2). Place the rotor in an empty, sterile petri dish.

10.3.3 Hold the rubber portion of the rotor with a flame-sterilized hemostat and remove a randomly chosen coupon from the rotor with flame-sterilized stainless steel dissecting tool and hemostat or coupon holder. Care should be given to not disturb the top coupon surface in any way until it is scraped. If other coupons are to be sampled, replace rotor in reactor to prevent the biofilm from drying.

10.3.4 Rinse the coupon to remove suspended cells. Orient the coupon at a 45° angle over a beaker that contains 20 mL sterile buffered water. Gently immerse coupon with a continuous motion into the buffered water then immediately remove the coupon.

10.3.5 Remove biofilm from the coupon. Scrape the top coupon surface for approximately 15 s using the flat end of a wooden applicator stick held perpendicular to the coupon surface. Rinse the stick by stirring it in the buffered water in the culture tube. Repeat the scraping and rinsing process 3 to 4 times, ensuring full coverage of the coupon surface.

10.3.6 Rinse the coupon by holding the coupon at a 60° angle over the culture tube and pipetting 1 ml of sterile buffered water over the surface of the coupon. The final volume in the culture tube is 10 mL.

NOTE 12—Each culture tube originally contains 9 mL of buffered water.

10.4 *Analyze the Biofilm Sample:*

10.4.1 Homogenize the scraped biofilm sample at 20 500 ± 5000 r/min for 30 s. If more than one biofilm sample is taken, rinse the homogenizer probe between each new sample as follows: Homogenize a dilution blank for 30 s at 20 500 + 5000 r/min, homogenize a tube containing 10 mL 70 % ethanol for 15 s, then remove the probe and let the probe sit in the ethanol tube for 1 min. Shake any remaining ethanol off the probe, reattach it and homogenize a dilution blank for 30 s. Homogenize a second dilution blank and then homogenize the next sample tube. Always repeat this cleaning process between samples.

NOTE 13—Homogenizing the sample disaggregates the biofilm clumps to form a homogeneous cell suspension. Improper disaggregation will result in an underestimation of the viable cells present in the sample (**5**).

10.4.2 Serially dilute the sample.

10.4.3 Plate each dilution in duplicate for colony growth using an accepted plating technique such as drop-plating (**6**), spread plating or spiral plating. (See Practice D5465.)

10.4.4 Incubate the plates for 24 ± 2 h at 36 ± 2°C.

10.5 *Cell Enumeration:*

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

10.5.2 Calculate the arithmetic mean of the replicate samples plated.

10.6 The $\log_{10}$ density for one coupon is calculated as follows:

$$\mathrm{LOG}_{10}\,(\mathrm{CFU/cm^2}) = \mathrm{LOG}_{10}\left[(X/B)(V/A)(D)\right]$$

where:

X = mean CFU,
B = volume plated,
V = volume scraped into,
A = surface area scraped, and
D = dilution.

NOTE 14—The equation in 10.6 is accurate only if the culture tube the biofilm was scraped into is referred to as the 10^0 dilution.

10.7 Calculate the overall biofilm accumulation by taking the mean of the $\log_{10}$ densities calculated in 10.6.

11. Precision and Bias

11.1 Randomization is used whenever possible to reduce the potential for systematic bias.

11.2 During development, the protocol was repeated 27 times. The internal reproducibility standard deviation for this protocol, when the biofilm density is based on a single coupon, was calculated to equal 0.5. The sources of variability were:

40 % attributable to technician-to-technician variability,
40 % attributable to experiment-to-experiment within technician variability, and
20 % attributable to coupon-to-coupon, within experiment variability.

12. Keywords

12.1 analysis; biofilm; coupon; growth reactor; *Pseudomonas aeruginosa*; reactor; sampling

REFERENCES

(1) Ellison, S. L. R., Rosslein, M., Williams, A. (Eds.), *Guide Quantifying Uncertainty in Anyalytical Measurement, 2nd Edition*, Eurachem/CITAC, 2000.

(2) Zelver, N., Hamilton, M., Pitts, B., Goeres, D., Walker, D., Sturman, P., Heersink, J.,"Measuring antimicrobial effects on biofilm bacteria: from laboratory to field," In: Doyle, R. J. (Ed.), *Methods in Enzymology – Biofilms*, Vol 310, Academic Press, San Diego, CA, 1999, pp. 608–628.

(3) Zelver, N., Hamilton, M., Goeres, D., Heersink, J., "Development of a Standardized Antibiofilm Test," In: Doyle, R. J. (Ed.), *Methods in Enzymology – Biofilms*, Vol 337, Academic Press, San Diego, CA, 2001, pp. 363–376.

(4) Rice, E.W., Baird, R.B. Eaton, A. D., Clesceri, L. S. (eds.), *Standard Methods for the Examination of Water and Wastewater,* 22nd Edition, American Public Health Association, American Water Works Assocation, Water Environment Federation, Washington D.C., 2012.

(5) Hamilton, M. A., Buckingham-Meyer, K., Goeres, D. M., "Checking the Validity of the Harvesting and Disaggregating Steps in Laboratory Tests of Surface Disinfectants," *Journal of AOAC International* , Vol 92, No. 6, 2009, pp. 1755–1762.

(6) Herigstad, B., Hamilton, M., Heersink, J., "How to Optimize the Drop Plate Method for Enumerating Bacteria," *Journal of Microbiological Methods* Vol 44, No. 2, 2001, pp. 121-129.