US Environmental Protection Agency
Office of Pesticide Programs

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Single Tube Method with Splashguard for Evaluating Disinfectant Activity against *Pseudomonas* Biofilm – 2015 Method Performance Study

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Single Tube Method with Splashguard for Evaluating Disinfectant Activity against *Pseudomonas* Biofilm

2015 Method Performance Study

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Section 1 **– Introduction**

The U.S. EPA is considering the use of the ASTM Single Tube Method (ASTM E2871-13) as an efficacy method to support the registration of antimicrobial products with biofilm claims. In 2014, the Office of Pesticide Programs Microbiology Laboratory Branch (MLB) performed a collaborative study to assess the method's performance. During this study, an unexpected level of variability in log reduction values was noted both within and between laboratories for both high efficacy treatments. Following discussions with the collaborators, it was determined that the variability may have been due to inadvertent contact and splashing of the carrier-associated inoculum onto the inner walls of the reaction tube during the deposition of the carrier. A cylindrical device, a splashguard, was developed by Montana State University to prevent/mitigate the problem. The splashguard device is now commercially available (BioSurface Technologies, Bozeman, MT).

In mid-2015, six laboratories evaluated the use of the splashguard with the same presumed highly efficacious sodium hypochlorite treatment evaluated in the 2014 study. Use of the splashguard was deemed successful in mitigating the risk of recovering un-exposed inoculum from the inner walls of the reaction tube. In this study, two sets of test chemicals, each with a high and low level of presumed efficacy, will be evaluated against a *Pseudomonas aeruginosa* biofilm to reassess the method's performance with the splashguard.

Section 2 **– Study Goals**

- Utilize standardized protocols for data generation.
- Conduct a replicated set of efficacy assays for the purpose of generating log reduction (LR) values and associated variance; specifically, repeatability and reproducibility standard deviations will be calculated for the purpose of assessing method performance. Furthermore, the data will be used to formulate testing criteria (i.e., required log reduction, number of replications and labs based on error rates).
- The data may be used to support revisions to the appropriate ASTM standard(s).
- A minimum of six labs will conduct the method against the sodium hypochlorite reference standard and a quaternary ammonium-based product with two levels of presumed efficacy for each test chemical.
- Control coupon counts and log reduction values following exposure to four disinfectant treatments with a range of efficacy are the main test variables.

Section 3 – **Collaborators**

The laboratories must have existing microbiology programs, appropriately trained personnel, and the capability of conducting the study within the established timeframe. Each laboratory will be encouraged to establish a technical team which will conduct all tests. Practice runs by each laboratory will be encouraged in advance of testing to ensure analyst proficiency in performing the method. The identity of the laboratories will be coded and will not be identified in the data summary or final reports. The laboratories are identified in Table 1.

Table 1. **Laboratories**

Laboratory Name
A
B
C
D
E
F
G

Section 4 – **Study Plan and Reminders**

- Study Design
    - The study design calls for evaluating the efficacy of four treatments as indicated in Table 2; the testing scheme is provided in Tables 3 and 4. Five treated coupons (per chemical treatment) and three control coupons (per test day per reactor) will be analyzed per test day.
    - A total of three replications (three test days) per treatment are required; thus a total of 6 days will be required assuming testing of one treatment set per reactor run. If desired, a minimum of 2 analysts may run both treatment sets in one day (total of 23 coupons).
    - The order of testing for each test day should be: 1) high efficacy, 2) low efficacy, and 3) controls. The controls should always be processed last to mitigate cross contamination and to expedite processing of the treated coupons. The identification of the rod and coupon position in the reactor is not required.
    - **Submit the first replicate of data to EPA prior to additional testing.**
    - Testing and data submission must be completed by the end of December 2015.
- Methods and Paperwork
    - Labs must strictly follow the provided SOPs: MB-19 dated 10/20/15 (CDC reactor) and MB-20 dated 10/22/15 (Single Tube Method) as the test method protocols.
    - Verify 30 minute residence time of CDC biofilm reactor following procedures in MLB SOP MB-19.
    - See Attachment 1 for pictures of technique sensitive steps.
    - Standardized test forms and data sheets will be provided by the EPA.
    - Reagent preparation sheets will be provided and must be filled in for each test substance per test day.
- Test Chemicals
    - Test chemicals and the Safety Data Sheets will be provided to each laboratory by the EPA.
    - A single lot of each test chemical will be used for testing at all labs.
    - Initiate use of a test substance within three hours of preparation.
    - Range of ppm for sodium hypochlorite solutions is ±10%. See media/reagent preparation sheets. Use of a Hach test kit or comparable analysis is required for measuring available chlorine for the two reference standard treatments on each test

day.

- Test Microbe
    - *P. aeruginosa* (ATCC 15442); obtained directly from a reputable supplier (e.g., ATCC). Use frozen stock cultures generated according to MLB SOP MB-19. A new test culture is required for each test day; thus, a new frozen stock culture must be used to initiate a new test culture for each day of the study.
- Equipment and Supplies
    - Degas sonicator for ~5 min on the day of use; during testing, sonicate tubes on the "normal" setting at 100% power.
    - Use visually screened borosilicate glass coupons (procured by each laboratory). Prepare the coupons by following the cleaning/screening procedure located in section 12.1 of MLB SOP MB-19.
    - Sterile splashguards will be used for only treated carriers; one splashguard per tube.
- Test Conditions
    - See Tables 3 and 4 for filtration/spread plating scheme. Adjustments to the scheme may be made after the first replication.
    - Control coupons are exposed to 4 mL SMDW instead of the chemical treatment.
    - The neutralizer is 36 mL 2X Dey/Engley broth; used for control and treated carriers.
    - All testing will be conducted at room temperature. Record room temperature each test day; recording of relative humidity is optional.
- Recovery
    - For filtration purposes, use hydrophilic polyethersulfone (PES) filter membranes (0.45 µm, 47 mm diameter) plated on R2A.
    - Laboratories may use disposable analytical filter units, magnetic filter funnels, or other comparable filtration apparatus (e.g., manifolds).
    - For spread plating, use 100 µL per plate, duplicate R2A plates.
    - Incubate all plates and filters (controls and treated) for 48±2 hours at 36±1°C.
    - Record counts from 0 to TNTC. For filters, the countable range is 0-200; for spread plating, the countable range is 0-300. In some instances, it is conceivable that results from plated and filtered samples will be zero or TNTC.

Table 2. Treatments and testing parameters.

Test Substance ID	Active Ingredient	Treatment ID	Dilution	Diluent	Contact Time
A	Sodium hypochlorite	1	5,000 ppm pH-adjusted NaOCl (pH ~7)	Sterile DI water	10 min
		2	200 ppm pH-unadjusted NaOCl (pH ~10)	Sterile DI water	10 min
B	Quat	3	Ready to use (no dilution)	N/A	10 min
		4	1:15 (1 part disinfectant + 14 parts diluent)	Sterile DI water	10 min

Table 3. Testing scheme assuming a total of 6 test days.

Test Days for One Replication	Treatment ID	# of Coupons	Recovery Step
1	1	5	Filter 10 mL from 10^{0}
	2	5	Spread plate 0.1 mL in duplicate from 10^{-1}, 10^{-2}, and 10^{-3}, filter remaining sample in 10^{-1}.*
	Controls	3	Spread plate 0.1 mL in duplicate from 10^{-4} and 10^{-5}
2	3	5	Filter 10 mL from 10^{0}
	4	5	Spread plate 0.1 mL in duplicate from 10^{-1}, 10^{-2}, and 10^{-3}, filter remaining sample in 10^{-1}.*
	Controls	3	Spread plate 0.1 mL in duplicate from 10^{-4} and 10^{-5}

*Filter sample remaining in 10^{-1} dilution tube after samples taken for direct plating (for at least the first replication).

Table 4. Testing scheme assuming a total of 3 test days.

Test Days for One Replication	Treatment ID	# of Coupons	Recovery Step
1	1	5	Filter 10 mL from 10^{0}
	3	5	Filter 10 mL from 10^{0}
	2	5	Spread plate 0.1 mL in duplicate from 10^{-1}, 10^{-2}, and 10^{-3}, filter remaining sample in 10^{-1}.*
	4	5	Spread plate 0.1 mL in duplicate from 10^{-1}, 10^{-2}, and 10^{-3}, filter remaining sample in 10^{-1}.*
	Controls	3	Spread plate 0.1 mL in duplicate from 10^{-4} and 10^{-5}

*Filter sample remaining in 10^{-1} dilution tube after samples taken for direct plating (for at least the first replication).

Section 5 – **Quality Assurance and Documentation**

- Maintain documents to ensure that all studies are supported by complete, accurate, consistent, and chronological records from initial collection of raw data to final analysis interpretation and reporting of results.
- The expected level of quality assurance is consistent with EPA Good Laboratory Practices.
- No specific certification is required for this study; however, staff performing the assays must be familiar with standard microbiological techniques such as aseptic transfer, serial dilutions, plate counts and microbe identification.
- Scientists and analysts involved in testing shall be knowledgeable of the STM procedure and capable of accurately and independently conducting the procedure.
- Pre-printed data sheets and test forms will be used. Record data in ink. Use a single line for correcting entries with the date and initials of the person making the correction and the reason for the change.
- Where possible, use standard forms for those operations which have become or will become routine, including test methodology, analytical procedures and calibration procedures.
- Selected electronic spreadsheets and email will be considered as official documentation and will be maintained and stored accordingly.
- Track all preparations of test chemicals, media and reagents using an assigned media preparation number.
- Maintain samples (test chemicals) to ensure their integrity; store test chemicals away from standards, media, and reagents to prevent cross-contamination.
- No official chain of custody documentation will be required for test chemicals evaluated in this project; however the laboratories will maintain specific information on source, identification, and volume received for all test chemicals.
- Inspect all supplies and materials considered "critical" to the quality of the research such as media, reagents, coupons, and test chemicals prior to use to ensure that the shipment has not been damaged or compromised in any way.
- For pre-sterilized lab supplies, the manufacturer's statement of sterility is acceptable for quality control documentation for sterility; no further testing is required. For growth media, conduct performance testing (sterility and suitability to support growth) a minimum of one time, preferably on the first batch prepared per lot.
- Upon completion of each study, a peer review of the data entry/tabulation will be performed by laboratory personnel. The peer-reviewed data should be forwarded to the Study Director.
- Deviations should be reported to the Study Director as soon as possible. Following consultation with the Study Director, the data will be deemed valid or invalid.
- Data may be rejected by the management of each laboratory or Quality Assurance Unit if the study is not performed correctly or if deviations to the procedure are not documented.
- Data may be rejected if microbial contamination occurs at an unacceptable level (if contamination is systemic or interferes with recording of results).

Section 6 – **Statistical Analysis**

The statistical analyses will provide the information described below. The responses of interest are the control coupon log CFU per coupon and the log reduction (LR). The statistical analysis will produce estimates of the repeatability standard deviation (SD) and the reproducibility standard deviation for each response for each treatment.

- **Substitutions** – In order to incorporate the data into the statistical analyses of the efficacy data, the following substitution rules will be automatically applied (i.e., these substitutions will be supplied by the statisticians and not by the lab technicians). When all zeros are observed, a 0.5 will be substituted at the lowest dilution, and then scaled up as any other observed CFU value at that dilution. When all TNTC values are observed, a 200 or 300 will be substituted at the highest dilution, and then scaled up accordingly.

- **Raw data plots** – the individual data points will be plotted for visual inspection to see trends and effects and to detect outliers or influential observations.

- **Analysis of control coupon mean log densities** – the log transformed CFU per control coupon will be analyzed using an analysis of variance (ANOVA). These results will describe the "normal range" of control coupon titers as well as estimates of the repeatability SD, the reproducibility SD, and the percentages of variance due to within-test sources, between test sources, and among-laboratory sources.

- **Log reduction (LR) value** – *LR is the primary quantitative response and most of the statistical work will focus on the LR data.* For each combination of organism and test chemical, a one factor, random effects model ANOVA will be conducted to estimate the repeatability SD (the estimate is denoted by S_r), the reproducibility SD (the estimate is denoted by S_R) and the percentages of variance attributable to intra-laboratory versus inter-laboratory sources.

- **Mean LR** – for each treatment, the mean LR will be calculated along with the associated standard error and confidence interval. The two efficacy levels associated with the reference standard will be used to measure responsiveness, a statistical trend test will determine whether the mean LRs increase significantly with increasing efficacy level.

- **Diagnostic plots and tests** – these will be performed routinely to check whether the observations conform to the mathematical assumptions underlying the ANOVA calculations.

- **Presentation of results** – tables and figures will be created to present the results in a report.

Section 7 – **Media preparation sheets and data collection forms**

Media Preparation Sheets

1. Acetic acid (5%)
2. 2X Dey/Engley neutralizing broth
3. Magnesium chloride stock solution
4. Phosphate buffer stock solution
5. Standard methods dilution water
6. R2A agar
7. Treatment #1 (5,000 ppm pH-adjusted NaOCl)
8. Treatment #2 (200 ppm pH-unadjusted NaOCl)
9. Treatment #3 (RTU quat product)
10. Treatment #4 (1:15 dilution of quat product)

Data Collection Forms:

1. Biofilm Organism Culture Tracking Form
2. Test Microbe Confirmation Sheet (Quality Control)
3. Biofilm Test Information Sheet
4. Biofilm Timing Form
5. Biofilm Dilution/Plating Tracking Form
6. Biofilm Results Form
7. Biofilm Test Microbe Confirmation Sheet
8. Spreadsheet

Attachment 1. **Testing Photographs**

Splashguard insert in 50 mL conical tube

Appropriate location of splashguard insert (arrow indicates appropriate position of bottom of insert in conical tube)

Position of rod over conical tube during carrier deposition (the rod must not touch the splashguard insert)

Removal of splashguard insert

Addition of disinfectant/ control fluid to conical tube

2015 Single Tube Method Collaborative Data Compilation

Log Density of Control Carriers

Lab	Test Chemical	Replicate	Log Density
A	Controls	1	9.017033
A	Controls	1	9.245513
A	Controls	1	8.905060
A	Controls	2	9.246409
A	Controls	2	8.815940
A	Controls	2	8.983913
A	Controls	3	9.421604
A	Controls	3	9.440909
A	Controls	3	9.204120
B	Controls	1	8.664471
B	Controls	1	8.602060
B	Controls	1	8.677939
B	Controls	2	8.812306
B	Controls	2	8.679593
B	Controls	2	8.730929
B	Controls	3	8.881851
B	Controls	3	8.881851
B	Controls	3	8.748188
C	Controls	1	9.047098
C	Controls	1	8.956867
C	Controls	1	8.961205
C	Controls	2	9.309630
C	Controls	2	9.230449
C	Controls	2	8.991226
C	Controls	3	8.752398
C	Controls	3	8.600081
C	Controls	3	8.736759
D	Controls	1	8.720535
D	Controls	1	8.686149
D	Controls	1	8.770182
D	Controls	2	8.741080
D	Controls	2	8.797456
D	Controls	2	8.867092
D	Controls	3	8.868163
D	Controls	3	8.735308
D	Controls	3	8.823118

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Log Density of Control Carriers

Lab	Test Chemical	Replicate	Log Density
E	Controls	1	9.133539
E	Controls	1	9.071882
E	Controls	1	9.086360
E	Controls	2	8.985549
E	Controls	2	8.964645
E	Controls	2	8.982271
E	Controls	3	8.867092
E	Controls	3	8.923338
E	Controls	3	8.966355
E	Controls	4	9.064458
E	Controls	4	9.079181
E	Controls	4	9.009374
E	Controls	5	9.181844
E	Controls	5	8.991226
E	Controls	5	9.017033
E	Controls	6	8.898127
E	Controls	6	9.019305
E	Controls	6	9.064458
F	Controls	1	9.447158
F	Controls	1	9.437751
F	Controls	1	9.521138
F	Controls	2	9.390935
F	Controls	2	9.732394
F	Controls	2	9.732394
F	Controls	3	9.414973
F	Controls	3	9.414973
F	Controls	3	9.462398
F	Controls	4	9.424882
F	Controls	4	9.387390
F	Controls	4	9.431364
G	Controls	1	9.230449
G	Controls	1	9.113943
G	Controls	1	9.292256
G	Controls	2	9.025306
G	Controls	2	8.832509
G	Controls	2	8.845098
G	Controls	3	9.365488
G	Controls	3	9.007049
G	Controls	3	8.835979

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Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
A	200 ppm pH-unadjusted NaOCl	1	6.408240
A	200 ppm pH-unadjusted NaOCl	1	6.866878
A	200 ppm pH-unadjusted NaOCl	1	7.079181
A	200 ppm pH-unadjusted NaOCl	2	7.079181
A	200 ppm pH-unadjusted NaOCl	2	7.079181
A	200 ppm pH-unadjusted NaOCl	2	6.063094
A	200 ppm pH-unadjusted NaOCl	2	5.363441
A	200 ppm pH-unadjusted NaOCl	2	7.031004
A	200 ppm pH-unadjusted NaOCl	3	7.079181
A	200 ppm pH-unadjusted NaOCl	3	7.079181
A	200 ppm pH-unadjusted NaOCl	3	7.079181
A	200 ppm pH-unadjusted NaOCl	3	3.917649
A	200 ppm pH-unadjusted NaOCl	3	5.871361
A	5,000 ppm pH-adjusted NaOCl	1	0.301030
A	5,000 ppm pH-adjusted NaOCl	1	0.301030
A	5,000 ppm pH-adjusted NaOCl	1	0.301030
A	5,000 ppm pH-adjusted NaOCl	1	0.301030
A	5,000 ppm pH-adjusted NaOCl	1	0.301030
A	5,000 ppm pH-adjusted NaOCl	2	0.301030
A	5,000 ppm pH-adjusted NaOCl	2	0.301030
A	5,000 ppm pH-adjusted NaOCl	2	0.301030
A	5,000 ppm pH-adjusted NaOCl	2	0.301030
A	5,000 ppm pH-adjusted NaOCl	2	0.301030
A	5,000 ppm pH-adjusted NaOCl	3	0.301030
A	5,000 ppm pH-adjusted NaOCl	3	0.301030
A	5,000 ppm pH-adjusted NaOCl	3	0.301030
A	5,000 ppm pH-adjusted NaOCl	3	0.301030
A	5,000 ppm pH-adjusted NaOCl	3	0.301030

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Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
A	Quat - 1:15	1	5.918602
A	Quat - 1:15	1	6.979548
A	Quat - 1:15	1	6.997386
A	Quat - 1:15	1	5.320335
A	Quat - 1:15	1	5.104735
A	Quat - 1:15	2	6.012454
A	Quat - 1:15	2	3.566062
A	Quat - 1:15	2	7.079181
A	Quat - 1:15	2	4.903090
A	Quat - 1:15	2	7.079181
A	Quat - 1:15	3	6.023065
A	Quat - 1:15	3	6.014750
A	Quat - 1:15	3	6.558709
A	Quat - 1:15	3	3.088941
A	Quat - 1:15	3	5.134699
A	Quat - RTU	1	0.301030
A	Quat - RTU	1	0.301030
A	Quat - RTU	1	0.301030
A	Quat - RTU	1	0.301030
A	Quat - RTU	1	0.301030
A	Quat - RTU	2	0.301030
A	Quat - RTU	2	0.301030
A	Quat - RTU	2	0.301030
A	Quat - RTU	2	0.301030
A	Quat - RTU	2	0.301030
A	Quat - RTU	3	0.301030
A	Quat - RTU	3	0.301030
A	Quat - RTU	3	0.301030
A	Quat - RTU	3	0.301030
A	Quat - RTU	3	0.301030

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Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
B	200 ppm pH-unadjusted NaOCl	1	1.356547
B	200 ppm pH-unadjusted NaOCl	1	1.356547
B	200 ppm pH-unadjusted NaOCl	1	5.906985
B	200 ppm pH-unadjusted NaOCl	1	5.584087
B	200 ppm pH-unadjusted NaOCl	1	2.611820
B	200 ppm pH-unadjusted NaOCl	2	0.866461
B	200 ppm pH-unadjusted NaOCl	2	2.622336
B	200 ppm pH-unadjusted NaOCl	2	1.042552
B	200 ppm pH-unadjusted NaOCl	2	2.343582
B	200 ppm pH-unadjusted NaOCl	2	1.410529
B	200 ppm pH-unadjusted NaOCl	3	5.441481
B	200 ppm pH-unadjusted NaOCl	3	2.833669
B	200 ppm pH-unadjusted NaOCl	3	7.006038
B	200 ppm pH-unadjusted NaOCl	3	3.072551
B	200 ppm pH-unadjusted NaOCl	3	4.791116
B	5,000 ppm pH-adjusted NaOCl	1	0.301030
B	5,000 ppm pH-adjusted NaOCl	1	0.301030
B	5,000 ppm pH-adjusted NaOCl	1	0.301030
B	5,000 ppm pH-adjusted NaOCl	1	2.301030
B	5,000 ppm pH-adjusted NaOCl	1	2.447158
B	5,000 ppm pH-adjusted NaOCl	2	0.301030
B	5,000 ppm pH-adjusted NaOCl	2	0.301030
B	5,000 ppm pH-adjusted NaOCl	2	0.301030
B	5,000 ppm pH-adjusted NaOCl	2	0.301030
B	5,000 ppm pH-adjusted NaOCl	2	0.301030
B	5,000 ppm pH-adjusted NaOCl	3	0.301030
B	5,000 ppm pH-adjusted NaOCl	3	0.602060
B	5,000 ppm pH-adjusted NaOCl	3	0.301030
B	5,000 ppm pH-adjusted NaOCl	3	0.301030
B	5,000 ppm pH-adjusted NaOCl	3	0.301030

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Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
B	Quat - 1:15	1	4.617435
B	Quat - 1:15	1	6.694206
B	Quat - 1:15	1	3.544068
B	Quat - 1:15	1	5.446039
B	Quat - 1:15	1	5.843418
B	Quat - 1:15	2	0.301030
B	Quat - 1:15	2	2.695765
B	Quat - 1:15	2	5.399516
B	Quat - 1:15	2	3.733124
B	Quat - 1:15	2	3.119975
B	Quat - 1:15	3	5.360008
B	Quat - 1:15	3	6.760422
B	Quat - 1:15	3	4.893106
B	Quat - 1:15	3	5.386742
B	Quat - 1:15	3	4.975641
B	Quat - RTU	1	2.903090
B	Quat - RTU	1	2.903090
B	Quat - RTU	1	0.301030
B	Quat - RTU	1	0.301030
B	Quat - RTU	1	0.301030
B	Quat - RTU	2	0.301030
B	Quat - RTU	2	0.301030
B	Quat - RTU	2	0.301030
B	Quat - RTU	2	0.301030
B	Quat - RTU	2	0.301030
B	Quat - RTU	3	0.301030
B	Quat - RTU	3	0.301030
B	Quat - RTU	3	0.301030
B	Quat - RTU	3	0.301030
B	Quat - RTU	3	0.301030

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Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
C	200 ppm pH-unadjusted NaOCl	1	0.565431
C	200 ppm pH-unadjusted NaOCl	1	0.301030
C	200 ppm pH-unadjusted NaOCl	1	0.301030
C	200 ppm pH-unadjusted NaOCl	1	0.301030
C	200 ppm pH-unadjusted NaOCl	1	0.301030
C	5,000 ppm pH-adjusted NaOCl	1	0.301030
C	5,000 ppm pH-adjusted NaOCl	1	0.301030
C	5,000 ppm pH-adjusted NaOCl	1	0.301030
C	5,000 ppm pH-adjusted NaOCl	1	0.301030
C	5,000 ppm pH-adjusted NaOCl	1	0.301030
C	5,000 ppm pH-adjusted NaOCl	2	0.301030
C	5,000 ppm pH-adjusted NaOCl	2	0.301030
C	5,000 ppm pH-adjusted NaOCl	2	0.301030
C	5,000 ppm pH-adjusted NaOCl	2	0.301030
C	5,000 ppm pH-adjusted NaOCl	2	0.301030
C	5,000 ppm pH-adjusted NaOCl	3	0.301030
C	5,000 ppm pH-adjusted NaOCl	3	0.301030
C	5,000 ppm pH-adjusted NaOCl	3	0.301030
C	5,000 ppm pH-adjusted NaOCl	3	0.301030
C	5,000 ppm pH-adjusted NaOCl	3	0.301030

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Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
C	Quat - 1:15	1	6.434569
C	Quat - 1:15	1	5.884950
C	Quat - 1:15	1	5.250863
C	Quat - 1:15	1	3.907997
C	Quat - 1:15	1	4.791116
C	Quat - 1:15	2	5.122960
C	Quat - 1:15	2	5.948057
C	Quat - 1:15	2	6.544068
C	Quat - 1:15	2	5.813520
C	Quat - 1:15	2	5.396358
C	Quat - 1:15	3	4.490086
C	Quat - 1:15	3	3.018749
C	Quat - 1:15	3	6.065138
C	Quat - 1:15	3	1.565431
C	Quat - 1:15	3	3.812913
C	Quat - RTU	1	0.301030
C	Quat - RTU	1	0.301030
C	Quat - RTU	1	0.301030
C	Quat - RTU	1	0.301030
C	Quat - RTU	1	2.903090
C	Quat - RTU	2	0.301030
C	Quat - RTU	2	0.301030
C	Quat - RTU	2	0.301030
C	Quat - RTU	2	0.301030
C	Quat - RTU	2	0.301030
C	Quat - RTU	3	0.301030
C	Quat - RTU	3	0.301030
C	Quat - RTU	3	0.301030
C	Quat - RTU	3	0.301030
C	Quat - RTU	3	0.301030

2015 Single Tube Method Collaborative Data Compilation

Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
D	200 ppm pH-unadjusted NaOCl	1	1.042552
D	200 ppm pH-unadjusted NaOCl	1	0.301030
D	200 ppm pH-unadjusted NaOCl	1	1.844185
D	200 ppm pH-unadjusted NaOCl	1	1.606824
D	200 ppm pH-unadjusted NaOCl	1	2.246672
D	200 ppm pH-unadjusted NaOCl	2	4.803705
D	200 ppm pH-unadjusted NaOCl	2	4.602060
D	200 ppm pH-unadjusted NaOCl	2	4.560667
D	200 ppm pH-unadjusted NaOCl	2	1.866461
D	200 ppm pH-unadjusted NaOCl	2	0.301030
D	200 ppm pH-unadjusted NaOCl	3	1.980404
D	200 ppm pH-unadjusted NaOCl	3	0.301030
D	200 ppm pH-unadjusted NaOCl	3	0.301030
D	200 ppm pH-unadjusted NaOCl	3	2.321306
D	200 ppm pH-unadjusted NaOCl	3	0.301030
D	5,000 ppm pH-adjusted NaOCl	1	0.301030
D	5,000 ppm pH-adjusted NaOCl	1	0.301030
D	5,000 ppm pH-adjusted NaOCl	1	0.301030
D	5,000 ppm pH-adjusted NaOCl	1	0.301030
D	5,000 ppm pH-adjusted NaOCl	1	0.301030
D	5,000 ppm pH-adjusted NaOCl	2	0.301030
D	5,000 ppm pH-adjusted NaOCl	2	0.301030
D	5,000 ppm pH-adjusted NaOCl	2	0.301030
D	5,000 ppm pH-adjusted NaOCl	2	0.301030
D	5,000 ppm pH-adjusted NaOCl	2	0.301030
D	5,000 ppm pH-adjusted NaOCl	3	0.301030
D	5,000 ppm pH-adjusted NaOCl	3	0.301030
D	5,000 ppm pH-adjusted NaOCl	3	0.301030
D	5,000 ppm pH-adjusted NaOCl	3	0.301030
D	5,000 ppm pH-adjusted NaOCl	3	0.301030

2015 Single Tube Method Collaborative Data Compilation

Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
D	Quat - 1:15	1	5.677939
D	Quat - 1:15	1	6.278754
D	Quat - 1:15	1	2.228189
D	Quat - 1:15	1	1.167491
D	Quat - 1:15	1	0.301030
D	Quat - 1:15	2	1.958607
D	Quat - 1:15	2	2.888026
D	Quat - 1:15	2	1.741522
D	Quat - 1:15	2	2.629889
D	Quat - 1:15	2	1.958607
D	Quat - 1:15	3	0.301030
D	Quat - 1:15	3	0.301030
D	Quat - 1:15	3	2.782915
D	Quat - 1:15	3	2.504950
D	Quat - 1:15	3	1.410529
D	Quat - RTU	1	0.301030
D	Quat - RTU	1	0.301030
D	Quat - RTU	1	0.301030
D	Quat - RTU	1	0.301030
D	Quat - RTU	1	2.584331
D	Quat - RTU	2	0.301030
D	Quat - RTU	2	0.301030
D	Quat - RTU	2	0.301030
D	Quat - RTU	2	0.301030
D	Quat - RTU	2	0.301030
D	Quat - RTU	3	0.301030
D	Quat - RTU	3	1.556303
D	Quat - RTU	3	0.301030
D	Quat - RTU	3	0.301030
D	Quat - RTU	3	1.716003

2015 Single Tube Method Collaborative Data Compilation

Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
E	200 ppm pH-unadjusted NaOCl	1	6.289021
E	200 ppm pH-unadjusted NaOCl	1	3.397940
E	200 ppm pH-unadjusted NaOCl	1	4.702865
E	200 ppm pH-unadjusted NaOCl	1	6.719030
E	200 ppm pH-unadjusted NaOCl	1	5.760857
E	200 ppm pH-unadjusted NaOCl	2	1.167491
E	200 ppm pH-unadjusted NaOCl	2	1.167491
E	200 ppm pH-unadjusted NaOCl	2	6.434569
E	200 ppm pH-unadjusted NaOCl	2	5.052029
E	200 ppm pH-unadjusted NaOCl	2	5.679593
E	200 ppm pH-unadjusted NaOCl	3	6.301030
E	200 ppm pH-unadjusted NaOCl	3	6.245513
E	200 ppm pH-unadjusted NaOCl	3	5.441481
E	200 ppm pH-unadjusted NaOCl	3	5.402652
E	200 ppm pH-unadjusted NaOCl	3	2.556657
E	5,000 ppm pH-adjusted NaOCl	1	0.301030
E	5,000 ppm pH-adjusted NaOCl	1	0.301030
E	5,000 ppm pH-adjusted NaOCl	1	0.301030
E	5,000 ppm pH-adjusted NaOCl	1	0.301030
E	5,000 ppm pH-adjusted NaOCl	1	0.301030
E	5,000 ppm pH-adjusted NaOCl	2	0.301030
E	5,000 ppm pH-adjusted NaOCl	2	0.301030
E	5,000 ppm pH-adjusted NaOCl	2	0.301030
E	5,000 ppm pH-adjusted NaOCl	2	0.301030
E	5,000 ppm pH-adjusted NaOCl	2	0.301030
E	5,000 ppm pH-adjusted NaOCl	3	0.301030
E	5,000 ppm pH-adjusted NaOCl	3	0.301030
E	5,000 ppm pH-adjusted NaOCl	3	0.301030
E	5,000 ppm pH-adjusted NaOCl	3	0.301030
E	5,000 ppm pH-adjusted NaOCl	3	0.301030

2015 Single Tube Method Collaborative Data Compilation

Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
E	Quat - 1:15	1	5.876638
E	Quat - 1:15	1	5.973128
E	Quat - 1:15	1	4.104735
E	Quat - 1:15	1	7.066699
E	Quat - 1:15	1	6.551450
E	Quat - 1:15	2	6.857332
E	Quat - 1:15	2	6.873902
E	Quat - 1:15	2	7.079181
E	Quat - 1:15	2	7.079181
E	Quat - 1:15	2	6.546543
E	Quat - 1:15	3	6.938520
E	Quat - 1:15	3	7.057666
E	Quat - 1:15	3	6.274158
E	Quat - 1:15	3	6.225309
E	Quat - 1:15	3	7.079181
E	Quat - RTU	1	0.301030
E	Quat - RTU	1	0.301030
E	Quat - RTU	1	0.301030
E	Quat - RTU	1	0.301030
E	Quat - RTU	1	0.301030
E	Quat - RTU	2	0.301030
E	Quat - RTU	2	0.301030
E	Quat - RTU	2	0.301030
E	Quat - RTU	2	0.301030
E	Quat - RTU	2	0.301030
E	Quat - RTU	3	0.301030
E	Quat - RTU	3	0.301030
E	Quat - RTU	3	0.301030
E	Quat - RTU	3	0.301030
E	Quat - RTU	3	0.301030

2015 Single Tube Method Collaborative Data Compilation

Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
F	200 ppm pH-unadjusted NaOCl	1	5.935416
F	200 ppm pH-unadjusted NaOCl	1	6.763428
F	200 ppm pH-unadjusted NaOCl	1	6.499687
F	200 ppm pH-unadjusted NaOCl	1	6.235528
F	200 ppm pH-unadjusted NaOCl	1	5.052029
F	200 ppm pH-unadjusted NaOCl	2	6.633468
F	200 ppm pH-unadjusted NaOCl	2	6.748188
F	200 ppm pH-unadjusted NaOCl	2	6.579784
F	200 ppm pH-unadjusted NaOCl	2	6.491362
F	200 ppm pH-unadjusted NaOCl	2	6.556303
F	200 ppm pH-unadjusted NaOCl	3	4.722035
F	200 ppm pH-unadjusted NaOCl	3	6.072551
F	200 ppm pH-unadjusted NaOCl	3	4.949833
F	200 ppm pH-unadjusted NaOCl	3	3.861697
F	200 ppm pH-unadjusted NaOCl	3	3.912850
F	5,000 ppm pH-adjusted NaOCl	1	0.301030
F	5,000 ppm pH-adjusted NaOCl	1	0.301030
F	5,000 ppm pH-adjusted NaOCl	1	0.301030
F	5,000 ppm pH-adjusted NaOCl	1	0.301030
F	5,000 ppm pH-adjusted NaOCl	1	0.301030
F	5,000 ppm pH-adjusted NaOCl	2	0.301030
F	5,000 ppm pH-adjusted NaOCl	2	0.301030
F	5,000 ppm pH-adjusted NaOCl	2	0.301030
F	5,000 ppm pH-adjusted NaOCl	2	0.301030
F	5,000 ppm pH-adjusted NaOCl	2	0.301030
F	5,000 ppm pH-adjusted NaOCl	3	0.301030
F	5,000 ppm pH-adjusted NaOCl	3	0.301030
F	5,000 ppm pH-adjusted NaOCl	3	0.301030
F	5,000 ppm pH-adjusted NaOCl	3	0.301030
F	5,000 ppm pH-adjusted NaOCl	3	0.301030

2015 Single Tube Method Collaborative Data Compilation

Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
F	Quat - 1:15	1	5.621365
F	Quat - 1:15	1	5.507611
F	Quat - 1:15	1	7.079181
F	Quat - 1:15	1	5.984732
F	Quat - 1:15	1	7.079181
F	Quat - 1:15	2	6.857332
F	Quat - 1:15	2	6.869232
F	Quat - 1:15	2	7.079181
F	Quat - 1:15	2	7.079181
F	Quat - 1:15	2	7.079181
F	Quat - 1:15	3	4.750999
F	Quat - 1:15	3	6.447158
F	Quat - 1:15	3	5.052029
F	Quat - 1:15	3	5.592076
F	Quat - 1:15	3	6.556303
F	Quat - RTU	1	0.301030
F	Quat - RTU	1	0.301030
F	Quat - RTU	1	0.301030
F	Quat - RTU	1	0.301030
F	Quat - RTU	1	0.301030
F	Quat - RTU	2	0.301030
F	Quat - RTU	2	0.301030
F	Quat - RTU	2	0.301030
F	Quat - RTU	2	0.301030
F	Quat - RTU	2	0.301030
F	Quat - RTU	3	0.301030
F	Quat - RTU	3	0.301030
F	Quat - RTU	3	0.301030
F	Quat - RTU	3	0.301030
F	Quat - RTU	3	0.301030

2015 Single Tube Method Collaborative Data Compilation

Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
G	200 ppm pH-unadjusted NaOCl	1	6.357935
G	200 ppm pH-unadjusted NaOCl	1	2.711559
G	200 ppm pH-unadjusted NaOCl	1	2.741522
G	200 ppm pH-unadjusted NaOCl	1	6.015512
G	200 ppm pH-unadjusted NaOCl	1	2.979797
G	200 ppm pH-unadjusted NaOCl	2	3.576655
G	200 ppm pH-unadjusted NaOCl	2	4.861697
G	200 ppm pH-unadjusted NaOCl	2	0.866461
G	200 ppm pH-unadjusted NaOCl	2	1.042552
G	200 ppm pH-unadjusted NaOCl	2	5.714482
G	200 ppm pH-unadjusted NaOCl	3	0.301030
G	200 ppm pH-unadjusted NaOCl	3	0.301030
G	200 ppm pH-unadjusted NaOCl	3	0.301030
G	200 ppm pH-unadjusted NaOCl	3	0.301030
G	200 ppm pH-unadjusted NaOCl	3	0.301030
G	5,000 ppm pH-adjusted NaOCl	1	0.602060
G	5,000 ppm pH-adjusted NaOCl	1	0.301030
G	5,000 ppm pH-adjusted NaOCl	1	0.301030
G	5,000 ppm pH-adjusted NaOCl	1	0.301030
G	5,000 ppm pH-adjusted NaOCl	1	0.301030
G	5,000 ppm pH-adjusted NaOCl	2	0.301030
G	5,000 ppm pH-adjusted NaOCl	2	0.301030
G	5,000 ppm pH-adjusted NaOCl	2	0.301030
G	5,000 ppm pH-adjusted NaOCl	2	0.301030
G	5,000 ppm pH-adjusted NaOCl	2	0.301030
G	5,000 ppm pH-adjusted NaOCl	3	0.301030
G	5,000 ppm pH-adjusted NaOCl	3	0.301030
G	5,000 ppm pH-adjusted NaOCl	3	0.301030
G	5,000 ppm pH-adjusted NaOCl	3	0.301030
G	5,000 ppm pH-adjusted NaOCl	3	0.301030

2015 Single Tube Method Collaborative Data Compilation

Log Density of Treated Carriers

Lab	Test Chemical	Replicate	Log Density
G	Quat - 1:15	1	2.800960
G	Quat - 1:15	1	2.738617
G	Quat - 1:15	1	2.810944
G	Quat - 1:15	1	2.672641
G	Quat - 1:15	1	2.735693
G	Quat - 1:15	2	6.824776
G	Quat - 1:15	2	6.309630
G	Quat - 1:15	2	6.779596
G	Quat - 1:15	2	3.815940
G	Quat - 1:15	2	2.255627
G	Quat - 1:15	3	5.799967
G	Quat - 1:15	3	5.719030
G	Quat - 1:15	3	5.957738
G	Quat - 1:15	3	4.872421
G	Quat - 1:15	3	0.301030
G	Quat - RTU	1	2.414973
G	Quat - RTU	1	2.606381
G	Quat - RTU	1	2.453318
G	Quat - RTU	1	2.556303
G	Quat - RTU	1	2.447158
G	Quat - RTU	2	0.301030
G	Quat - RTU	2	0.301030
G	Quat - RTU	2	0.301030
G	Quat - RTU	2	0.301030
G	Quat - RTU	2	0.301030
G	Quat - RTU	3	0.301030
G	Quat - RTU	3	0.301030
G	Quat - RTU	3	0.301030
G	Quat - RTU	3	0.301030
G	Quat - RTU	3	0.301030

2015 Single Tube Method Collaborative Data Compilation

Log Reduction for Treated Carriers

Lab	Test Chemical	Replicate	Log Reduction
A	200 ppm pH-unadjusted NaOCl	1	2.271102
A	200 ppm pH-unadjusted NaOCl	2	2.492240
A	200 ppm pH-unadjusted NaOCl	3	3.150234
A	5,000 ppm pH-adjusted NaOCl	1	8.754839
A	5,000 ppm pH-adjusted NaOCl	2	8.714391
A	5,000 ppm pH-adjusted NaOCl	3	9.054514
A	Quat - 1:15	1	2.991747
A	Quat - 1:15	2	3.287427
A	Quat - 1:15	3	3.991512
A	Quat - RTU	1	8.754839
A	Quat - RTU	2	8.714391
A	Quat - RTU	3	9.054514
B	200 ppm pH-unadjusted NaOCl	1	5.284959
B	200 ppm pH-unadjusted NaOCl	2	7.083850
B	200 ppm pH-unadjusted NaOCl	3	4.208326
B	5,000 ppm pH-adjusted NaOCl	1	7.517901
B	5,000 ppm pH-adjusted NaOCl	2	8.439913
B	5,000 ppm pH-adjusted NaOCl	3	8.476061
B	Quat - 1:15	1	3.419123
B	Quat - 1:15	2	5.691060
B	Quat - 1:15	3	3.362113
B	Quat - RTU	1	7.306303
B	Quat - RTU	2	8.439913
B	Quat - RTU	3	8.536267
C	200 ppm pH-unadjusted NaOCl	1	8.634480
C	5,000 ppm pH-adjusted NaOCl	1	8.687360
C	5,000 ppm pH-adjusted NaOCl	2	8.876072
C	5,000 ppm pH-adjusted NaOCl	3	8.395383
C	Quat - 1:15	1	3.734491
C	Quat - 1:15	2	3.412109
C	Quat - 1:15	3	4.905949
C	Quat - RTU	1	8.166948
C	Quat - RTU	2	8.876072
C	Quat - RTU	3	8.395383

2015 Single Tube Method Collaborative Data Compilation

Log Reduction for Treated Carriers

Lab	Test Chemical	Replicate	Log Reduction
D	200 ppm pH-unadjusted NaOCl	1	7.317369
D	200 ppm pH-unadjusted NaOCl	2	5.575091
D	200 ppm pH-unadjusted NaOCl	3	7.767903
D	5,000 ppm pH-adjusted NaOCl	1	8.424592
D	5,000 ppm pH-adjusted NaOCl	2	8.500846
D	5,000 ppm pH-adjusted NaOCl	3	8.507833
D	Quat - 1:15	1	5.594942
D	Quat - 1:15	2	6.566546
D	Quat - 1:15	3	7.348773
D	Quat - RTU	1	7.967932
D	Quat - RTU	2	8.500846
D	Quat - RTU	3	7.973784
E	200 ppm pH-unadjusted NaOCl	1	3.704759
E	200 ppm pH-unadjusted NaOCl	2	5.018694
E	200 ppm pH-unadjusted NaOCl	3	3.893701
E	5,000 ppm pH-adjusted NaOCl	1	8.777672
E	5,000 ppm pH-adjusted NaOCl	2	8.617899
E	5,000 ppm pH-adjusted NaOCl	3	8.782138
E	Quat - 1:15	1	3.062959
E	Quat - 1:15	2	2.135410
E	Quat - 1:15	3	2.282139
E	Quat - RTU	1	8.676458
E	Quat - RTU	2	8.721608
E	Quat - RTU	3	8.696076
F	200 ppm pH-unadjusted NaOCl	1	3.371465
F	200 ppm pH-unadjusted NaOCl	2	3.016753
F	200 ppm pH-unadjusted NaOCl	3	4.726988
F	5,000 ppm pH-adjusted NaOCl	1	9.167652
F	5,000 ppm pH-adjusted NaOCl	2	9.317544
F	5,000 ppm pH-adjusted NaOCl	3	9.129752
F	Quat - 1:15	1	3.364160
F	Quat - 1:15	2	2.437960
F	Quat - 1:15	3	3.734832
F	Quat - RTU	1	9.317544
F	Quat - RTU	2	9.129752
F	Quat - RTU	3	9.113515

2015 Single Tube Method Collaborative Data Compilation

Log Reduction for Treated Carriers

Lab	Test Chemical	Replicate	Log Reduction
G	200 ppm pH-unadjusted NaOCl	1	5.050951
G	200 ppm pH-unadjusted NaOCl	2	5.688601
G	200 ppm pH-unadjusted NaOCl	3	8.768475
G	5,000 ppm pH-adjusted NaOCl	1	8.850980
G	5,000 ppm pH-adjusted NaOCl	2	8.599941
G	5,000 ppm pH-adjusted NaOCl	3	8.768475
G	Quat - 1:15	1	6.460445
G	Quat - 1:15	2	3.703857
G	Quat - 1:15	3	4.539468
G	Quat - RTU	1	6.716589
G	Quat - RTU	2	8.599941
G	Quat - RTU	3	8.768475

The 2015 Collaborative Study on the Single Tube Method (with Splashguard) for Evaluating Disinfectant Activity against *Pseudomonas* Biofilm: *Method Performance Assessment*

(Version for docket #EPA-OPP-2016-0357)

Study factors

- 7 labs (encoded as A-G)
- 1 microbe: *P. aeruginosa*
- 4 treatments:

NaOCl at 2 efficacy levels:
200 ppm (low), 5000 ppm pH-adjusted (high)

Quat at 2 efficacy levels:
1:15 (low), ready to use (RTU - high)

Study overview

- The 4 treatments were conducted side-by-side on each test day in each laboratory with the exceptions noted on Slide 5.
- 5 labs each performed 3 tests, 1 lab performed 6 tests, and 1 lab performed 4 tests for a total of 25 independent tests.
- Viable cells were enumerated on 3 untreated control carriers and 5 treated carriers per treatment for each test with the exceptions noted on Slide 5.

Terms

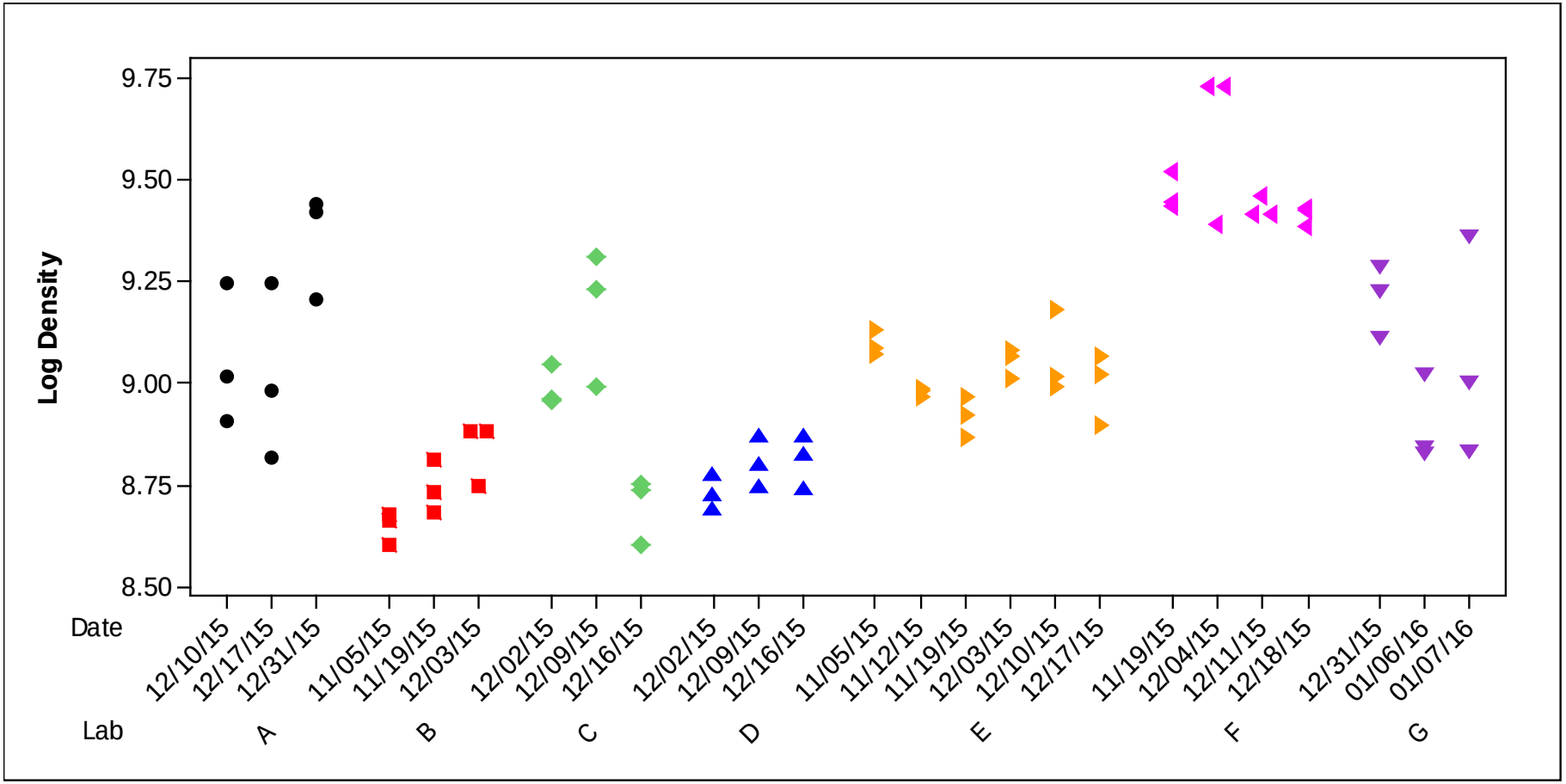
- Control LD: $\log_{10}$(CFU/carrier) for each control carrier.
- Treated LD: $\log_{10}$(CFU/carrier) for each treated carrier.
- *TestLD*: mean of the 3 Control LDs for a single test.
- LR: difference between the *TestLD* and the mean of the 3 Treated LDs enumerated in the same test.
- CS_r and S_r: *repeatability* SD (within-lab) for controls and LRs respectively.
- CS_R and S_R: *reproducibility* SD (among-lab) for controls and LRs respectively.
- CI: *confidence interval*, an interval that contains the true parameter (i.e., mean *TestLD* or mean LR) with a specified level of confidence (e.g., 95%).
- TI: *expectation tolerance interval*, an interval that contains a certain percentage (e.g., 95%) of results from single tests (e.g., LRs) on the average (Mee, *Technometrics*,1984).

Data issues

- Lab A's first test (on 12/10) only included 3 coupons treated with 200 ppm of NaOCl.
- Lab C conducted a single test (on 12/2) using 200 ppm of NaOCl. The other 2 replicate tests of 200ppm NaOCl were excluded from statistical analyses.
- Lab E applied both efficacy levels of NaOCl on 3 test days (11/5, 11/19 and 12/10) then both efficacy levels of Quat on 3 other test days (11/12, 12/3, and 12/17). Thus, Lab E conducted 6 tests.
- Lab F applied both efficacy levels of NaOCl 1 test day (11/19), and both efficacy levels of Quat on a separate test day (12/18). All levels of both chemicals were applied on 2 additional test days (12/4 and 12/11) for a total of 4 test days.
- Outlier detection methods* identified some data as being unusually large or small. These were included in the statistical analyses because they were found to be valid by EPA.

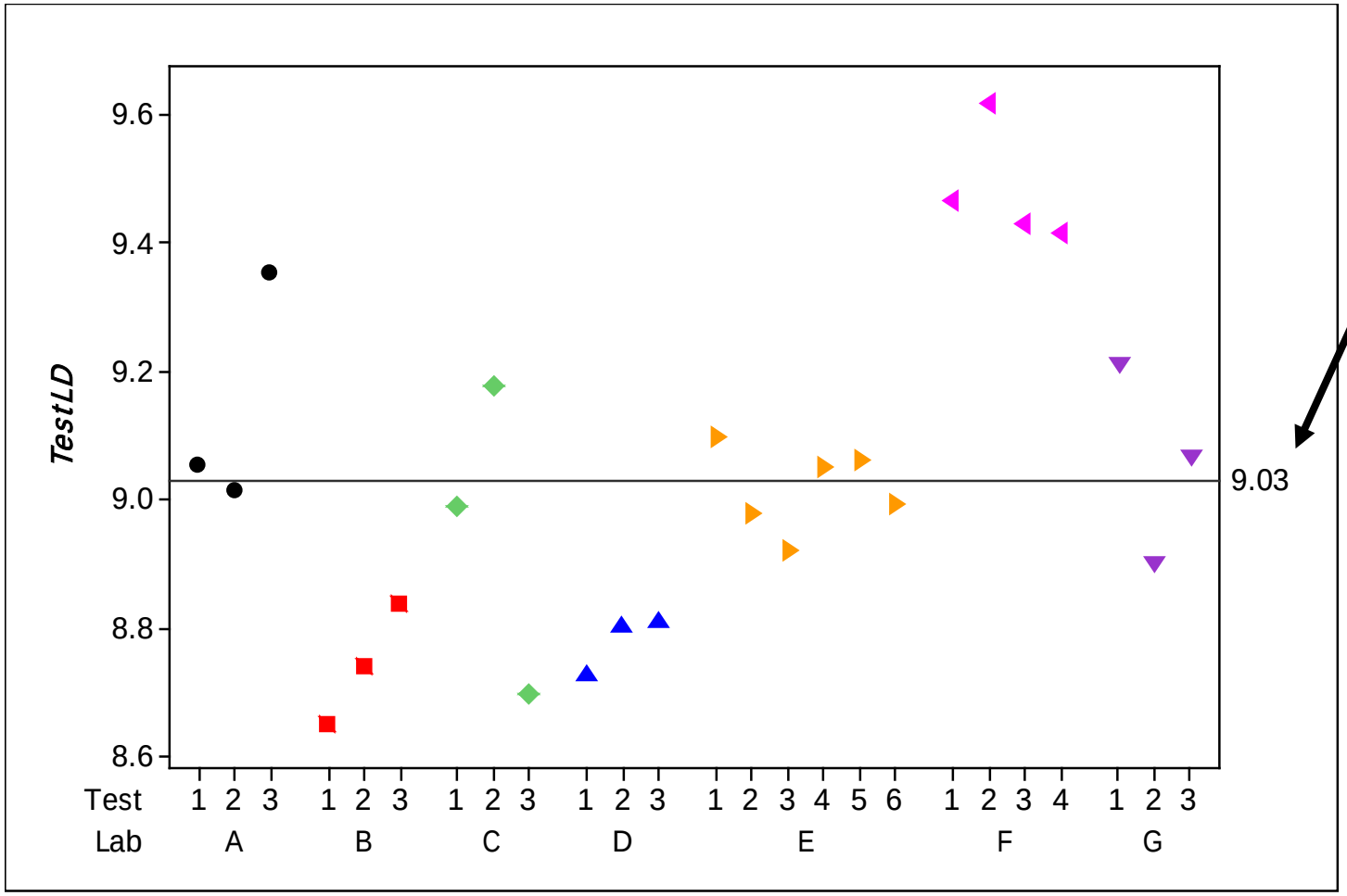
* Outlier detection was conducted via individual value plots, residual versus fits plots, and normal probability plots.

Figure 1. Control LDs – *P. aeruginosa*



Each point is 1 of 3 Control LDs from a single test.

Figure 2. *TestLDs*



Overall Mean

All labs met the requirement that, on each test day, *TestLD* ≥ 8.0

Each point is the *TestLD* = mean of the 3 Control LDs from a single test.

Results for *TestLD*

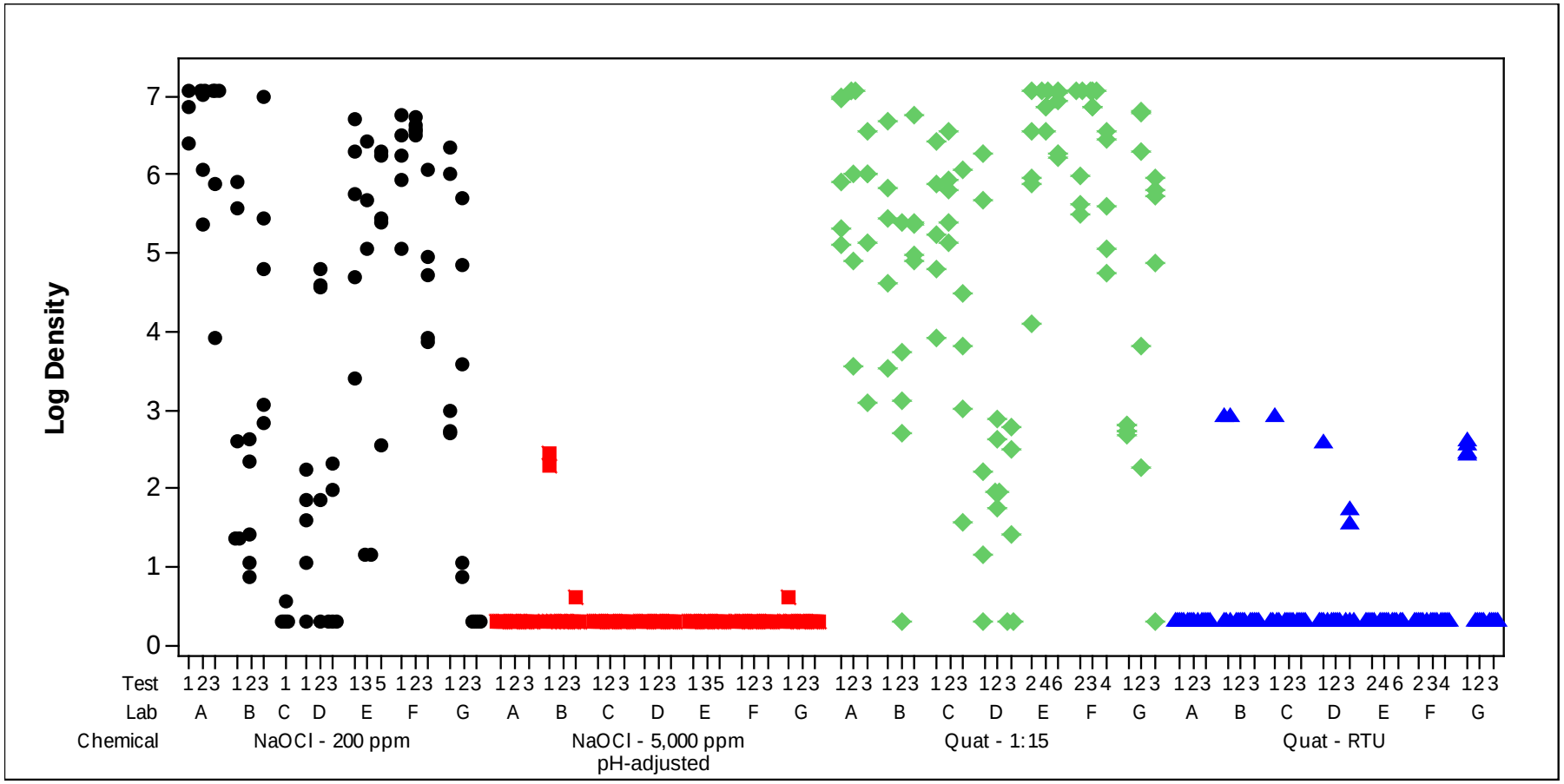
Table 1. Mean of the *TestLDs*

Mean *TestLD*	SEM	95% LCL	95% UCL	Geometric mean
9.03	0.0942	8.80	9.26	1.06×10^9

Table 2. Variability of the *TestLDs*

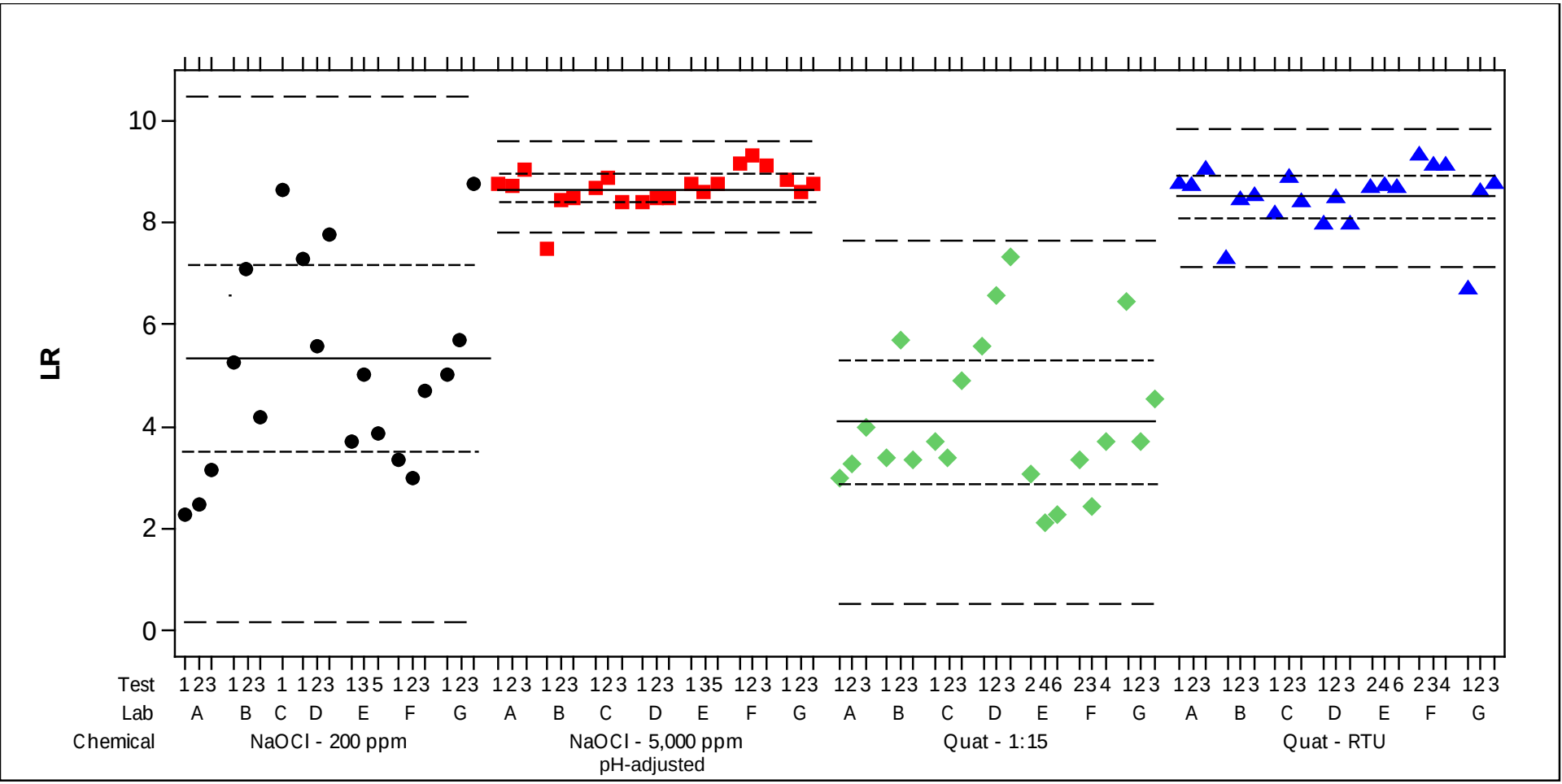
			Variance components			Percentage of total variance	
Mean *TestLD*	CS_r	CS_R	Var_{Lab}	Var_{Test}	$Var_{WithinTest}/3$	Among Lab	Within Lab
9.03	0.1300	0.2721	0.0571	0.0127	0.0042	77%	33%

Figure 3. Treated LDs – *P. aeruginosa*



Each point is 1 of 5 treated LDs from a single test.

Figure 4. LRs – *P. aeruginosa*



- Each point is a log reduction for a single test.
- Solid horizontal lines indicate the mean LR for each treatment.
- Short-dashed lines are 95% CIs for the true mean LR for each treatment.
- Long-dashed lines are 95% TI's for the LR from a single test.

Results for LR (1 of 3)

Table 3. Mean of the LRs

Treatment	Mean LR	SEM	95% CI		95% TI	
			Lower limit	Upper limit	Lower limit	Upper limit
NaOCl 200ppm	5.34	0.7427	3.52	7.16	0.14	10.5
NaOCl 5000ppm, pH adjusted	8.64	0.1230	8.38	8.98	7.80	9.57
Quat 1:15	4.10	0.4956	2.88	5.31	0.55	7.64
Quat RTU	8.50	0.1647	8.09	8.90	7.14	9.85

Table 4. Responsiveness to product efficacy;
LR responsiveness is LR for high conc. – LR for low conc. treatment

Chemical	LR responsiveness	SEM	95% LCL	95% UCL	*p*-value
NaOCl	3.33	0.8115	1.35	5.32	0.0031
Quat	4.40	0.6232	2.88	5.92	0.0002

Table 4 shows that the STM was statistically significantly responsive to the change in chemical concentrations

Results for LR (2 of 3)

Table 5. Variance of the LRs*

Year	Treatment	Mean LR	S_r	S_R	Variance components		Percentage of total variance	
					Var_{Lab}	Var_{Test}	Among Lab	Within Lab
2015	**NaOCl 200ppm**	5.34	1.236	2.182	3.23	1.53	68%	32%
	NaOCl 5000ppm, pH-adjusted	8.64	0.2465	0.3826	0.086	0.0607	59%	41%
	Quat 1:15	4.10	0.4956	1.517	1.43	0.873	62%	38%
	Quat RTU	8.50	0.5396	0.6197	0.093	0.291	24%	76%

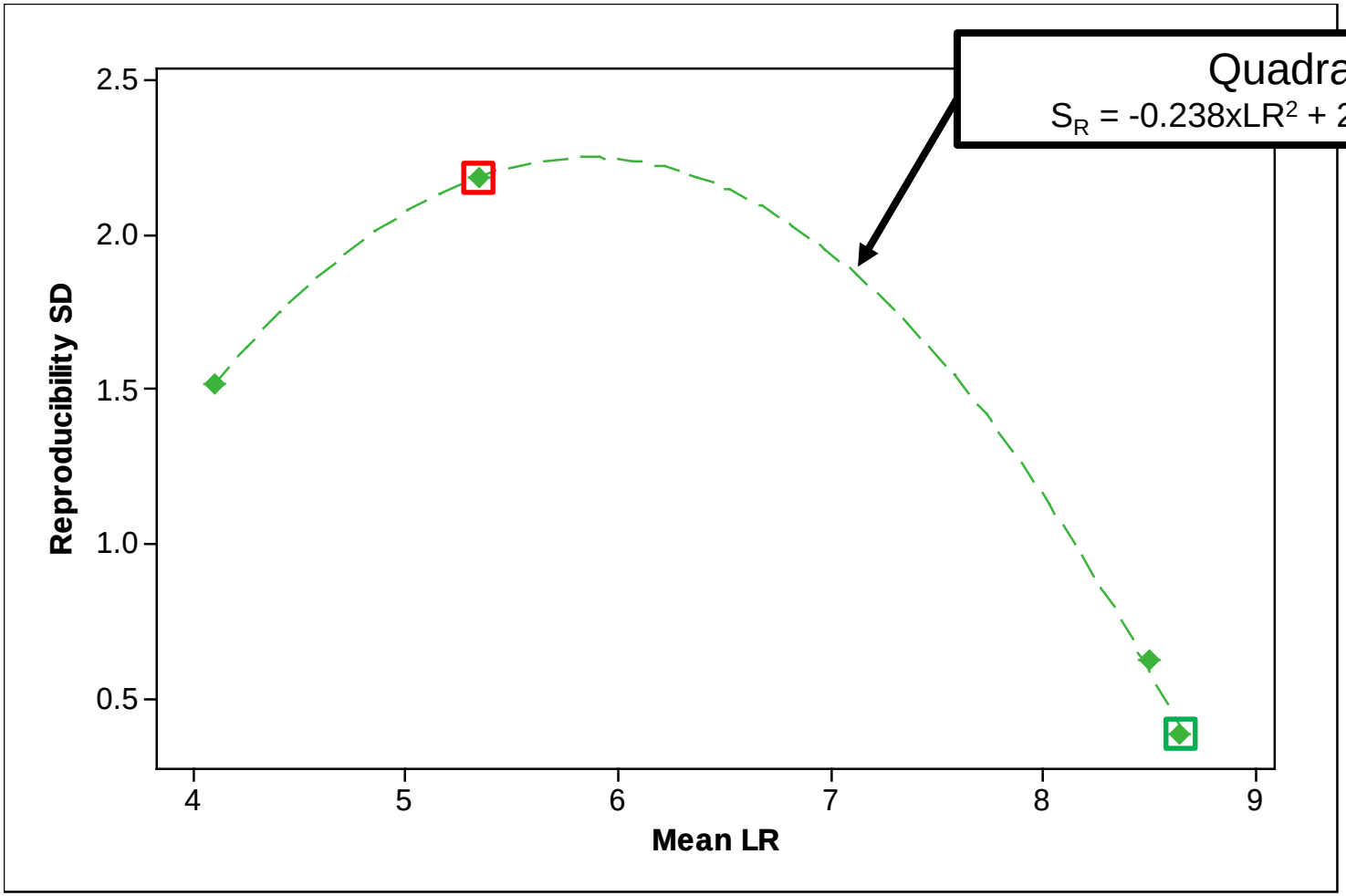
LARGEST VARIABILITY

smallest variability

*The study protocol specifies the statistical methods used to analyze the collaborative study data.

Results for LR (3 of 3)

Figure 5. S_R as a quadratic function of the mean LR for the STM



LARGEST VARIANCE

smallest variance

Conclusions

For control *TestLDs*:

- The mean *TestLD* = 9.03 and the reproducibility SD was CS_R = 0.2721.
- The biggest (77%) component of the variance was due to lab-to-lab sources.

For LR*s*:

- mean LR = 4.10 and reproducibility SD was S_R = 1.52 for Quat 1:15
- mean LR = 5.34 and reproducibility SD was S_R = 2.18 for NaOCl 200ppm
- mean LR = 8.50 and reproducibility SD was S_R = 0.620 for Quat RTU
- mean LR = 8.64 and reproducibility SD was S_R = 0.383 for NaOCl 5000 ppm, pH-adjusted

Responsiveness:

- STM was statistically significantly responsive to the change in concentration for both products tested ($p < 0.003$).

Appendix: Table 6. *P. aeruginosa* data

				LR			
Lab	Date	Test	*TestLD*	NaOCl 200ppm	NaOCl 5000ppm, pH-adjusted	Quat 1:15	Quat RTU
A	12/10/15	1	9.06	2.27	8.75	2.99	8.75
A	12/17/15	2	9.02	2.49	8.71	3.29	8.71
A	12/31/15	3	9.36	3.15	9.05	3.99	9.05
B	11/05/15	1	8.65	5.28	7.52	3.42	7.31
B	11/19/15	2	8.74	7.08	8.44	5.69	8.44
B	12/03/15	3	8.84	4.21	8.48	3.36	8.54
C	12/02/15	1	8.99	8.63	8.69	3.73	8.17
C	12/09/15	2	9.18	--	8.88	3.41	8.88
C	12/16/15	3	8.70	--	8.40	4.91	8.40
D	12/02/15	1	8.73	7.32	8.42	5.59	7.97
D	12/09/15	2	8.80	5.58	8.50	6.57	8.50
D	12/16/15	3	8.81	7.77	8.51	7.35	7.97
E	11/05/15	1	9.10	3.70	8.78	--	--
E	11/12/15	2	8.98	--	--	3.06	8.68
E	11/19/15	3	8.92	5.02	8.62	--	--
E	12/03/15	4	9.05	--	--	2.14	8.72
E	12/10/15	5	9.06	3.89	8.78	--	--
E	12/17/15	6	8.99	--	--	2.28	8.70
F	11/19/15	1	9.47	3.37	9.17	--	--
F	12/04/15	2	9.62	3.02	9.32	3.36	9.32
F	12/11/15	3	9.43	4.73	9.13	2.44	9.13
F	12/18/15	4	9.41	--	--	3.73	9.11
G	12/31/15	1	9.21	5.05	8.85	6.46	6.72
G	01/06/16	2	8.90	5.69	8.60	3.70	8.60
G	01/07/16	3	9.07	8.77	8.77	4.54	8.77

Appendix: Table 7. Summary by Lab

	TestLD		NaOCl 200ppm		NaOCl 5000ppm, pH-adjusted		Quat 1:15		Quat RTU	
Lab	Mean	CS_r	Mean LR	S_r	Mean LR	S_r	Mean LR	S_r	Mean LR	S_r
A	9.14	0.186	2.64	0.46	8.84	0.19	3.42	0.51	8.84	0.19
B	8.74	0.095	5.53	1.45	8.14	0.54	4.16	1.33	8.09	0.68
C	8.95	0.242	8.63	*	8.65	0.24	4.02	0.79	8.48	0.36
D	8.78	0.046	6.89	1.16	8.48	0.05	6.50	0.88	8.15	0.31
E	9.02	0.066	4.21	0.71	8.73	0.09	2.49	0.50	8.70	0.02
F	9.48	0.093	3.71	0.90	9.20	0.10	3.18	0.67	9.19	0.11
G	9.06	0.156	6.50	1.99	8.74	0.13	4.90	1.41	8.03	1.14

* Since only a single validated test for 200ppm NaOCl was conducted at lab C, it was not possible to calculate a repeatability SD.

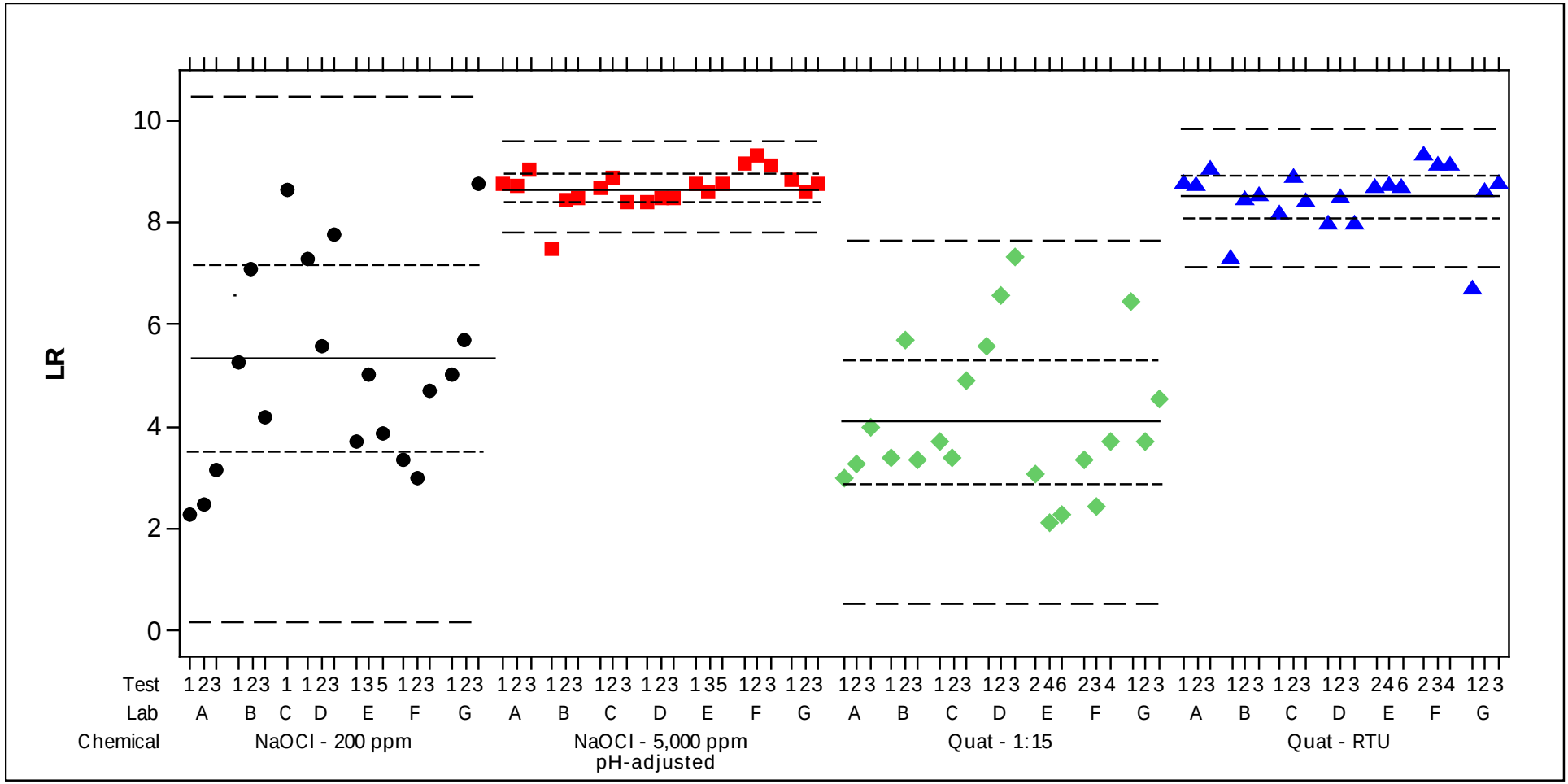
The 2015 Collaborative Study on the Single Tube Method (with Splashguard) for Evaluating Disinfectant Activity against *Pseudomonas* Biofilm: *Testing Criteria*

(Version for docket #EPA-OPP-2016-0357)

Terms:

- LR: difference between the *TestLD* and the mean of the 5 treated LDs enumerated in the same test
- S_r: *repeatability* SD (within-lab) for LRs
- S_R: *reproducibility* SD (among-lab) for LRs
- PS: *performance standard*
- ineffective products: have mean LR ≤ $LR_{ineffective}$ and SD = $SD_{ineffective}$ where $LR_{ineffective} = 5$ and $SD_{ineffective}$ is calculated from the low efficacy treatments in the recent collaborative study.
- pass-error percentage: the percentage of ineffective products that will incorrectly pass the PS
- highly effective products: have mean LR ≥ LR_{high} and SD = SD_{high}. Two cases are considered: (A) $LR_{high} = 7$ and SD_{high} is predicted by a quadratic model; (B) $LR_{high} = 8$ and SD_{high} is calculated from the high efficacy treatments in the recent collaborative study
- fail-error percentage: the percentage of highly effective products that will incorrectly fail the PS

Figure 1. LRs – *P. aeruginosa*



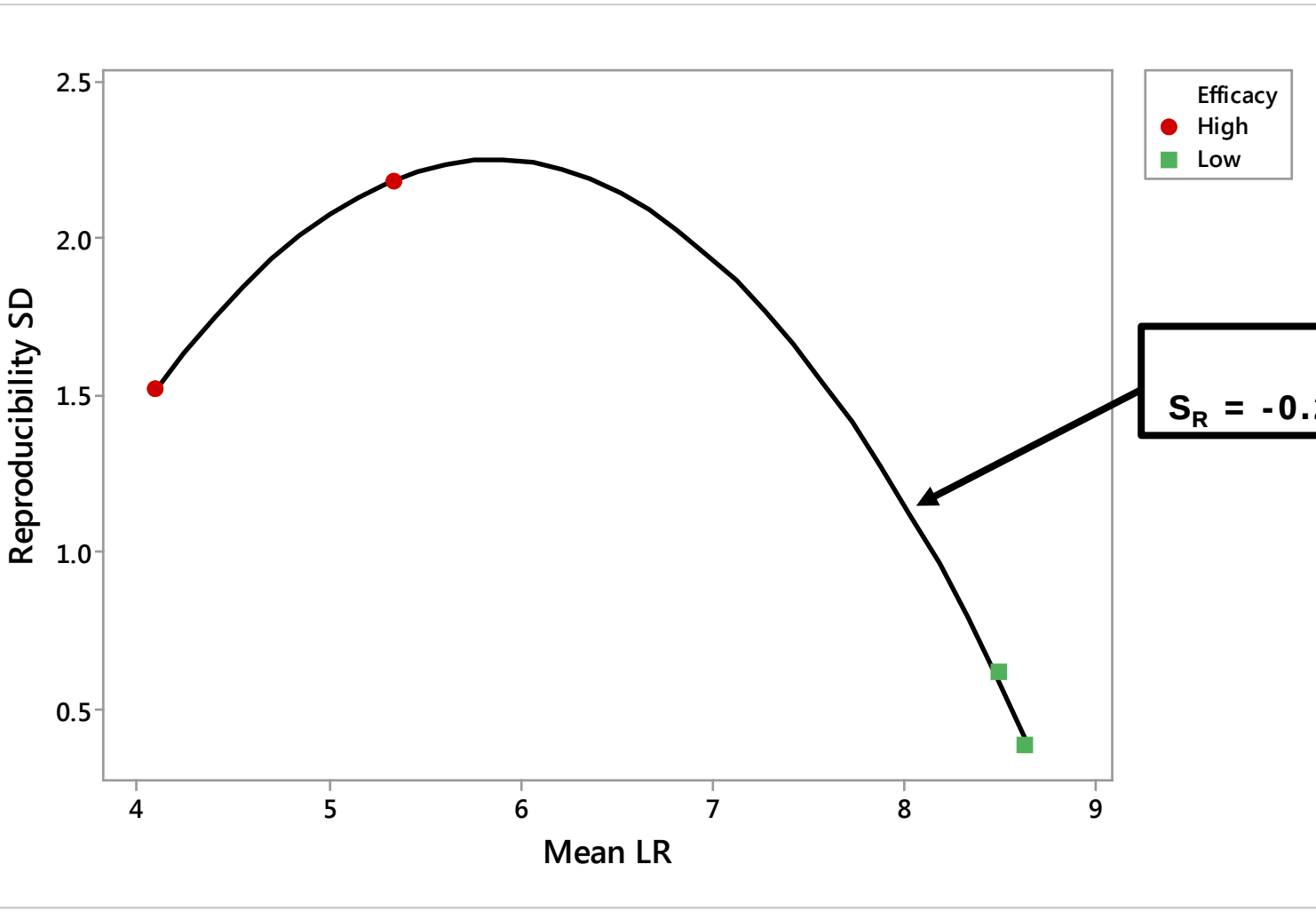
- Each point is a log reduction for a single test.
- Solid horizontal lines indicate the mean LR for each treatment.
- Short-dashed lines are 95% CIs for the true mean LR for each treatment.
- Long-dashed lines are 95% TI's for the LR from a single test.

Table 1. Variance of the LRs

Year	Treatment	Mean LR	S_r	S_R	Variance components		Percentage of total variance	
					Var_{Lab}	Var_{Test}	Among Lab	Within Lab
2015	**NaOCl 200ppm**	5.34	1.236	2.182	3.23	1.53	68%	32%
	NaOCl 5000ppm, pH-adjusted	8.64	0.2465	0.3826	0.086	0.0607	59%	41%
	Quat 1:15	4.10	0.4956	1.517	1.43	0.873	62%	38%
	Quat RTU	8.50	0.5396	0.6197	0.093	0.291	24%	76%

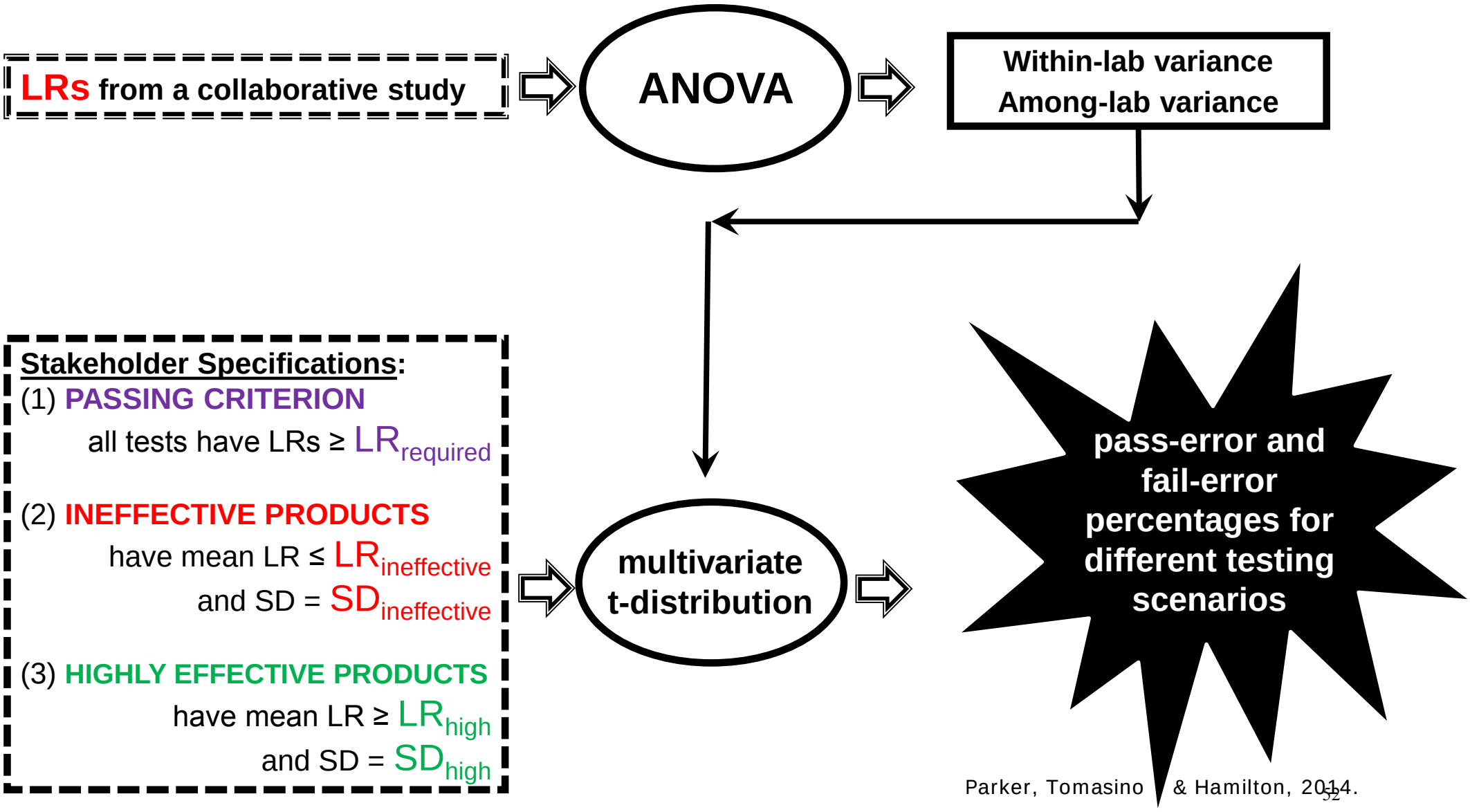
Used for PS calculations

Figure 2. Reproducibility vs. mean LR



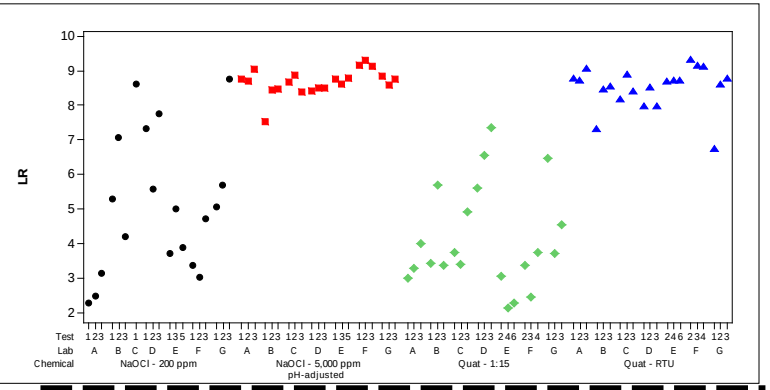
Each point is a reproducibility SD for a single product.

A statistical tool to assess performance standards:



Parker, Tomasino & Hamilton, 2014.

A statistical tool to assess performance standards:



Stakeholder Specifications:

(1) **PASSING CRITERION**

all tests have LRs ≥ 6.0

(2) **INEFFECTIVE PRODUCTS**

have mean LR ≤ 5.0

and SD = 1.78

(pooled across low efficacy Quat 1:15 and 200ppm NaOCl)

(3) **HIGHLY EFFECTIVE PRODUCTS**

Two cases:

(A) mean LR ≥ 7.0 and SD = 1.95

(predicted by quadratic equation in Fig. 2)

(B) mean LR ≥ 8.0 and SD = 0.51

(pooled across high efficacy Quat RTU and 5000ppm NaOCl, pH-adjusted)

ANOVA

Year	Treatment	Mean LR	S_r	S_R	Variance components		Percentage of total variance	
					Var_{Lab}	Var_{Test}	Among Lab	Within Lab
2015	**NaOCl 200ppm**	5.34	1.236	2.182	3.23	1.53	68%	32%
	NaOCl 5000ppm, pH-adjusted	8.64	0.2465	0.3826	0.086	0.0607	59%	41%
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	Quat RTU	8.50	0.5396	0.6197	0.093	0.291	24%	76%

multivariate t-distribution

pass-error and fail-error percentages for different testing scenarios

A picture describing how the variances were calculated …

Stakeholder Specifications:

(1) **PASSING CRITERION**

all tests have LRs ≥ 6.0

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(predicted by quadratic equation in Fig. 2)

(B) mean LR ≥ 8.0 and SD = 0.51

(pooled across high efficacy Quat RTU and 5,000ppm NaOCl, pH-adjusted)

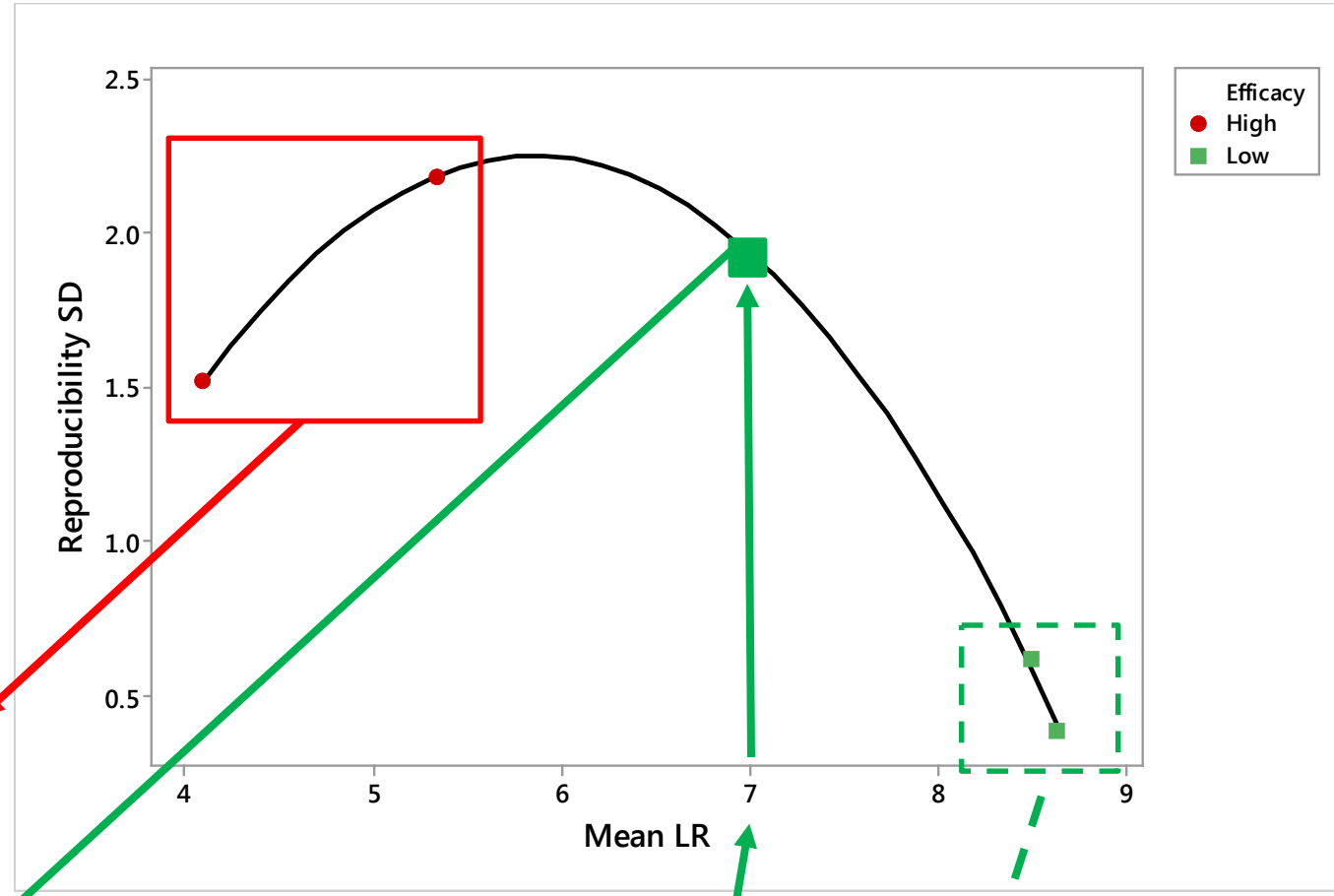

Table 2. Predicted error rates for 2-bug PS with $LR_{required} = 6$ fixed

"PASS-ALL-TEST" PS SPECIFICATION					ERROR RATES			CONFIDENCE AND POWER		
LR in each test must be larger than:	Number of Labs	Number of Tests in each lab for each microbe	Number of Control carriers in each test	Number of Treated carriers in each test	Pass-error percentage for ineffective products with mean LR ≤ 5 and SD = 1.78	A. Fail-error percentage for highly effective products with mean LR ≥ 7 and SD = 1.95	B. Fail-error percentage for highly effective products with mean LR ≥ 8 and SD = 0.513	Confidence	A. Power when mean LR ≥ 7 and SD = 1.95	B. Power when mean LR ≥ 8 and SD = 0.513
6.0	1	1	3	5	11.8%	59.1%	0.0%	88.2%	40.9%	100.0%
6.0	1	1	3	10	11.4%	46.9%	0.0%	88.6%	53.1%	100.0%
6.0	1	3	3	5	2.5%	71.7%	0.0%	97.5%	28.3%	100.0%
6.0	1	3	3	10	2.7%	69.6%	0.0%	97.3%	30.4%	100.0%
6.0	1	6	3	5	0.9%	82.6%	0.1%	99.1%	17.4%	99.9%
6.0	1	6	3	10	1.0%	80.3%	0.0%	99.0%	19.7%	100.0%
6.0	3	1	3	5	0.2%	85.5%	0.1%	99.8%	14.5%	99.9%
6.0	3	1	3	10	0.2%	84.5%	0.0%	99.8%	15.5%	100.0%

The assumption driving the calculations in Table 2 is that the variability of the second bug is the same as that for *P.a.*

If the variance of the second bug, such as *S.a.*, is actually less than the variance of *P.a.*, then the error rates in Table 2 are upper bounds for the error rates for a 2-bug PS.

Conclusions

- Increasing the number of treated carriers per test does not have a substantial effect on either the pass-errors or fail-errors. This is because the carrier-to-carrier variability is small in comparison the to test-to-test and lab-to-lab sources of variability (Table 2).
- Given the specification that highly efficacious products have a mean LR = 7, the fail-error percentages remain high across a variety of testing scenarios. This is because S_R is predicted to be 1.95 for products that have a mean LR of 7 (slide 8).
- For products with a mean LR = 7, fail-error percentages increase to at least 70% for multi-test PSs against 2 microbes (one being *P.a.*) that require a LR of 6 for each test (Table 2).
- Products with a mean LR = 8 will pass any of the multi-test, multi-microbe, PSs presented here at least 99% of the time.

References

- A. Parker, M. Hamilton, and S. Tomasino. A Statistical Model for Assessing Performance Standards for Quantitative and Semi-Quantitative Disinfectant Test Methods. *JAOAC International*, 97(1): 58-67, 2014.
- S. Tomasino, A. Parker, and M. Hamilton. Use of Statistical Modeling to Reassess the Performance Standard for the AOAC Use-Dilution Methods (955.15 and 964.02). *JAOAC International*, 97(1): 68-77, 2014.